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DATA ON THE EFFECTS OF THE PROBIOTIC "LACTO SACC"

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The effects of the probiotic "Lacto Sacc" on the lactic acid concentration, pH value and microbiological status of the intestinal content and faeces, as well as the ileal digestibility of different nutrients and essential amino acids were studied in growing pigs into which a T-cannula had been inserted surgically at the proximal part of the duodenum and at the distal part of the ileum. No differences were found between the control and the experimental group in the measured parameters of the chyme samples taken from the duodenum. As a result of "Lacto Sacc" feeding, lactic acid concentration of the ileal content increased from 4.77% to 7.22% while its pH value decreased significantly ($P<0.05$) from 7.39 to 6.42. The *Streptococcus* count was also significantly ($P<0.01$) elevated. At the same time, the *Escherichia coli* count decreased significantly ($P<0.001$) as a function of the elevated lactic acid concentration. The ileal digestibility of nutrients and essential amino acids improved by 1.3–16.1%. The favourable change observed in the microbiological status is attributed to the higher lactic acid concentration and the resulting lower pH, which adversely affect the survival of *E. coli*. Further studies are needed to reveal the factors responsible for the improved digestibility of nutrients.

Key words: *Escherichia coli, Streptococcus, Lactobacillus*, pH value, ileal digestibility, probiotic, pig

Since the introduction of ever stricter restrictions on the use of antibiotics as growth promotants, the attention of specialists has been increasingly focused on probiotics, growth-promoting preparations containing natural basic materials which exert a favourable effect on the health status of animals. These preparations contain different bacterial strains (most frequently *Lactobacillus* and *Streptococcus* spp.), possibly their metabolic products, and yeast cultures as well as enzymes.

The effects of probiotics are complex and in many respects insufficiently known. After entering the intestinal tract, they grow to high numbers and by their stabilizing effect exerted on the normal intestinal microflora, they reduce the risk of colonization by pathogens. They achieve this by reducing the pH of the intestinal content, occupying the superficial binding sites of the mucous membrane and,

in certain cases, expressly by their antibacterial effect (Potsubay, 1983; Fuller, 1989; Vanbelle et al., 1990). Numerous authors have observed a reduction in the incidence of enteric disorders caused by *E. coli* or *Salmonella* spp. as a result of feeding different *Lactobacillus* strains (Gilliland and Speck, 1977; Márai, 1982; Robinson et al., 1984; Fuller, 1989).

Favourable stabilization of the gut microflora results in improved digestibility and utilization of the different nutrients. This phenomenon is the basis of the growth-promoting effect of these preparations. By improving the intestinal microflora, probiotics inhibit the multiplication of not only the pathogenic bacteria but also of those which impairing the utilization of nutrients by using them up (Freter, 1962). Through the action of their enzymes, probiotics facilitate the utilization of nutrients and exert a detoxifying effect through metabolizing adverse decomposition products such as ammonia, amines, indole, and skatole (Vanbelle et al., 1990).

Probiotics are thought to enhance the local and systemic immune responsiveness of the organism (Vanbelle et al., 1990). When fed to pigs, the yeast-containing probiotic designated Ascogen improved the efficiency of the humoral immune response elicited by vaccination. In fattening pigs the preparation significantly increased the white blood cell count and blood iron as well as haemoglobin concentration (Glawischnig and Pedit, 1992).

In this work, the effects of "Lacto Sacc", a combined probiotic containing yeast culture, enzymes, *Lactobacillus* and *Streptococcus* species on the pH and lactic acid concentration of the intestinal content and on the composition of the intestinal microflora were studied, together with its influence on the ileal digestibility of crude protein and certain amino acids.

Materials and methods

The experiment was carried out at the Department of Physiology and Animal Nutrition and at the Central Laboratory of the Faculty of Animal Science, Pannon University of Agricultural Science, Kaposvár. The test material was the probiotic "Lacto Sacc", developed by Alltech in 1982, which contains microcapsulated *Lactobacillus acidophilus* and *Streptococcus faecium* bacteria, enzymes and *Saccharomyces cerevisiae* (strain 1026) yeast culture.

Eight growing pigs (barrows of the Duroc breed) weighing 30–35 kg were used in the experiment.

Several chyme collection methods are available for studying the digestibility of nutrients by intestinal segment. In this work the so-called T-fistula procedure (Kubovics et al., 1989) requiring the least severe surgical intervention was

applied. As our aim was to obtain intestinal content from both the proximal and the distal segment of the small intestine, two fistulas were inserted surgically into each experimental animal: one into the duodenum, 10–12 cm from the pylorus, and another into the ileum, about 15–20 cm proximal to the ileocaecal valve.

Ten days before surgery the animals were transferred to individual pens where they remained until post-operation day 12, during the healing period. The pigs were fasted for 24 h before the operation, while on the day of surgery they received only drinking water and chamomile tea (100 ml). From day 2 after the operation the pigs received increasing amounts of an easily digestible feed. In a transition period starting on day 5, the pigs received a grower diet, while from day 10 they were fed a mash corresponding to their age and body mass, twice daily.

In the 10-day preliminary feeding period and in the 5-day sampling period the pigs were fed an intensive piglet diet designated TA 8030, in an amount of 2 × 800 g per day, mixed in 2 l of water on each occasion. The composition of the diet is presented in Table 1. "Lacto Sacc" was mixed in the diet of the experimental pigs at a dose rate of 0.2% (200 g/100 kg of feed). Thus, the daily *Lactobacillus* and *Streptococcus* intake was around 10^7 while the yeast intake was in the magnitude of 10^9.

At the beginning of the sampling period the animals were placed into individual metabolic cages where their feed consumption was measured and their faeces collected over a period of five days. During that period, samples were taken twice from the intestinal content through the duodenal and the ileal cannula. The chyme and fresh faecal samples were collected in a sealable plastics container and were immediately transported to the Central Laboratory where they were processed for microbiological examination without delay.

Test parameters. (1) The *pH value* of the fresh intestinal content and faecal samples was determined with a manual automatic pH meter (OP-110, Radelkis, Hungary). The *lactic acid content* of the same samples was determined with a Beckman UV 5260 spectrophotometer after the following processing. After previous centrifugation of the intestinal content, 4 ml of the liquid phase was measured into a centrifuge tube, 0.2 ml of 85% phosphoric acid was added, then the mixture was shaken and centrifuged. Ten µl of the centrifuged filtrate was measured into a polished test tube. One ml of water, 3 ml of concentrated sulphuric acid (during cooling with icy water) and 100 µl of 20% ethanolic hydroquinone solution were added. After shaking, the solution was heated in hot water for 20 min. The absorbance was measured in a 1-cm cuvette at 405 nm against a blind.

(2) The fresh samples were assayed also for the following parameters. The *total aerobic and anaerobic germ count* and the *E. coli count* were determined as described in Hungarian Standard MSz 6977-79 entitled "Examination of seeds

and hulled products for human consumption and for feeding purposes". The *Lactobacillus* spp. were cultured on "Micro assay culture" agar (Difco), in anaerobic thermostat, at 37 °C for 48 h. Streptococci were cultured on Edwards' medium, in aerobic thermostat, at 37 °C for 48 h. The germ count was expressed in log10 values to permit a better comparison. The objective of the studies was to monitor changes induced by the administration of "Lacto Sacc" in the number of lactic acid producing bacteria, rather than to perform a qualitative or quantitative determination of the intestinal microflora.

Table 1

Chemical composition and essential amino acid content of the diet

Nutrient content		Essential amino acid content g AA/100 g protein	
Dry matter, %	88.5		
	in % of dry matter		
Crude protein	20.9	Arginine	6.8
Crude fat	2.4	Histidine	2.8
Crude fibre	2.8	Isoleucine	1.8
Crude ash	6.1	Leucine	4.8
N-free extr.	67.8	Lysine	4.1
Acid-detergent fibre	5.2	Methionine	2.6
Ca	0.98	Phenylalanine	4.1
P	0.61	Threonine	1.9
ME	MJ/kg feed 13.41	Valine	2.9

(3) For determining the ileal digestibility of nutrients and amino acids, the feed and the ileal content were assayed for the following parameters. The *dry matter, crude protein, crude fat, crude fibre* and *crude ash* content of individual samples and the quantity of *N-free extract* were determined according to Hungarian Standard MSz 6830-77 entitled "Determination of the nutritive value of feeds". The *amino acid composition* of the samples was determined by an LKB 4101 type automatic amino acid analyzer. The acid-detergent fibre content was determined by the method of Van Soest, using a Tecator-Fibertec apparatus suitable for measuring fibre fractions. That parameter of the feed, representing an indigestible feed component excreted in its full quantity, served as marker.

Namely, the total quantity of the intestinal content could be calculated from the known quantity of the sample taken from the ileal content, its acid-detergent fibre content, and the acid-detergent fibre content of the feed.

(4) Statistical analysis of the results was done by regression analysis (Sváb, 1981).

Results

The pH value and lactic acid content of the samples are shown in Table 2. The pH of the duodenal content was between 4.6 and 6.3 in both the treated and the control animals: there was no difference between the two groups. Marked differences were demonstrable between the control and the treated animals in the pH of the ileal content (in the control animals the pH of the ileal content was 7.39, while "Lacto Sacc" induced a significant ($P<0.05$) decrease of the pH to 6.42.

Table 2

pH value and lactic acid content of the samples

		pH			Lactic acid content (%) in 100% dry matter content		
		Duodenum	Ileum	Faeces	Duodenum	Ileum	Faeces
Treated group	n	9	6	9	9	6	9
	$\bar{x}$	5.48	6.42	7.33	3.03	7.22	0.18
	CV%	7.9	10.0	6.7	21.8	38.0	34.6
Control group	n	4	4	3	4	4	3
	$\bar{x}$	5.34	7.39	7.54	2.39	4.77	0.21
	CV%	9.10	3.5	5.6	12.7	19.7	28.0

The pH of the faeces was 7.33 and 7.54, respectively, and corresponded to the average value published in the literature (Jonsson, 1985). No significant differences were demonstrable between the two groups in that respect.

The lactic acid concentration measured in the duodenum was only slightly (0.64%) higher in the treated than in the control group.

Table 3

Results of microbiological examination of the samples (germ count, lg/g sample)

		Lactobacillus			*Streptococcus*			*E. coli*			Total aerobe count			Total anaerobe count		
		D[1]	I[2]	B[3]	D	I	B	D	I	B	D	I	B	D	I	B
Treated group	n	9	6	9	9	6	9	9	6	9	9	6	9	9	6	9
	$\bar{x}$	5.04	7.09	7.43	5.40	9.10	8.64	5.46	7.84	6.87	5.80	8.52	7.18	6.36	8.30	8.36
	± s	0.38	0.35	0.30	0.30	0.16	0.57	0.40	0.62	0.45	0.31	0.62	0.38	0.48	0.37	0.31
Control group	n	4	4	3	4	4	3	4	4	3	4	4	3	4	4	3
	$\bar{x}$	5.09	7.18	7.71	5.46	8.59	9.22	5.57	8.30	7.11	5.62	8.50	6.99	5.77	8.54	7.65
	± s	0.20	0.21	0.19	0.38	0.22	0.26	0.47	0.30	0.38	0.24	0.16	0.22	0.32	0.15	0.27

[1] in intestinal content taken from the duodenum
[2] in intestinal content taken from ileum
[3] in faeces

Table 4
Published values of the composition of the intestinal microflora in swine (germ count, lg/g sample)

		Lactobacillus	*Streptococcus*	Coliforms	Total aerobe count	Total anaerobe count
Duodenum	(1)	6.9	5.1	3.4		
	(2)				6.5	6.7
	(3)	6–8	5	5	7	7
Ileum	(1)	8.2	7.4	6.3		
	(2)				7.9	8.3
	(3)	7–9	6	4–8	8	8
	(4)	8.1	8.0	6.9	8.1	8.4
Faeces	(1)	8.5	7.9	7.0		
	(2)	8–9	8–9	7–10	9.3	9.9
	(3)	7–11	6–9	5–8	8–9	9–10
	(4)	8.2	8.0	7.9	8.6	9.4
	(5)	9	8.5	*E. coli*: 6–6.5		
	(6)	9.5	8–9.4	*E. coli*: 6.6–8.0		

(1) Kovács et al. (1972); (2) Amtsberg (1984); (3) Jonsson (1985); (4) Wittenbrink et al. (1984); (5) Pesti (1963); (6) Gärtner et al. (1973)

As expected, a much bigger difference was found in that respect for the ileal chyme in which the lactic acid content was 7.22% in the group treated with "Lacto Sacc", as compared to 4.77% in the control animals. Due to the rather high standard deviation this difference is not statistically significant.

The lactic acid content of the faeces was lower in both groups (0.18 and 0.21%, respectively), with no major difference being demonstrable between the groups.

The results of the microbiological examination of the intestinal content and faeces are contained in Table 3. Comparing the obtained germ counts with data of the literature (Table 4) a similar tendency can be observed. In the proximal part of the small intestine *Lactobacillus* and *Streptococcus* spp. were dominant, the *E. coli* count and the total aerobe as well as total anaerobe count were markedly lower than in the distal part of the ileum. On the basis of the lactic acid concentration we expected a more marked difference between the treated and the control group in the number of lactobacilli and streptococci cultured from the ileal chyme. However, a significant ($P<0.01$) difference was found only in the *Streptococcus* count which increased from lg 8.59 (control group) to lg 9.10 ("Lacto Sacc" treated group).

Table 5

Changes induced in ileal digestibility (%) by probiotics

	Control (n=4)		"Lacto-Sacc" (n=4)	
	$\bar{x}$	± s	$\bar{x}$	± s
Dry matter	68.4	3.1	69.7	3.3
Crude protein	66.7	5.4	72.4	6.8
Crude fat	32.6	5.7	44.3	8.1
N-free extract	78.5	3.7	79.5	3.2
ARG	72.7	4.0	81.7	8.7
HIS	68.3	6.3	78.7	4.4
ILE	43.3	14.5	58.8	14.8
LEU	63.3	6.4	74.6	8.7
LYS	61.8	9.0	65.1	17.3
MET	65.8	11.3	81.9	3.4
PHE	72.2	5.9	78.7	5.9
THR	41.6	14.2	55.7	15.7
VAL	65.6	5.0	73.6	9.7

A difference was demonstrable between the treated and control animals also in the *E. coli* count which was lg 7.84 and lg 6.87 in the ileal chyme and faeces of the treated pigs, respectively, as compared to lg 8.30 and lg 7.11 in respective samples of the control group.

The results obtained for the ileal digestibility of certain nutrients and essential amino acids are presented in Table 5. As a result of treatment, the digestibility of the following nutrients improved: dry matter (1.3%), crude protein (5.7%), crude fat (1.7%), and N-free extract (1.0%). The ileal digestibility of certain essential amino acids also increased by 3.3–16.1%: this improvement was the most pronounced for isoleucine, methionine and threonine. Because of the relatively low number of data. the differences between values obtained for the experimental and control group were not significant.

Discussion

The normal intestinal flora of pigs consists of 400–500 bacterium species numbering approx. 10^{10}. The composition of the intestinal flora varies with intestinal segment but is relatively constant within a given individual and intestinal segment. Individual variation may be substantial, depending on the pH of the intestinal juice, motility of the intestines, and the nutrients present in the intestinal content, etc. (Gedek, 1987).

Lactic acid producing bacteria, which are present in the intestinal microflora in large numbers also under normal conditions, have an important role in the maintenance of small intestinal pH, which provides natural protection against the excessive multiplication of certain bacteria, e.g. enteropathogenic *E. coli* (Márai, 1982; Chapman, 1988). In our experiment, the lactic acid concentration increased as a result of "Lacto Sacc" treatment significantly reduced the *E. coli* count present in the ileum ($r = -0.71$; $P<0.001$). This may have been due to the higher *Streptococcus* and *Lactobacillus* count and the decreasing pH, which adversely affect their growth. Favourable changes occurred in the ratio of *E. coli* to the total aerobe count: in the treated animals this ratio was lower (7.84/8.52 in the ileum and 6.87/7.18 in the faeces) than in the controls (8.30/8.50 in the ileum and 7.11/6.99 in the faeces).

Ushe and Nagy (1985) studied whether *Streptococcus faecium* M74 could inhibit the small intestinal colonization of enterotoxigenic *E. coli*. They found that although the number of streptococci in the ileum did not increase considerably, it significantly reduced the rate of colonization by enterotoxigenic *E. coli*. They could not demonstrate a direct antibacterial or enterotoxin-neutralizing effect. As streptococci showed a weaker adherence to the intestinal epithelium than did

enterotoxigenic *E. coli*, the above findings could not be explained by a blocking of the surface binding sites: rather, it could be attributed to the higher lactic acid concentration and the resulting lower pH induced by streptococci.

The experiment conducted by Nagy and Ushe (1985) in mice brought similar results: *Streptococcus faecium* M74 did not inhibit colonization by enterotoxigenic *E. coli*.

On the basis of the above findings it appears that preparations containing lactic acid producing bacterium strains can exert their protective effect against different enteropathogenic bacteria if they are fed over a prolonged period, i.e. if a constantly high germ count is ensured. Therefore, the stability of probiotics is a fundamental requirement: the lactic acid producing bacteria contained in them should be able to maintain their viability and multiply in large numbers (Vanbelle et al., 1990).

From data reported in the literature it appears that the protective effect of probiotics can be attributed to the lower pH value induced by them in the small intestine. This lower pH represents unfavourable conditions for the growth of not only *E. coli* but other enteropathogenic bacteria as well. Prohászka and his coworkers reported in several papers (Prohászka and Baron, 1982; Prohászka and Lukács, 1984; Prohászka, 1986; Prohászka et al., 1990) that in pigs fed CCM the intestinal concentration of non-dissociated volatile fatty acids increased, and the consequent pH decrease reduced the *E. coli* and *Salmonella* count in the distal segment of the ileum and in the colon. In their opinion, the protective effect was assured at a pH value between 5.5 and 6.6.

Several reports have indicated that treatment with probiotics increased the body mass gain and improved the feed conversion rate (Jernigan et al., 1985; Jost and Bracher-Jakob, 1991). It would be interesting to investigate if probiotics can modify the activity of certain digestive enzymes of the intestinal juice. The question whether the enhanced activity of proteolytic enzymes (chymotrypsin, trypsin) can account for the measurable improvement of protein and amino acid digestibility constitutes the subject of our future work.

Thorough studies on the digestive-physiological and microbiological aspects of the subject would provide us with an opportunity to obtain a deeper understanding of the action mechanism of probiotics. In addition to giving a sound scientific explanation for the physiological phenomena underlying the improvement of production indices, such studies would show us in what age group and production environment can probiotics be used with the best result in pig nutrition.

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LACK OF EFFECT OF OXYTOCIN GIVEN INTO THE AORTA ABDOMINALIS ON CORPUS LUTEUM FUNCTION IN CATTLE

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The aim of present study was to infuse moderate doses of oxytocin into the aorta abdominalis at different stages of the oestrous cycle in cattle to investigate its effect on the secretory function of the corpus luteum (CL). Mature heifers (n=12) were synchronized with a luteolytic dose of Oestrophan. One day before the experiments the animals had cannulae inserted into the aorta abdominalis through the coccygeal artery, with the tip placed cranial to the origin of the ovarian artery. This allowed direct application of the drug into the reproductive tract. In a preliminary experiment (n=4) we found that 40 IU of oxytocin given for 5 h would be a more suitable dose to enhance the peripheral concentration of this peptide slightly over its physiological pulses. This dose of oxytocin was infused in Experiment 1 (n=4) once daily for 5 h on days 10, 11 and 12 of the oestrous cycle and in the same way on days 4, 8, 12 and 16 in Experiment 2. In all of these trials oxytocin affected progesterone or testosterone secretion measured in the peripheral blood. Oxytocin given into the aorta abdominalis acts not only on the CL but also on the uterus, oviduct, blood vessels in the reproductive tract etc. Hence the response of CL can be masked by the effect of oxytocin on these tissues. It is, therefore, assumed that oxytocin acts on the bovine CL inside the ovary but not as a systemic hormone.

Key words: Oxytocin, progesterone, corpus luteum, cattle, oestrous cycle

The role of ovarian oxytocin (OT) is still undefined (Pickering et al., 1990). It was suggested to be involved in luteolysis (McCracken et al., 1984). There have been also suggestions that OT may have a stimulatory effect on progesterone secretion, both *in vivo* (Mares and Casida, 1963) and *in vitro* (Tan et al., 1982). However, further studies using dispersed luteal cells (Rodgers et al., 1985; Przała et al., 1986; Barrett and Wathes, 1990) delivered discrepant data on the role of ovarian OT. It was found that OT had no effect, or that it stimulated or inhibited progesterone secretion by luteal cells in different species. However, if OT was infused directly into the CL in cattle in a microdialysis system, it stimulated pro-

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gesterone secretion (Miyamoto and Schams, 1991) mainly during the early luteal phase. On the other hand, it inhibited progesterone release during pregnancy in ewes (Sernia et al., 1991). Moreover, noradrenaline infused into the aorta abdominalis has been proved to cause a violent rise in both OT and progesterone release in heifers (Kotwica et al., 1991; Skarzyński and Kotwica, 1993; Jaroszewski and Kotwica, 1994), but in most cases the rise of OT preceded progesterone elevation. These data suggest that an intact ovary is required to show the real function of ovarian OT.

Therefore, in the present studies moderate doses of OT were infused into the aorta abdominalis at different stages of the oestrous cycle in cattle to investigate its effect on CL secretory function.

Materials and methods

Animals and surgery

Mature heifers (n=12) weighing 350–450 kg with controlled lengths of the oestrous cycle were used. After rectal palpation, heifers were synchronized with 500 μg of Oestrophan (Spofa). The first signs of oestrus were accepted as day zero of the cycle. One day before the experiments, the animals had polyvinyl cannulae (OD 1.7 mm, ID 1.2 mm) inserted into the aorta abdominalis through the coccygeal artery, with the tip placed cranial to the origin of the ovarian artery (Kotwica et al., 1990). This procedure allows the direct application of drugs into the reproductive tract. Such local delivery permitted the use of a lower dose of drug, thus avoiding side effects. A second catheter was inserted into the jugular vein to collect blood samples.

Preliminary experiment

To establish the peripheral concentration of OT at the upper level of physiological pulses (i.e. approximately 70 pg/ml), four different doses of OT (25, 40, 60 or 100 IU) were infused for 4 h to four heifers on day 11 of the oestrous cycle. Blood was collected from the jugular vein every 15 min 1 h before, during and 1 h after OT infusion.

Experiment I

On the basis of data from the preliminary experiment, a dose of 40 IU was chosen for further studies. The time of infusion was extended to 5 h and was preceded with a 5-h long saline infusion – control period. Three heifers were infused in this manner on days 10, 11 and 12 of the cycle i.e. at the time of the highest OT contents in the bovine CL (Fuchs, 1988).

Experiment II

Five heifers were infused as in Experiment I on days 4, 8, 12 and 16 of the oestrous cycle. Blood samples (8 ml) were collected into tubes containing 30 μM of EDTA and 1 mg of aspirin in all experiments. Subsequently, blood was chilled in an ice-bath and centrifuged (1,000 *g*) for 10 min at 4 °C. The obtained plasma was stored at –20 °C.

Hormone determination

Progesterone was determined in the whole plasma by specific RIA as described previously (Kotwica et al., 1990). Anti-ovine progesterone antiserum (GDN No 337; donated by G. D. Niswender) used in this assay was characterized by Gibori et al. (1977). Sensitivity of the assay was 15 pg/tube i.e. 0.3 ng/ml of plasma. Intra- and interassay coefficients of variation were 8.1 and 15.9%, respectively. The relationship between real and determined amounts of four different concentrations of progesterone added to the plasma samples is expressed by the linear regression equation $y=1.034x-0.13$, where "x" represents the real value and "y" is the value obtained.

Testosterone was determined according to the procedure described by Kotwica and Williams (1981) using rabbit antiserum (BSz/88/702) which cross-reacted 55.5% with 5α-androstan-17β-ol-3-one, 8.36% with 4-androstan-11β-ol-3,17-dione, 0.47% with 5β-androstane-3α-17β-diol, 0.09% with epiandrosterone, 0.08% with 5α-androstane-3,17-dione, 0.03% with androsterone, progesterone, 0.02% with 5β-androstane-3α-17β-dione and less than 0.01% with oestradiol-17β, oestrone, dihydroisoandrosterone, 5α-pregnan-3α-ol-20-one and 5α-pregnan-3β-ol-20-one. The efficiency of extraction was on average above 85% and the data were corrected for procedural losses. The sensitivity was 1.3 pg/tube i.e. 3 pg/ml. Intra- and interassay coefficients of variation were 11.3 and 16.0% respectively. The reliability of the procedure is expressed by the linear regression equation $y=0.89x+1.69$. OT was extracted from 0.5 ml of plasma in duplicates using ice-chilled acetone. Rabbit OT antiserum (R-1, donated by G. Kotwica) was characterized earlier (Kotwica and Skarzyński, 1993). The efficiency of extraction was above 85% and final data were corrected for procedural loses. The sensitivity of the method was 3 pg/ml. Intra- and interassay variations were 7.5 and 14.6%, respectively. The precision of the procedure is expressed by the linear regression equation ($y=0.99x+0.14$).

Luteinizing hormone (LH) was estimated according to Ziecik et al. (1979). USDA-bLH-I-1 was used as the standard and USDA-bLH-B-5 was used for radioiodination performed according to Bryant and Greenwood (1968). The sensitivity of the assay was 0.3 ng/ml and intra- and interassay coefficients of variation were 10.2 and 18.2%, respectively. The precision of the method for the

measurement of 2, 6 and 30 ng/ml (n=8) is illustrated by the linear regression equation (y=1.01x+0.23).

Data analysis

The amount of OT in peripheral blood during the infusion of this peptide on days 10–12 was measured by calculating the area under the curve above mean basal concentrations before infusion. Differences between means (±SEM) were analyzed using one-way analysis of variance (InStat, GraphPAD).

Results

In the preliminary experiment all infused doses of OT caused a marked ($P<0.001$) increase of this peptide in peripheral circulation (Fig. 1). A dose of 40 IU was chosen for further studies. There were, however, no significant changes ($P>0.05$) in progesterone secretion during and after treatment compared to the period before infusion (data not given).

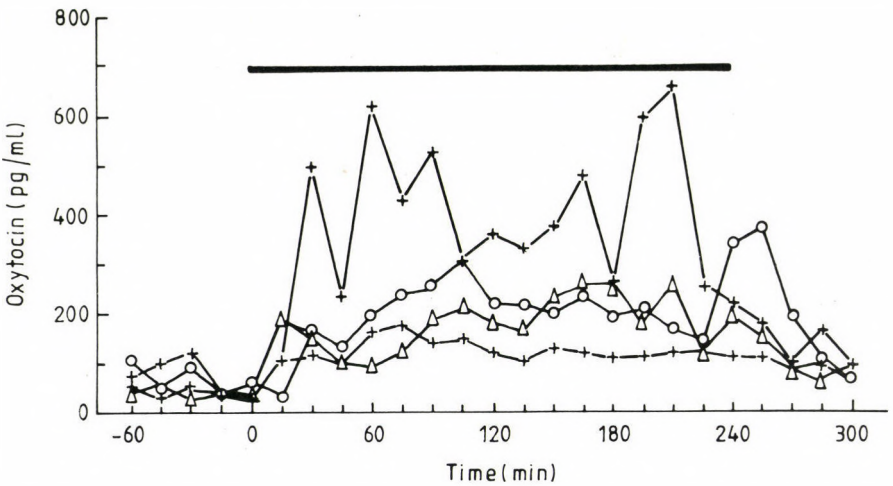

Fig. 1. Plasma oxytocin evoked by infusion into the aorta abdominalis of 25 (—+—), 40 (—Δ—), 60 (—O—) or 100 (—+—) IU of OT (solid horizontal bar) within 4 h on day 11 of the oestrous cycle in heifers (n=4)

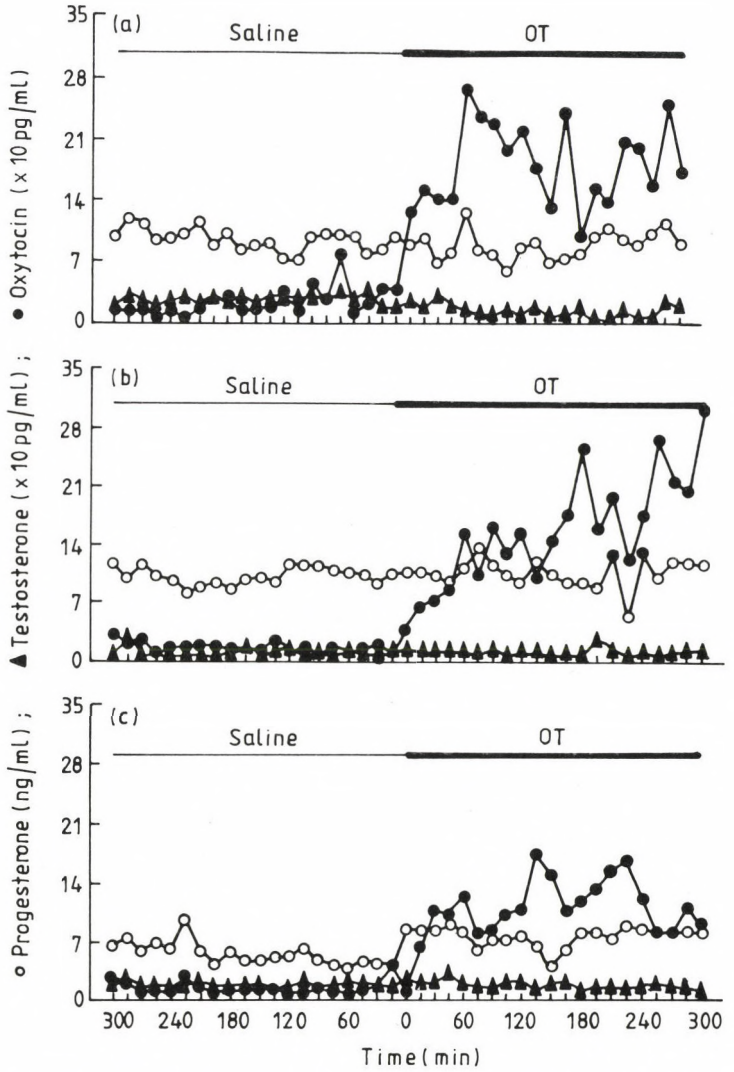

Fig. 2. Progesterone, testosterone and oxytocin concentrations in the peripheral blood during infusion of oxytocin (40 IU/5 h/day) into the aorta abdominalis on days 10 (a), 11 (b) and 12 (c) of the oestrous cycle in one representative heifer

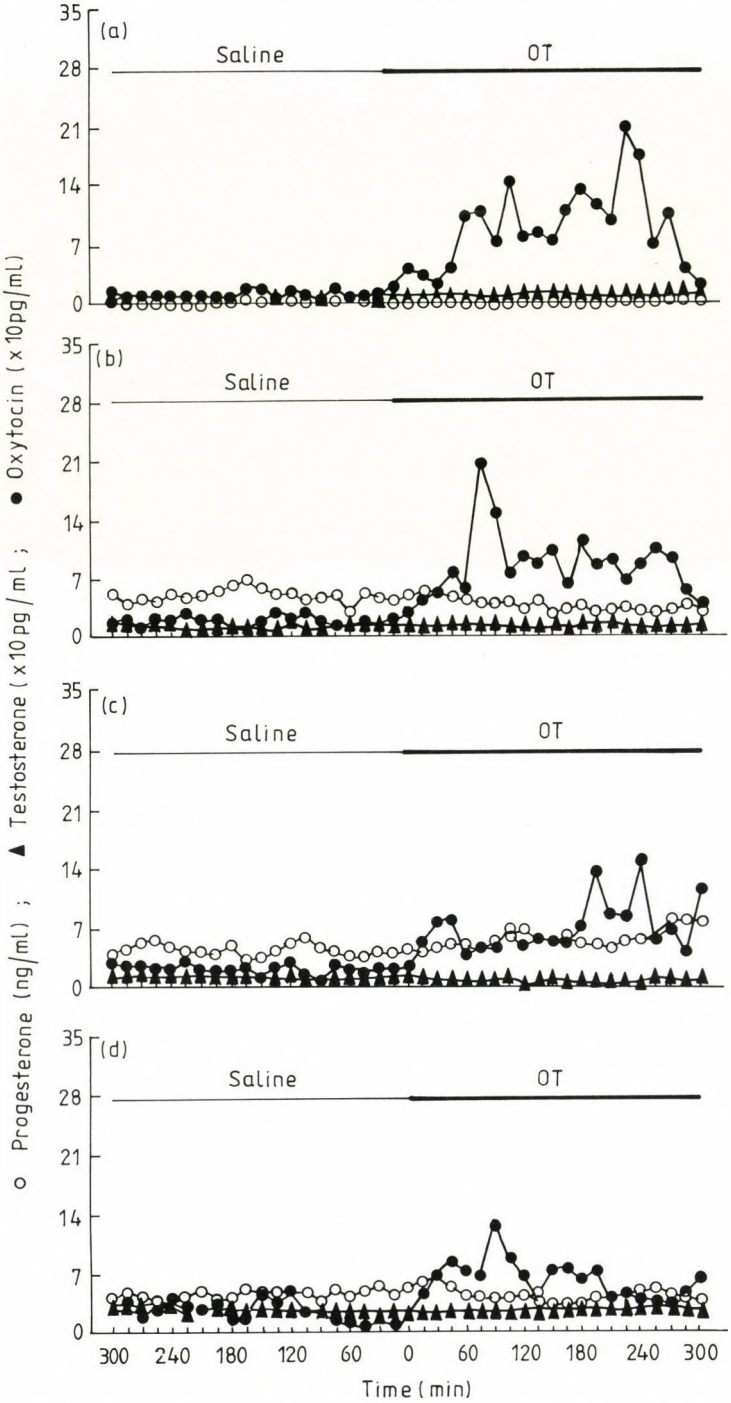

Fig. 3. Progesterone, testosterone and oxytocin concentrations in the peripheral blood during infusion of oxytocin (40 IU/5 h/day) into the aorta abdominalis on days 4 (a), 8 (b), 12 (c) and 16 (d) of the oestrous cycle in one representative heifer

Table 1

Influence of oxytocin infusion (40 IU/5 h/heifer) on progesterone (P_4) and testosterone (T_4) response in three heifers on days 10–12 of the oestrous cycle

Day of oestrous cycle	Increase		Decrease		No change	
	P_4	T_4	P_4	T_4	P_4	T_4
10	–	1^{**}	–	2^{*}	3	–
11	–	–	1^{*}	1^{*}	2	2
12	1^{**}	–	1^{*}	–	1	3

$^{**}P<0.001$; $^{*}P<0.01$
Numbers 1, 2, 3 represent the number of heifers

Table 2

Influence of oxytocin infusion (40 IU/5 h/heifer) into the aorta abdomonalis on progesterone (P_4) and testosterone (T_4) response in the peripheral blood in heifers (n=5) on days 4, 8, 12 and 16 of the oestrous cycle

Day of oestrous cycle	Increase		Decrease		No change	
	P_4	T_4	P_4	T_4	P_4	T_4
4	1^{*}	1	1^{*}	–	3	4
8	1^{**}	1	2^{*}	–	2	4
12	2^{**}	1	–	1^{**}	3	3
16	–	–	2^{**}	1^{*}	3	4

$^{**}P<0.001$; $^{*}P<0.01$
Numbers 1, 2, 3, 4 represent the number of heifers

In Experiment I, the infusion of 40 IU OT increased peripheral concentrations of this peptide up to approximately 50–200 pg/ml (Fig. 2). Progesterone and testosterone were secreted in a variable fashion; we observed an increase, decrease or lack of steroid reaction during OT challenge on the studied days of the cycle (Table 1). Similarly, different responses with respect to secretion of steroids were found during OT infusion in Experiment II, on days 4, 8, 12 and 16 of the cycle (Fig. 3; Table 2).

LH plasma concentration was determined in four heifers in the preliminary experiment and in 2 heifers of Experiment II on day 16 of the cycle. In all samples studied, the LH concentration was less than 1 ng/ml (data not given) and hence this hormone was not analyzed further in these studies.

Discussion

The results of studies carried out *in vivo* (Mares and Casida, 1963) and *in vitro* (Tan et al., 1982) suggested that OT may have a stimulatory effect on progesterone secretion in cattle. Walters et al. (1984) observed concomitant pulses of ovarian oxytocin and progesterone in the mid-luteal but not in the early luteal phase in cows. It was also found that infusion of OT directly into the CL of cows stimulates progesterone secretion in a dose-dependent manner (Miyamoto and Schams, 1991). Finally, it was proved that noradrenaline increases enhance both OT and progesterone release in heifers, but in most cases the elevation of OT preceded that of progesterone (Kotwica et al., 1991). These results clearly indicate that OT is involved in progesterone secretion. Moreover, testosterone level was found to be higher in the blood of goats treated with OT (Homeida and Khalafalla, 1990), suggesting that testosterone is involved in OT-affected CL function in this species.

We were, however, unable to find any regularity in the secretion of both steroid hormones studied in this experiment, although peripheral OT concentration was enhanced to 50–200 pg/ml for 5 h. Therefore, it is reasonable to assume that during OT infusion, variability in steroid plasma concentrations arises from their natural pulsatile secretion, rather than being caused by OT.

OT infused into the aorta abdominalis supposedly exerts an influence upon target cells other than the CL, e.g. the uterus (Oyedipe et al., 1984) or blood vessels in the reproductive tract (Katusic et al., 1984). Hence the final effect of OT is both the sum and result of its effect on these tissues.

Gilbert et al. (1989) found in cattle that continuous infusion of OT from the mid-luteal stage of the cycle prolonged the length of the luteal phase but an increase in progesterone secretion was not observed. A similar infusion of OT from days 14–16 of the cycle in cattle had no effect on the length of the oestrous cycle (Kotwica et al., 1988). This latter work indicates that oxytocin may prolong the life-span of CL only if infused continuously from the mid-luteal phase. Supposedly, this may depend on the presence of OT receptors in the bovine CL, since their highest concentrations can be observed on days 8–12 and are almost 10 times lower on days 15–18 of the cycle (Okuda et al., 1992).

We assume therefore that OT can really affect CL function during a short term only if given directly into the ovary, acting then as an autocrine/paracrine factor.

Acknowledgements

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BACTERIOLOGICALLY CONFIRMED CASES OF OVINE EPIDIDYMO-ORCHITIS CAUSED BY *Brucella ovis* IN SUB-CARPATHIA

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During the eradication of *Brucella ovis* infection from five large breeding ram flocks of Sub-Carpathia (The Ukraine), the genital organs of 55 rams culled because of seropositivity in the agar-gel precipitation (AGP) test and ELISA were subjected to gross pathological, histopathological and bacteriological examination. The results of these examinations, as well as the properties of *B. ovis* strains isolated for the first time in the region are reported. Thirty-three out of the 55 pairs of epididymides and testicles (60%) showed gross lesions (chronic epididymitis associated with the formation of spermatocele, sperm granuloma or abscess). From the 55 pairs of epididymides and testicles examined, a total of 26 *B. ovis* strains were isolated: of them, 24 (92.3%) originated from breeding rams while 2 (7.7%) from ram hoggets kept together with the breeding rams. Seven out of the 26 *B. ovis* strains (26.9%) were cultured from the epididymides of rams which did not show palpable epididymal and/or testicular lesions. It is emphasized that chronic inflammatory processes were observed in both epididymides even if the clinically palpable epididymo-orchitis occurred unilaterally. Three out of the 26 *B. ovis* strains were derived from AGP-positive but ELISA-negative, while 5 from ELISA-positive but AGP-negative rams. The remaining *B. ovis* strains were isolated from the genital organs of rams found seropositive both by the AGP test and by ELISA. The cultural, morphological and most important biochemical properties of the 26 Sub-Carpathian *B. ovis* strains were identical with those of the reference strain designated Weybridge 63/290 (NCTC 10512). Differences existed between the 26 strains in urease activity as well as in sensitivity to thionine and basic fuchsin. The 10 Sub-Carpathian *B. ovis* strains proved sensitive to ampicillin, streptomycin, oxytetracycline, chloramphenicol, erythromycin, gentamicin, Sumetrolim, oxacillin and levomycetin but were resistant to bacitracin and vancomycin.

Key words: *Brucella ovis*, ram, epididymo-orchitis, bacteriology, Sub-Carpathia

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The occurrence of *Brucella ovis* infection of rams in Sub-Carpathia (The Ukraine) has been known for more than a decade. By serological tests the infection has been diagnosed in several sheep flocks of the Carpathian breed. In the flocks where the infection was demonstrated, clinically apparent epididymo-orchitis was observed among the breeding rams. In spite of this fact, attempts to isolate the pathogen had failed until the start of this study.

This paper presents the results of the pathomorphological and bacteriological examination of the genital organs of rams culled during the eradication of *B. ovis* infection from five large breeding ram flocks of Sub-Carpathia (Dénes and Babkin, 1991; Dénes et al., 1993), as well as the properties of the isolated *B. ovis* strains.

Materials and methods

Pairs of epididymides and testicles derived from 55 castrated or emergency-slaughtered rams that had proved seropositive for *B. ovis* infection by the AGP test and/or by ELISA, submitted for examination from five sheep flocks in the years 1990–1991, were subjected to gross pathological, histopathological and bacteriological examination. Serological tests for *B. ovis, B. abortus, B. melitensis* and *Chlamydia psittaci* infection were carried out as described earlier (Dénes et al., 1993).

Smears prepared from the content of formations that appeared to be an abscess or spermatocele on gross inspection were stained according to Stamp and Gram, and examined with an oil-immersion lens at a magnification of × 1,250.

Smears were prepared from the cut surface of epididymides and testicles even if these organs did not exhibit any gross lesions. Particular attention was paid to the caudal part of the pairs of epididymides whose sperm content was examined by microscopy and by bacteriological methods. These investigations were aimed primarily at the isolation of pathogens most frequently involved in the aetiology of genital infections in rams, viz. *B. ovis, Actinobacillus seminis, Histophilus ovis* and *Corynebacterium (Actinomyces) pyogenes* (Hajtós, 1990).

For the culturing of bacteria, Albimi agar, liver-broth-peptone-glucose-glycerol agar and, based upon favourable experience gained in Hungary earlier (Hajtós, 1988; 1990), blood agar plates containing 10% defibrinated bovine or ovine blood and 0.5% yeast extract. The cultures were incubated at 37 °C, in 10% CO_2 atmosphere, for one week. Bacterial growth was checked at 1, 3, 5 and 7 days after the inoculation.

The cultured *B. ovis* strains were identified on the basis of the characteristics proposed by Meyer (1982) and Alton et al. (1988). The bacterial colonies were tested by agglutination using Brucella monospecific A, M and R sera ('N. F.

Gamalei" Research Institute of Epidemiology and Microbiology, Moscow, Russia). For the comparative bacteriological examinations the reference strain designated Weybridge 63/290 (NCTC 10512) was used. The sensitivity of the reference strain and of our 10 *B. ovis* isolates to different antibiotics (penicillin, oxytetracycline, streptomycin, etc.) was evaluated by the disc diffusion method using a Resistest disc series (HUMAN Serum Production and Medicine Manufacturing Company Ltd., Budapest), after incubation at 37 °C for 120 h on blood agar.

Organ samples taken from the affected epididymides and testicles for histological examination were fixed in 10% neutral formaldehyde solution, then paraffin-embedded sections were prepared and stained with haematoxylin and eosin.

Results

Thirty-three out of the 55 pairs of epididymides and testicles examined (60%) showed gross lesions (chronic epididymitis accompanied by spermatocele, sperm granuloma or abscess formation; Table 1).

By the microscopic examination of sperm smears made from the cut surface of epididymides, inflammatory changes could be demonstrated in the epididymides on both sides even if gross inspection revealed only unilateral epididymitis. The sperm smears contained inflammatory cells, mainly neutrophilic granulocytes, in a number corresponding to the severity of the lesions.

In 11 out of the 26 bacteriologically positive cases (42.3%) the pathogen was successfully isolated from the caudal part of both the right and the left epididymis irrespective of the gross lesions. In smears prepared from the exudate of abscesses and spermatocele phagocytosed or extracellularly located *B. ovis* bacteria could also be observed. The sperm smears of bacteriologically negative epididymides of seropositive rams free from the clinical signs of disease also contained inflammatory cells.

Histopathological examination of the testicles revealed extensive, circumscribed inflammatory-necrotic areas surrounded by zones showing angiofibroblast tissue proliferation and infiltration by mononuclear cells. In the necrotic areas, focal calcification could be seen (Figs 1 and 2). Sections of the seminiferous tubules exhibited the degeneration and necrosis of germinal epithelial cells and the stoppage of spermatogenesis. In the epididymides, extensive inflammatory-necrotic areas suggestive of abscess formation (Fig. 3) were observed. In the wall of the abscesses pus cells, necrotic, disrupted cell debris, and giant cells were seen. The mucous membrane of the epididymal tubules showed vacuolar degeneration of the epithelial cells, infiltration by mononuclear inflammatory cells, while their lu-

men contained masses of partially degenerated spermiocytes. Signs of fibrosis could be seen around the tubules.

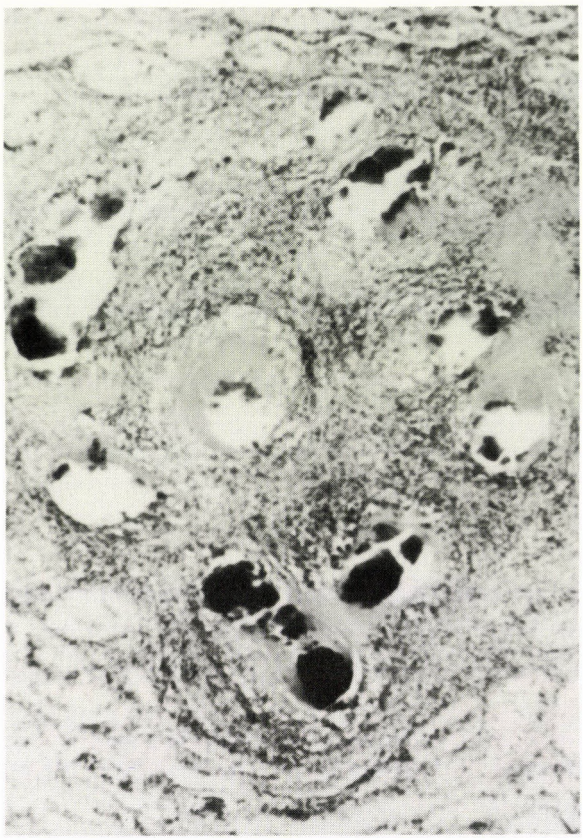


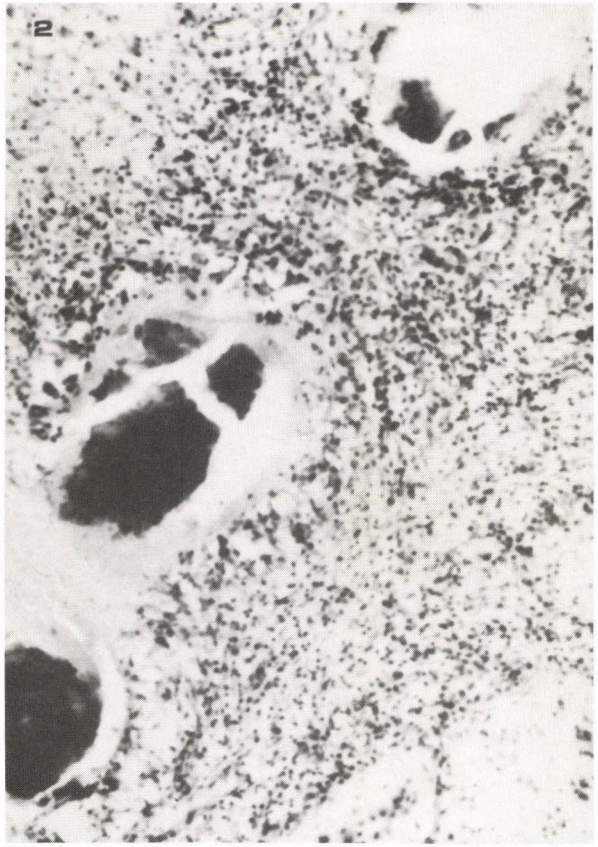
Fig. 1. Circumscribed inflammatory-necrotic area in the testicle, with proliferation of angiofibroblast tissue and focal calcification. Haematoxylin and eosin (H.–E.), × 35

By bacterial culture, a total of 26 *B. ovis* strains were isolated from the 55 pairs of epididymides and testicles. Twenty-four (92.3%) of the strains were isolated from breeding rams while the remaining two (7.7%) from ram hoggets kept together with the former. Seven out of the 26 *B. ovis* strains (26.9%) were cultured from the epididymides of rams without visible or palpable epididymal and/or testicular lesions. Three strains were derived from AGP-seropositive but ELISA-seronegative rams, while 5 strains from ELISA-seropositive but AGP-seronegative rams (Dénes et al., 1993). The other *B. ovis* strains were isolated from the genital organs of rams that had been found seropositive by both the AGP test and by ELISA (Table 1).

Table 1

Pathomorphological and bacteriological findings for the pairs of epididymides and testicles derived from the 55 rams seropositive for *Brucella ovis* infection

Group of rams	No. of test samples	Gross lesion		Bilateral histopathol-ogical lesions	Distribution of *B. ovis* strains isolated from the seropositive rams				
		Unilateral	Bilateral		No. of strains	Symptom-less	AGP-negative	ELISA-negative	AGP+ ELISA-positive
Breeding rams	35	18	5	35	24	6	5	3	16
Ram hog-gets	20	8	2	20	2	1	-	-	2
Total	55	26	7	55	26	7	5	3	18



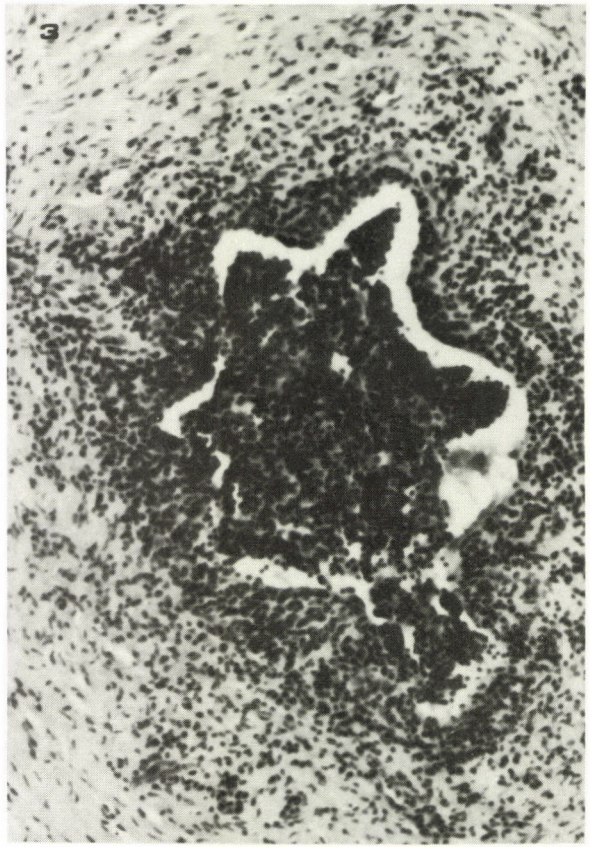


Fig. 2. Detail of Fig. 1 at 63-fold magnification. Note infiltration of the proliferating angiofibroblast tissue with mononuclear cells and degeneration of the germinal epithelium along with signs of aspermatogenesis in a section of the neighbouring tubule

Fig. 3. Infiltration of the mucous membrane of the epididymal tubule with inflammatory cells. Note a mass of partially degenerated spermiocytes in the lumen. H.–E., × 63

The isolated *B. ovis* bacteria were Gram-negative coccoid rods. They stained only partially by the modified Köster staining, formed no spores, and were immotile. They were agglutinated by the monospecific R serum within 1 min. None of the 26 *B. ovis* tested could grow in the absence of CO_2.

The characteristics of the isolates are presented in Table 2.

Table 2

Properties of the 26 *Brucella ovis* isolates

Catalase	+	Growth*		
Oxidase	–	thionine	1:25,000	– (+3)
OF test (glucose)	–		1:50,000	– (+8)
Indole	–		1:100,000	+
Urease	– (+4)	basic fuchsin	1:50,000	– (+6)
$NO_3^- \rightarrow NO_2^-$	–		1:100,000	+
H_2S formation	–	Agglutination		
Methyl red reaction	–	with monospecific A serum		–
Voges-Proskauer test	–	with monospecific M serum		–
		with monospecific R serum		+

*– : did not grow; + : growth+

According to the antibiotic sensitivity tests, the reference strain and the 10 test strains proved sensitive to ampicillin, streptomycin, oxytetracycline, chloramphenicol, erythromycin, gentamicin, Sumetrolim, oxacillin and levomycetin but were resistant to bacitracin and vancomycin. The reference strain was moderately sensitive to penicillin and nitrofurantoin, while among our isolates there were strains which proved sensitive, moderately sensitive and resistant to these antibiotics.

Discussion

The cultural, morphological and biochemical properties of the 26 *B. ovis* strains isolated by us in Sub-Carpathia were identical with the main properties of the reference strain designated Weybridge 63/290. By SDS-PAGE, no differences were found between the electrophoretograms of two randomly selected *B. ovis* strains isolated by us and that of the reference strain (Gálné (Mrs) et al., 1991). At

the same time, differences were observed between the *B. ovis* strains of Sub-Carpathian origin in urease activity as well as in sensitivity to thionine and basic fuchsin (Table 2). The reference strain grew well on media containing these dyes and proved to be urease-negative. Earlier, Corbel and Thomas (1985) made a similar observation in their comparative study of *B. ovis* strains originating from different parts of the world.

According to results of the antibiotic sensitivity tests, the 10 Sub-Carpathian strains proved to be sensitive to antibiotics widely used in the practice but were resistant to bacitracin and vancomycin. These results are consistent with the antibiogram of *B. canis* of similarly R colony type (Weber et al., 1982), as well as with that of the South-African *B. ovis* reference strain (Van Tonder, 1979).

It should be mentioned that, of all the culture media used, *B. ovis* could be isolated only on blood agar plates containing 10% defibrinated bovine or ovine blood and 0.5% yeast extract, which finding supports the earlier experience gained in Hungary in this respect (Hajtós, 1988, 1990). Later on our *B. ovis* strains cultured on blood agar were successfully adapted to Albimi agar and to liver-broth-peptone-glucose-glycerol agar.

No other bacteria involved in the aetiology of genital infections in rams (Hajtós, 1990) were isolated from the 55 pairs of epididymides and testicles examined. We mention here that the genital organs of two ram hoggets seropositive for *B. ovis* infection and culled because of epididymo-orchitis yielded bacterial strains whose cultural and morphological properties resembled those of *Histophilus ovis* described also in Hungary (Hajtós et al., 1986). However, the biochemical properties of these strains could not be determined, as they died out on the blood agar in 5 days.

The gross and histopathological findings were similar to those reported for *B. ovis* infection by others (Walker et al., 1986; Searson, 1986, 1987). It is worth emphasizing that inflammatory processes were observed bilaterally, in both epididymides, even if the lesions were unilateral clinically and gross pathologically (Table 1). This is in agreement with the observations made by Searson (1986, 1987) in Australia.

Walker et al. (1986) claim that chronic inflammation of the caudal part of the epididymis, accompanied by spermatocele or sperm granuloma formation, are typical of bacterial epididymitis in general and not specific of any one pathogen; therefore, reliable diagnosis must be based on the isolation of the pathogen.

By our studies supported by the isolation of the pathogen, we have clearly proved that ovine epididymo-orchitis caused by *B. ovis* occurs in Sub-Carpathia.

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PASSAGE OF SOME HEAVY METALS, NITRATES AND MYCOTOXINS IN THE FOOD CHAIN

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I. General Aspects

Since the early 'fifties of this century, trends of increasing production and profit have affected the natural environment (soil–water–air) of mankind to such a degree that now seems to threaten the harmonic coexistence of man and his immediate surroundings.

The prevailing tendencies of environmental damage have already endangered human health. Therefore, measures to be taken for the protection of health and environment are nowadays practically inseparable.

The greatest impact on the natural environment has been obviously due to the increasing use of chemicals, whose introduction in food production has in fact greatly increased the output, but has simultaneously accounted for a deterioration of quality that is now readily obvious in the entire food chain (soil–plant–animal–man).

Chemical residues in foods have long been a matter of great concern, not much for their mere presence in food items, as for their potential health hazards. The deleterious effects of chemical food contaminants vary greatly with the biological properties of the chemical compounds used for that purpose.

This can account for the still drastically conflicting opinions on the use of chemicals in food production. While some experts rigorously oppose the use of any chemical, others would rather advocate it, for lack of a firm proof of direct deleterious effects, and seem thereby to disregard some long-verified risks, such as (1) chronic toxicosis concomitant upon accumulation of chemical residues in human or animal tissues; (2) potential mutagenic, cancerogenic or teratogenic effects of the chemical residues; (3) adverse effects of the residues on reproductive abilities and (4) on the cardiovascular and cerebral systems, etc.

This controversy can be reconciled only by a strictly scientific approach to clarification of the cause–effect relationships, with special regard to the establishment of safe maximum permissible levels for chemical residues, because many

chemical compounds are in fact indispensable for the normal course of the physiological events.

However, certain other chemical substances, such as heavy metals, nitrates, nitrites and mycotoxins are not usually physiological compounds, although their maximum tolerated levels and toxic concentrations fall into fairly close ranges. The forthcoming review was compiled in an attempt to summarize the results of the authors' decade-long investigations along this line.

II. Experiments

The investigations in question were conducted under identical or at least highly similar conditions in samples obtained from soils, plants, animals and humans and do, therefore, reflect the passage of the residues studied through the entire food chain, with special regard to the trends of residue formation, verified by biometric analyses.

1. Studies into cadmium overload

We used cadmium (Cd) as experimental model to follow up the passage of heavy metals through the food chain with regard to the facts that the environmental Cd concentration tends to increase year by year, the motility (transfer factor) of Cd increases about 10 times over that of other heavy metals (for example lead), and excessive Cd is being accumulated in the tissues (biological half-life: 30 years).

The Cd levels present in foods depend on the amount of Cd emission from industrial and/or mining plants, refuse burning units, fuels and fertilizers used, as well as on the extent of use of composted urban garbage for dunging in rural agricultural areas.

The passage of Cd from soils into plants is also influenced by several factors, but above all by the pH of the soil. In model experiments the drop of pH by one grade step was always associated with an increase in plant Cd incorporation by about one order of magnitude.

In our studies the Cd content and pH of the soil showed a close, significant correlation with the Cd content of the plants grown on that soil, and with the Cd levels measured in the blood serum and pigmented hairs of the cows fed on those plants as well. The Cd levels tended to increase proportionally with the decrease in soil pH.

The mean Cd concentrations measured in the soil samples studied varied between 0.8 and 3.2 mg/kg, and were always higher in soils treated with fertilizers

than in those not treated. The bulk of Cd was always present in the superficial layers of the soils.

As to plant Cd levels, identical concentrations in the soil (range: 0.08–0.31 mg/kg dry matter) were associated with peak levels in onions and corn cobs, and relative minima in hays and cereals.

The dairy cows fed these fodder plants showed peak serum Cd levels prior to calving, followed by a gradual decrease in serum Cd until 15 days post partum. The Cd content of cow's milk increased significantly over the lactation peak immediately after calving, whereas the urine Cd level tended to drop significantly relative to the prepartal value.

Obviously, the ruminant organism tended to eliminate (mobilize) that noxious element prior to parturition, if required, also in the milk; in view of this, the use of Cd-loaded cow's milk for human or animal nutrition deserves special consideration.

Cows ingesting Cd-contaminated fodder plants may take in 1.3–2.6 mg Cd daily; with a productive lifespan of 3–4 years in mind this can account for a marked accumulation of Cd that may share responsibility for reproduction disorders ensuing later in life.

Analyses of the visceral organs of wildlife (roe deer, red deer, wild boars) indigenous in regions with a high Cd contamination of soil revealed peak Cd concentrations in the kidneys and to a lesser extent in the liver and muscles of the wild living animals studied. The renal Cd levels of boars (average: 3.27 mg/kg) increased over those of red deer (average: 0.77 mg/kg).

Humans take in Cd partly by inhalation, partly by ingestion of Cd-containing food items. About 20–25 per cent of inhaled Cd is being deposited in the pulmonary tissues. Thus smoking represents an important source of Cd exposure. Among the food items the cereals (bread and farinaceous products) and the meat of older cattle, pigs, wild ruminants or boars may serve as major source of human Cd exposure.

According to data of the literature, excessive intake of Cd may play a role in the development of renal and general hypertension, arteriosclerosis, reproductive disorders and, last but not least, also in neoplasia. The International Agency for Research on Cancer (IARC) has classified Cd with the potential cancerogenic agents. Sample surveys (Takács, 1985) demonstrated excessive Cd levels in the kidneys of subjects who had died of cancer relative to the levels demonstrated in those of victims of case fatalities of the same sex and age groups.

2. Studies on nitrate overload

Pertinent reports prognosticate that nitrate overloading of the environment threatens with an imminent catastrophe.

Our own model experiments along this line demonstrated that the nitrate overload of soils, water and plants tended to increase under the same conditions of nitrate supply with a decrease in the pH (acidification), owing to pH-dependent changes in nitrate reduction that took 50, 45, 24 and 11 days at pHs of 6.7, 7.2, 7.6 and 8.6, respectively, indicating a linear increase in the pH range between 5.0 and 8.0.

Lime treatment of soils with a low organic matter content failed to shorten the time period of nitrate reduction, whereas an increase in the amount of the organic component (e.g. by addition of cellulose) had a marked accelerating effect. However, above a certain limit of added organic component, the pH had little influence, if any, on the rate of nitrate reduction.

The toxicity of soils contaminated by metal salts (100 mg/kg Cu^{2+}) also seemed to change as the function of soil pH. For example, while *Azotobacter ochrococcum* organisms failed to grow in the pH range 5.5–6.5, growth inhibition was negligible between pH 7.0 and 7.5 and failed to take effect altogether between pH 7.5 and 8.5.

It also deserves mention that while nitrate-to-nitrite transformation was negligible in the soil pH range 5.5–6.5, it took place at a measurable rate between pH 7.0 and 7.5, and vigorously between pH 7.5 and 8.5.

According to pertinent investigations, nitrate reduction in soils depends in a decreasing sequence on organic matter content, pH, heavy metal concentration(s), and last but not least on the intricate interactions between these factors.

Our own experimental observations suggest that reduction of nitrate ions at soil pHs higher than 7.0 gives rise to formation of a bactericidal principle that acts on soil pathogens. For example, decrease in the survival time of *Escherichia coli* and *Salmonella typhimurium* organisms in soil samples showed a parallelism with the decrease in nitrate reduction rate (increase in organic matter content). The bactericidal principle (now under analysis) seems to be unrelated to the earlier established such properties of a *Pseudomonas* strain.

The passage of nitrates in soils may, depending on soil pH and on the chemical structure of the nitrate compounds, cover a distance of us to several hundred meters and may thus also contaminate water. Waters and culinary plants that contain high (500–4500 mg/kg) nitrate concentrations may give rise to methaemoglobinaemic conditions of varying severity, which may be fatal or fetopathogenic during intrauterine life. Continuous intake of greater amounts of NO_3 may potentially promote the formation of nitrosamines, which are well-known cancerogenic compounds.

3. Mycotoxin studies

In Hungary, *Fusarium* infections of agricultural plants and a cyclic recurrence of endemic outbreaks of fusariosis have accounted for a continuous presence of mycotoxins in the environment. During the last 10 years, presence of one or more mycotoxins has been demonstrated in 17 per cent of fodders on the average. In 1991, mycotoxins were detected in 56, 80 and 86 per cent of corn, wheat and compound feed samples, respectively. Recently, evidence has been presented of the frequent contamination of wheat by deoxynivalenol, which was detected in 54 per cent of the samples examined. In 1992, mycotoxin contamination was demonstrated in 77.6 and 88.9 per cent of wheat grain and compound feed samples, respectively.

So-called store-room moulds (*Aspergillus* or *Penicillium* species) that infect susceptible cereals under inadequate conditions of storage, have become known since the middle 'seventies. The intensity of such mould infections depends on the extent of storage failures.

Economic losses from mycotoxicoses amount to billions of dollars or other currencies in both plant and animal production, and associated with these losses is the adverse impact of mycotoxins on human health, of which relatively little is known for the time being.

III. Principal Damages of Fungal Toxins

1. Mycotoxic damages in plant production

In Hungary, various species of *Fusarium* fungi affect agricultural plants in different stages of development, causing thereby endemic outbreaks of fusariosis, which account for major economic losses. Decrease in the market value of the infected plants may amount to 10–30 per cent, depending on the intensity of infection and on the extent of the outbreak. Economic losses result not only from a reduced output, but also from an increase in grain contamination, as well as quality deterioration of sowing seeds and, last but not least, poorer bakery qualities.

The extent of damage caused by *Fusarium* fungi depends on the susceptibility and sensitivity of the cultivated wheat and corn species (hybrids), as well as on meteorological factors and on the impact of the applied agricultural techniques. Susceptibility plays a leading role in the case of infection by *Fusarium* fungi.

The moulds use up considerable amounts of plant nutrients (carbohydrates and proteins) for their growth. Tests performed to this end have indicated an up to 20 per cent decrease in the value of the starch equivalent of corn. The fungi seem to use up almost selectively those essential amino acids, which are most valuable

for animal nutrition. The performance of pigs and poultry fed on mould-infected feeding stuffs tends to decrease, owing to lack of the limiting amino acids and to deterioration of the biological value of protein components.

2. Mycotoxic damages in livestock production

Economic losses from mycotoxicoses affecting domestic birds and animals are consequent upon limited performance, reproductive disorders and impairment of functions of the immune system.

Limited performance is due primarily to the emetic and feed refusal effects of mycotoxins (mainly of the trichothecenes) as well as to inhibition of protein synthesis, disturbance of protein incorporation and a toxic injury of the gastrointestinal mucosa and of parenchymatous organs.

The degree of liver injury depends on the amount of toxin intake and the time of toxin ingestion. Susceptibility to mycotoxic damage may be increased by regular toxin intake.

Fusariotoxins produce the greatest losses by their adverse effects on reproductive events. Two main types of fusariotoxins have been distinguished by dissimilar mechanisms of action, such as (a) oestrogenic (F_2) toxins, above all zearalenone, and (b) trichothecene (T_2, HT_2) toxins, of which deoxynivalenol, diacetoxyscirpenol, nivalenol and Fusarenone-X have been most frequently encountered in Hungary.

Fusariotoxins may affect both male and female animals.

In males, zearalenone impairs spermatogenesis; all animal species and also humans are sensitive to it. The extent of disorders in spermatogenesis depends on the quantity of the toxin and on duration of its effect.

Females are susceptible to both oestrogenic and trichothecene toxins. Minor concentrations of zearalenone do not interfere with the ovarian function, but give rise to a persistent uterine oedema accompanied by marked cell proliferation. The consequent asynchronism of ovarian and uterine functions inhibits the implantation of the ovum and accounts thereby for an uterus-dependent infertility.

Toxin T-2 (and other trichothecenes) arrest the cyclic function of the ovary, prevent ovulation and cause a multiple cystic degeneration of the ovaries that accounts for ovary-dependent infertility. In avian females ingestion of trichothecenes blocks in turn follicle maturation and within 3–4 days also the rupture of the follicle, which ultimately results in a reduced egg production.

Last but not least, trichothecene toxins also affect the immune system.

IV. Food Hygienic and Public Health Aspects of Mycotoxicoses

Relatively little is known about mycotoxicoses in humans, although serious "epidemics", identified only in these days as mycotoxic conditions, had already been reported centuries ago.

Endemic occurrences of *Claviceps purpurea* infections (Claviceps mycotoxicosis; ergotism), with thousands of fatal cases, had not infrequently taken place in the Middle Ages.

Major food hygienic problems have generally been caused by aflatoxicosis. Aflatoxin M_1, that is eliminated in cow's milk, has been identified as a cancerogenic agent. Fortunately, since the temperate climate does not favour the conditions required for aflatoxin production and imported feeding stuffs are rigorously checked for such contamination, there is little if any risk of aflatoxicosis in this part of the world.

However, ochratoxicosis occasionally presents serious public health problems also in Hungary. Since several fungus species of the genera *Aspergillus* and *Penicillium* are producers of ochratoxin, sources of contamination are continuously present. Humans may become contaminated both directly (by ingestion of mould-infected food items of plant origin) and indirectly, by ingestion of animal products. The risk of ochratoxicosis is increased by the circumstance that the elimination of ochratoxin from the human organism takes several months, during which even micro-doses of ingested toxin may accumulate in the tissues.

Ochratoxin causes damage to the liver and the kidneys, and may develop teratogenic, mutagenic and, according to recent studies, probably also cancerogenic effects. A disease called "Balkan endemic nephropathy" has long been known among rural populations in Bulgaria, the former Yugoslavia and in Romania.

Hygienic measures intended to minimize the damages of porcine nephropathy have up to now been introduced in Denmark. Since, however, in Hungary pork has been a major item in the meat diet of the population, regulations to this end would be of immediate interest in this country, too.

With regard to the frequent presence of ochratoxin A in cereals and in the kidneys of pigs for slaughter, we analyzed 100 human blood samples obtained from the Municipal Hospital in Szolnok for such contamination. Fifty-two per cent of these random samples did in fact contain ochratoxin, at the average level of 0.92 ng/ml.

Susceptibility to mycotoxins has shown a close correlation with age. Newborn babies are most susceptible and the mycotoxin content of the maternal colostrum is a reliable indicator of the degree of infantile exposure. Among 96

randomly collected colostrum samples examined by us, 38 contained ochratoxin A at concentrations up to 7.63 ng/ml.

We demonstrated in earlier studies on pigs that zearalenone was able to pass across the placental barrier and to damage the fetus *in utero*. The changes caused by intrauterine toxin exposure may persist later in life. The toxin accumulated in the maternal tissues during gestation was rapidly eliminated in the colostrum after farrowing. This observation prompted us to examine human colostrum samples, to ascertain whether or not zearalenone exposure could occur during human pregnancy as well. Nineteen out of 68 samples collected in a provincial hospital (28%) contained either zearalenone or its alpha- or beta-metabolite. There is reason to postulate that in such cases zearalenone may damage not only the fetus, but also the ovaries of the mother. This problem deserves greater attention, also with regard to the recent poor population growth rates in Hungary.

Fusariotoxicosis caused by trichothecene toxins had been first reported by Woronin in 1891, who described mass incidence of a disease termed "staggering grains" among humans in Eastern Siberia. The disease was caused by consumption of mouldy cereals and was clinically characterized by dizziness, headache, shivering, salivation, vomiting and visual disturbances.

In Hungary, above all the food items prepared from mycotoxin-contaminated cereals may acquire a public health significance. Trichothecene (mainly T-2 and deoxynivalenol) contamination of the wheat samples examined by us not infrequently exceeded the risk level for humans.

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A MOUSE MONOCLONAL ANTIBODY REACTING WITH SWINE LEUKOCYTES: A FLOW CYTOMETRICAL AND IMMUNOHISTOCHEMICAL STUDY

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A mouse monoclonal antibody (Mab) was prepared by immunization of Balb/c mice with porcine peripheral blood mononuclear cells. Its reactivity was studied by flow cytometry and immunohistochemistry. By flow cytometry 75–90% of blood lymphocytes and 93–97% of blood granulocytes showed positivity, while all monocytes were labelled. The specificity of the Mab was investigated further by immunohistochemical methods using different tissues of swine. Both cortical and medullary thymocytes strongly expressed the target antigen of Mab 335-2. Intensive staining of T cell dependent areas in the lymph nodes, spleen and tonsils was also observed. Many positive cells occurred in the red pulp of the spleen. In the non-lymphoid tissues no organ-specific staining was observed, except for the connective tissue which contained scattered positive cells. Kupffer's cells and some glial cells reacted with this Mab. These results suggest that this novel Mab recognizes a major antigen present on immature and mature T cells; however, subpopulations of myeloid cells (neutrophils, monocytes, and some types of macrophages) share this antigen.

Key words: swine leukocytes, flow cytometry, cell surface antigens, immunohistochemistry

The development of monoclonal antibody production brought about rapid progress in the study of cell surface structures of the human and murine immune system. Much less is known about this issue in domestic animals, especially in swine (Lunney and Pescovitz, 1987). The development of such monoclonal antibodies may have importance not only for studies in veterinary science but it also might lead to a better understanding of immune functions in general.

Among the relatively few data available on the porcine immune system

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there are some unusual ones. It has long been known that porcine lymph nodes have an inverted structure (Hoshi et al., 1986; McFarlin and Binns, 1973) and that the efferent lymph is acellular (Binns, 1982; Binns et al., 1985). These data, together with the homing of a high proportion of g/d T cells to lymph nodes, have revealed a specific way of lymphocyte recirculation in swine (Saalmüller et al., 1990). Another interesting fact is that the majority of resting T cells of swine constitutively and strongly express MHC II antigens (Lunney and Pescovitz, 1987; Thistlethwaite et al., 1983), which points to the possibility of an additional way of antigen presentation and T cell activation.

The study of these differences is important because the pig is an experimental animal often used in human medicine, first of all in tissue (discordant xeno-) transplantation and in experimental surgery (Tumbleson, 1986; Platt and Bach, 1991).

All these facts prompted us to prepare monoclonal antibodies suitable for the characterization of porcine immunocompetent cells. One of our antibodies, Mab 335-2, showed a strong T cell reactivity combined with a reactivity with some myeloid cells, too. The flow cytometric reactivity and immunohistochemical staining pattern of Mab 335-2 are described in the present paper.

Materials and methods

Animals. Tissue specimens were collected from 6-month to 1-year-old, healthy slaughterhouse pigs of the Hungarian Large White breed.

Antibodies. The mouse monoclonal antibody 335-2 (IgG2a,κ) was prepared by immunization of Balb/c mice with porcine peripheral blood mononuclear cells and selected on the basis of its cytotoxic activity (Magyar et al., manuscript in preparation). Mab 74-22-15 (swine monocyte/macrophage/granulocyte specific mouse IgG1,κ; Pescovitz et al., 1984) was a kind gift from Dr. Armin Saalmüller. Swine IgM (μ-chain) specific Mab 2B5-13 (IgG1,κ) was prepared and characterized in our laboratory. Z 28 (influenza haemagglutinin specific mouse IgG2a,κ, kindly provided by Dr. Éva Rajnavölgyi) was used as irrelevant Mab. Biotin-conjugated rabbit anti-mouse IgG and ABC-kit were obtained from Vector Laboratories.

Flow cytometry. Swine leukocytes were separated from heparinized blood on Ficoll gradient (density: 1.078 g/cm^3) or by dextran sedimentation (MW: 500,000; 5% solution in phosphate-buffered saline (PBS), pH 7.4). Erythrocytes were lysed with ammonium chloride solution (0.17 M ammonium chloride solution, buffered with 0.16 M Tris, pH 7.2). The yield of living leukocytes was higher than 92% in all cases. The separated mononuclear cells were suspended in

PBS containing 1% bovine serum albumin (BSA). For indirect staining, 10^6 cells were spun down in microcentrifuge tubes, and 100 ml titrated supernatant of the monoclonal antibodies was added to the pellet. After 30-min incubation on ice the cells were washed with PBS and 100 ml of FITC-labelled secondary antibody (FITC-labelled rabbit anti-mouse Ig, rabbit $F(ab)'_2$, DAKO) was added to them. The cells were incubated on ice for 30 min in the dark, then washed with PBS. Flow cytometry was performed on FacStar (Becton-Dickinson) or Cytoron (Ortho).

Immunohistochemical staining of the tissues. Six to eight μm thick cryostat sections of porcine thymus, submandibular and mesenteric lymph node, pharyngeal tonsil, spleen, liver, stomach, intestines, salivary gland, pancreas, thyroid gland, skin, spinal cord, urinary bladder, skeletal and heart muscle, kidney and lungs were mounted on poly-L-lysine (Sigma) coated slides and immediately fixed with acetone for 1 min. The slides were stored at room temperature for two weeks or at –20 °C for months. Before staining the slides were rehydrated in PBS for 10 min. Endogenous peroxidase (ENPO) activity was developed prior to immunohistochemical staining with 0.5 mg/ml DAB (SIGMA) in PBS containing 0.03% H_2O_2. Developing ENPO activity before immunohistochemical staining is the mildest procedure in preserving antigenic activity; therefore we chose this method instead of blocking ENPO activity with H_2O_2 (Oláh and Glick, 1992). The slides were washed three times in PBS, incubated with hybridoma supernatants containing Mabs 335-2, 2B5-13 or 74-22-15 for 30 min, repeatedly washed three times in PBS, incubated with the biotin-labelled second antibody (diluted 1:200) for 30 min, washed again three times in PBS and covered with the freshly prepared ABC (preformed avidin–biotin complex) for 30 min. After repeated washing in PBS we used 0.25 mg/ml 4-chloro-1-naphtol (SIGMA) in PBS containing 0.03% H_2O_2, for visualization, for 10–15 min. Using the two chromogens, the nonspecific ENPO activity (brown staining of 3,3'-diaminobenzidine (DAB), mainly cytoplasmic) could be clearly differentiated from the membrane staining of Mabs (blue colour of 4-chloronaphtol). After rinsing in PBS the slides were mounted in glycerine-gelatine (DAKO). Photographs were taken with Olympus BH-2 microscope and NP 15 films (ORWO).

Results

Flow cytometric studies

The forward and side scatter plot of swine blood leukocytes is shown in Fig. 1. Normally the populations of lymphocytes and monocytes weakly overlap.

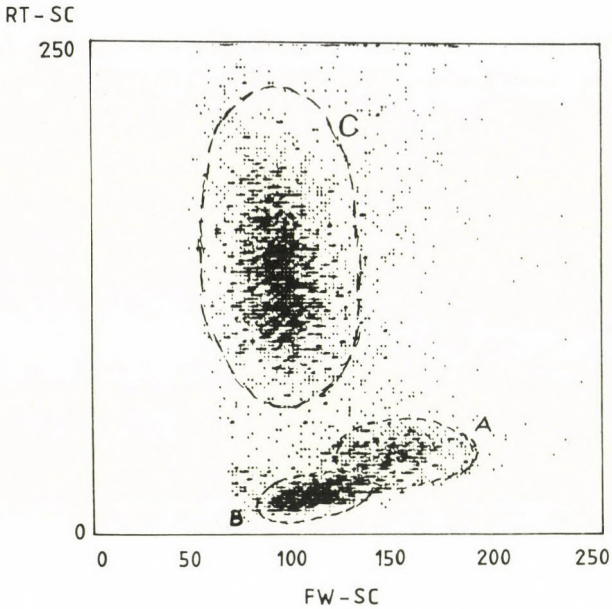


Fig. 1. Dot plot of normal swine blood leukocytes (FW-SC: forward scatter, RT-SC: side scatter). Population A refers to monocytes, B to lymphocytes and C to granulocytes. Erythrocytes and platelets are gated out (left, lower corner). The monocyte and lymphocyte populations are in general weakly overlapping and this reflects most probably the activation status of lymphocytes. For analysis we chose blood samples with minimal overlapping

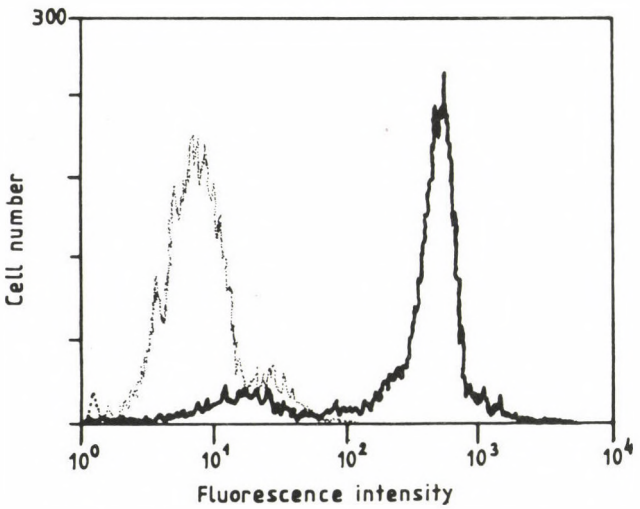


Fig. 2. Reactivity of blood lymphocytes with Mab 335-2 (solid line) or with irrelevant mab Z 28 (dotted line)

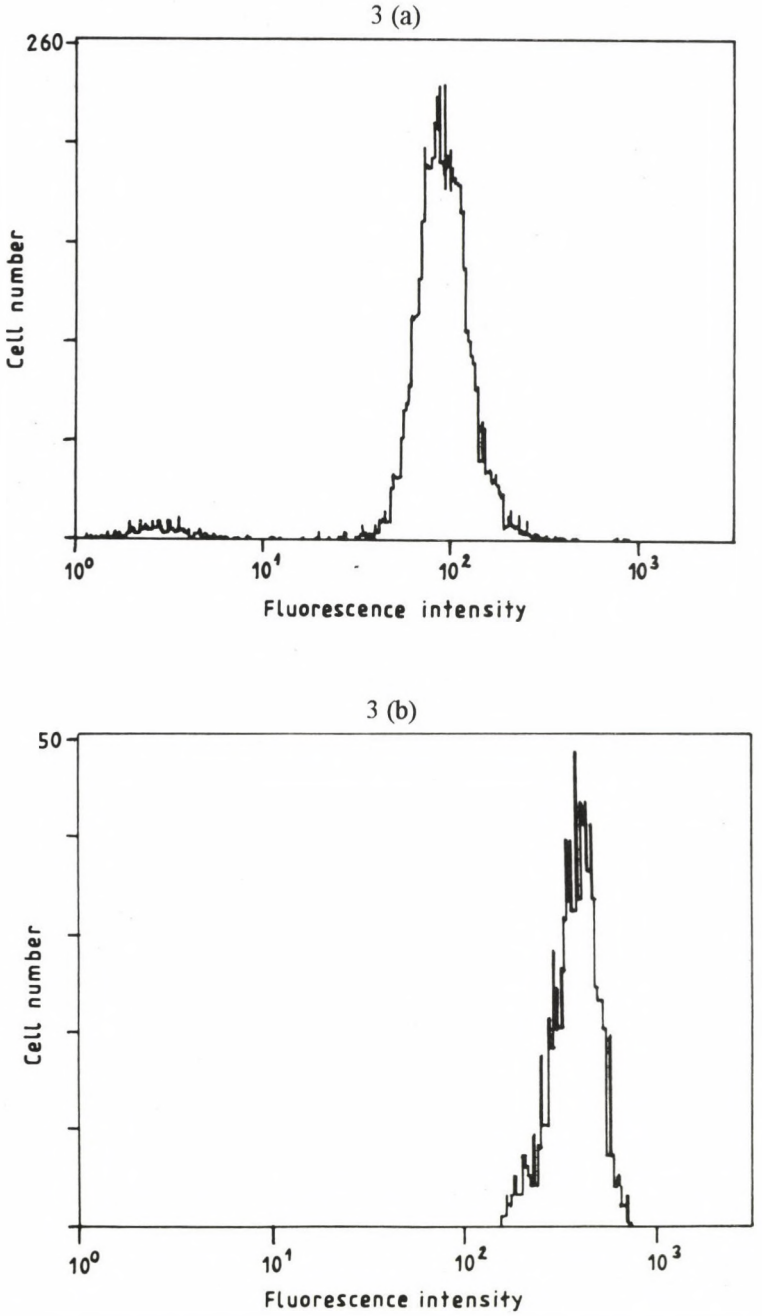


Fig. 3. Reactivity of blood myeloid cells with Mab 335-2: (a) granulocytes, (b) monocytes

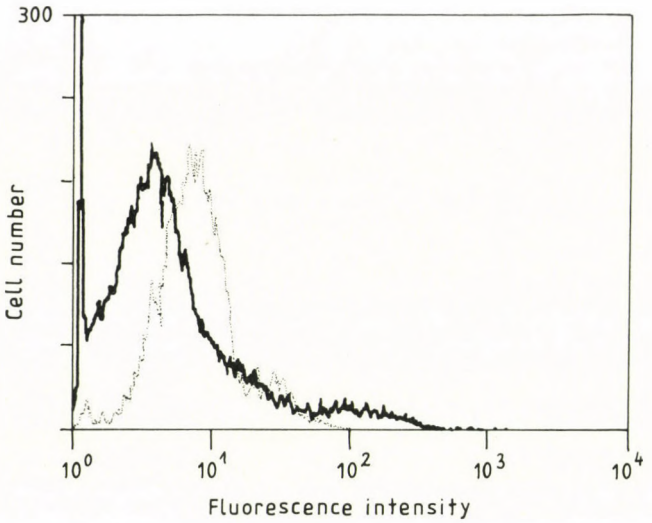
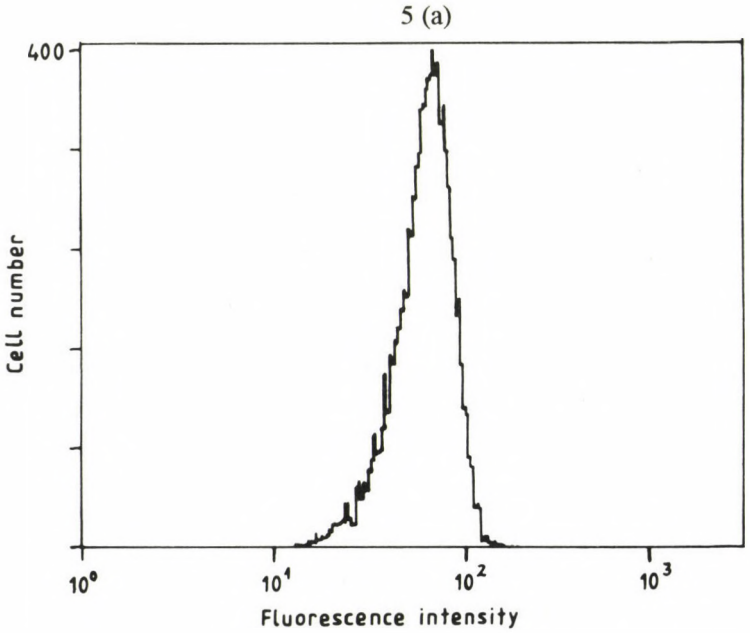


Fig. 4. Histogram of blood lymphocytes with anti-swine IgM Mab, 2B5-13 (solid line) or irrelevant Mab, Z 28 (dotted line)



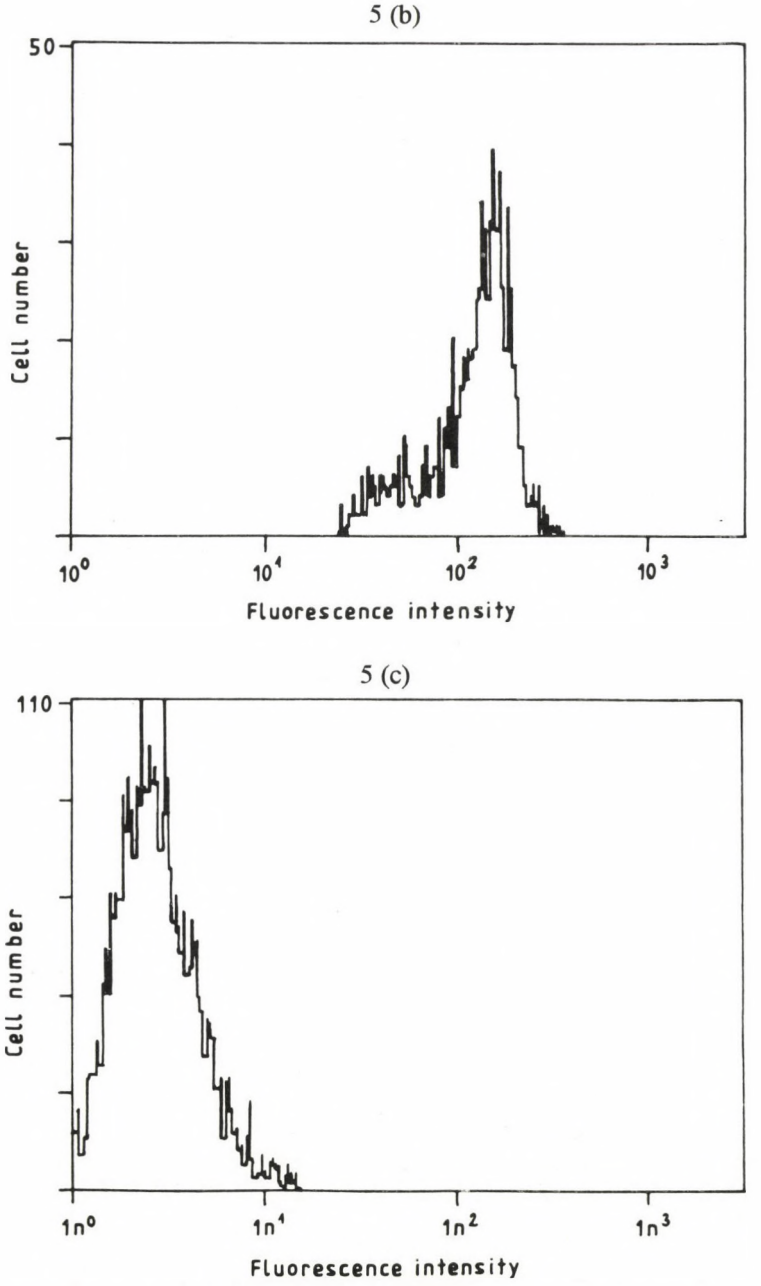


Fig. 5. Reactivity of leukocytes with Mab 74-22-15: (a) granulocytes, (b) monocytes, (c) lymphocytes

Staining with Mab 335-2 (n=20) resulted in 75–90% positive cells among lymphocytes (Fig. 2), 93–97% among granulocytes (Fig. 3a), while all monocytes were positive (Fig. 3b). The irrelevant Mab Z 28 did not give considerable reactions. The swine IgM-specific 2B5-13 Mab reacted with 10–20% of blood lymphocytes (Fig. 4). Some positivity could be observed among myeloid cells, probably due to Igs bound to the Fc-receptor *in vivo*. Mab 74-22-15 reacted with all the granulocytes and monocytes (Fig. 5a and 5b, respectively), but not with other blood cell types (Fig. 5c).

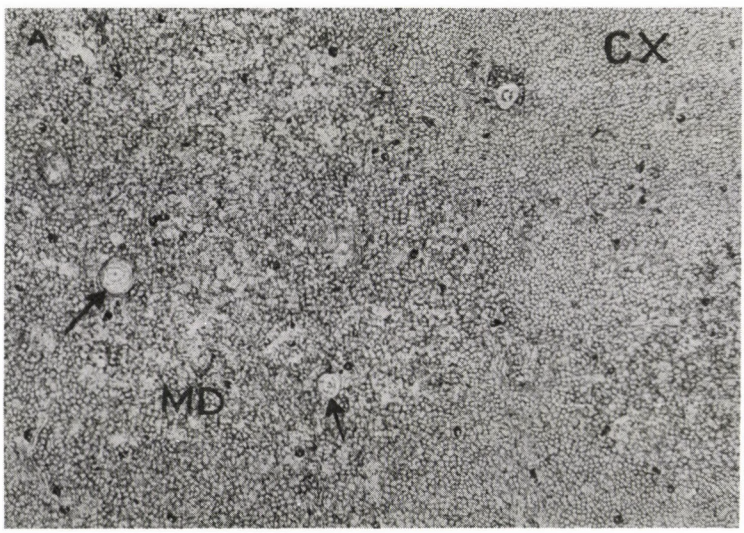
Immunohistochemical reactivity of Mab 335-2

Lymphoid organs. Mab 335-2 stained all the cortical and most of the medullary thymocytes. Hassal's corpuscles were negative (Fig. 6a).

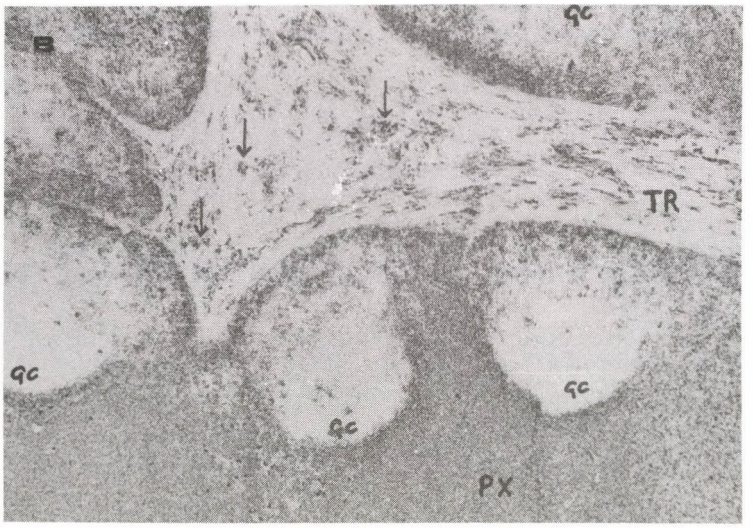
The T-dependent areas of the peripheral lymphoid organs, i.e. the paracortex (Fig. 6b) and the periarteriolar lymphoid sheath (PALS, Fig. 6c) were positive for Mab 335-2. The majority of germinal center (GC) cells do not express this antigen but scattered 335-2-positive cells may occur in the GC (Fig. 7).

In the lymph nodes, the dense lymphoreticular tissue located in close proximity to the subcapsular sinus was free of 335-2 positive cells (not shown). The splenic marginal zone surrounding the PALS revealed mainly positive cells. Positive cells were scattered throughout the red pulp. Ellipsoids were negative (Fig. 6c).

6 (a)



6 (b)



6 (c)

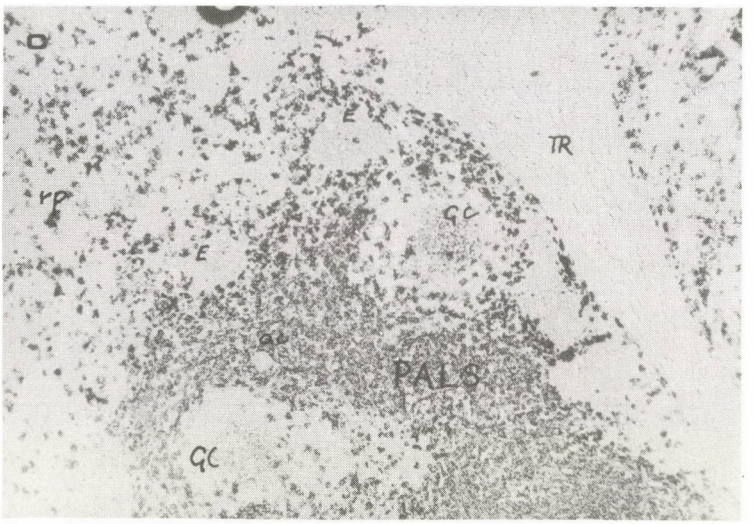


Fig. 6. Immunohistochemical staining of swine thymus (a), submandibular lymph node (b) and spleen (c) with Mab 335-2.
(a) CX: cortex, MD: medulla, Hassal's corpuscle (arrow).(Magnification: × 100)
(b) PX: paracortex, GC: germinal center,TR: trabecula, eosinophils (arrow). (× 40)
(c) PALS: periarteriolar lymphoid sheath, ac: arteria centralis, GC: germinal center, TR: trabecula, E: ellipsoid, rp: red pulp. The mild positivity in GCs is due to cross-reaction of secondary antibody with swine Igs (× 100)

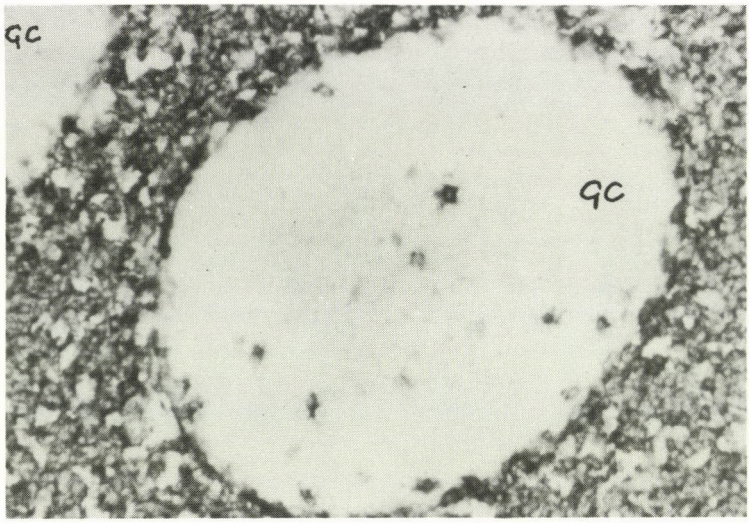


Fig. 7. Higher magnification of 335-2 stained lymph node showing a lymphoid follicle with germinal center. The latter contains a few positive cells scattered in it (× 200)

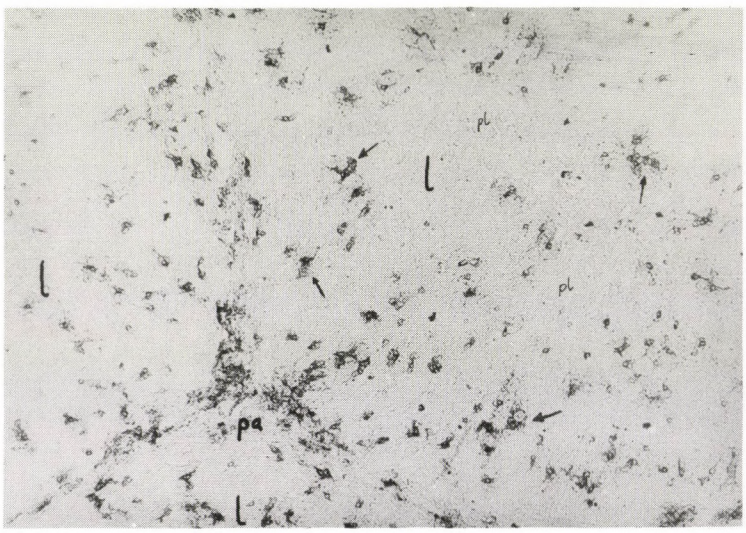


Fig. 8. Mab 335-2 stains Kupffer's cells in the liver. l: hepatic lobule, v: central venule, pl: plates of hepatic cells, pa: portal area, Kupffer's cells (arrow). (× 100)

Non-lymphoid organs. Organ-specific cells of the non-lymphoid tissues were negative. Kupffer's cells (Fig. 8) and some glial cells expressed this antigen, and the connective tissue of all organs contained some positive cells.

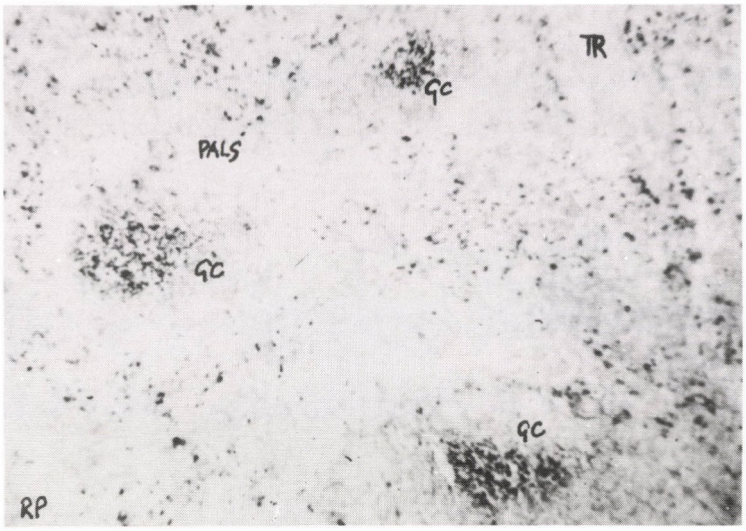


Fig. 9. Detection of IgM-positive cells in the spleen with Mab 2B5-13. This cryostat section is the consecutive one to that shown in Fig. 6c. Abbreviations: see Fig.6c (× 40)

Comparative studies

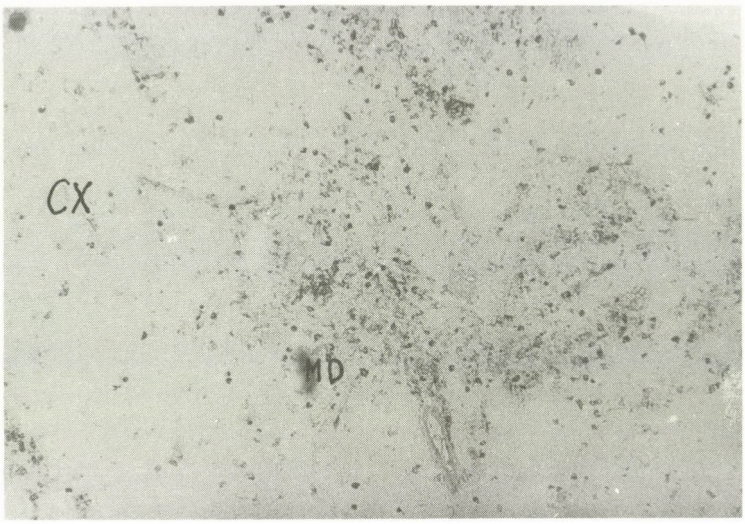
Using the Mab 2B5-13 to detect IgM-expressing B lymphocytes and plasma cells we could confirm on serial sections that the 335-2 negative areas of lymphoid organs are indeed GCs or other B-dependent areas (Fig. 9).

The anti-monocyte/macrophage/granulocyte Mab 74-22-15 does not react with lymphoid cells. It stains few cells with processes in the thymic cortex, while the thymic medulla shows an extensive reticular network with few, strongly positive cell bodies (Fig. 10a). Elongated cells of the subcapsular area and trabeculae are positive only in the lymph nodes (not shown). In the spleen, the white pulp with the marginal zone does not stain. The red pulp contains strongly positive cells that form a network with their processes. The ellipsoids are strongly positive (Fig. 10b). The Kupffer's cells do not react with Mab 74-22-15.

Discussion

Our results indicate an interesting binding specificity of Mab 335-2. This monoclonal antibody reacts only with swine lymphoid and myeloid cells and does not show cross reactivity with immunocompetent cells of human or murine origin (data not shown). The positive lymphocytes are T cells. Both cortical and me-

10 (a)



10 (b)

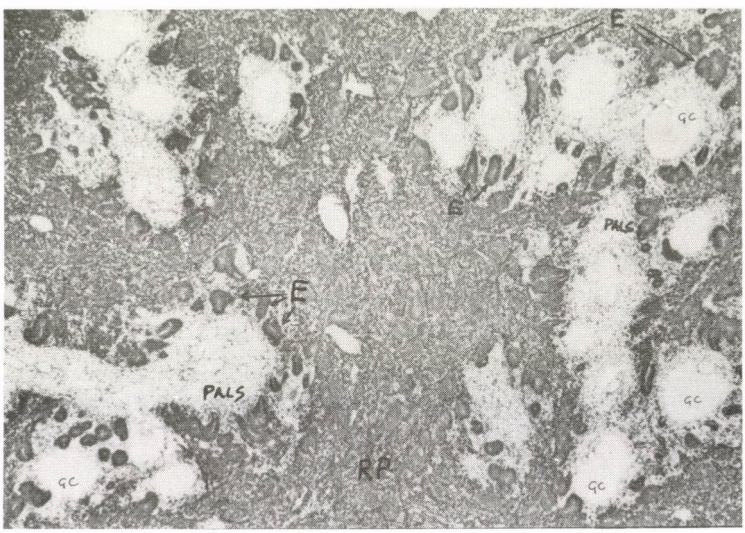


Fig. 10. Immunohistochemical reactivity of Mab 74-22-15 on swine thymus (a, × 100) and spleen (b, × 40). Abbreviations: see Fig. 6a and 6c, respectively

dullary thymocytes react with Mab 335-2, proving that it could recognize immature and mature T cells as well. The reactivity with mature T cells is further supported by the facts that T-dependent areas in all peripheral lymphoid organs show strong positive staining and the majority of blood lymphocytes are positive. The Mab seems to recognize both CD4+ and CD8+ cells, since all the cells of T-dependent areas are positive. The staining intensity, as demonstrated by the results of immunohistochemical and flow cytometric analysis, is strong and invariable on thymocytes and T cells, i.e. Mab 335-2 detects a major cell surface antigen whose expression does not depend on the differentiation or functional status of the cell (the latter is supported by the fact that the expression of the target antigen of Mab 335-2 does not change upon activation of T cells with ConA, data not shown). Germinal center cells do not react with this Mab, and other B-dependent areas (e.g. medullary cords) contain mainly negative cells, showing that B cells are negative. The few positive cells scattered in GCs most probably represent T_h cells (Rouse et al., 1982).

According to the results of our flow cytometric experiments, a significant number of blood granulocytes and monocytes carries the target antigen. About 93–97% of blood granulocytes are positive with Mab 335-2 and the remaining 3–7% negative cells are eosinophils (Magyar et al., manuscript in preparation) that have strong endogenous peroxidase activity. The monocytes are all positive, but the reactivity of macrophages is not so unequivocal. Using our Mab we observed positive staining of Kupffer's cells and some glial cells, but the former do not react with the swine monocyte/macrophage/granulocyte specific Mab 74-22-15. The connective tissue of all the tested organs contains positive cells which are most probably recirculating T cells, although the staining of tissue macrophages cannot be excluded either. For the lack of well-characterized Mabs the positive cells in the red pulp of the spleen could not identified.

Summarizing our results, we developed a Mab that reacts with both the CD4+ and CD8+ populations of swine T cells and their precursors, as well as with some cells of myeloid origin (monocytes and some granulocytes and macrophages). The unique distribution of the target antigen resembles that of CD43 (leukosialin, Borche et al., 1987) or one of the low molecular weight forms of CD45 recognized by UCHL-1 monoclonal antibody in man (Akbar et al., 1988; Thomas and Lefrancois, 1988). However, based on the results of biochemical and functional studies of the cell surface structure recognized by this Mab 335-2 (Magyar et al., manuscript in preparation) it seems unlikely that it is the swine analogue of CD43 or CD45RO. Consequently, the CD characterization of the 335-2 target antigen remains to be elucidated. Otherwise, this pattern of reactivity is similar to that of Mabs 8/1 (Saalmüller et al., 1987) and 76-6-7 (Pescovitz et al., 1984) that detect the swine differentiation marker swC1a (Lunney, 1993).

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INFLUENCE OF MANAGEMENT TECHNOLOGY AND PARTURITION ON ANTIBODY LEVELS IN COWS WITH BOVINE LEUKOSIS

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Sera from 97 Holstein-Friesian cows kept in isolation (herd I) were tested on 9 occasions with an interval of 90 days. The cows had previously given a positive reaction with bovine leukaemia virus antigen in the agar gel immunodiffusion (AGID) test. During the period of loose housing (at tests 1–3) 0–3 cows per test (0–3.1%) gave a negative reaction. When the same animals were kept in the tie-in system (at tests 4–7) the number of negative reactors varied between 9 and 26 (16.9–20.1%). Twenty-eight cows of herd II were tested serologically by the AGID test on a total of 9 occasions from day 60 before term up to postpartum day 90. The lowest antibody level was obtained at calving, when 7 cows gave a negative result in the AGID test. In three cows the decline of antibody level was so pronounced that their serum was negative even after postpartum day 60. One cow was negative even at the end of the test period (on postpartum day 90). The variation in serum antibody level was demonstrable also by ELISA; however, by that test none of the animals gave a negative result. The antibody level demonstrable in the milk reached its peak at calving and then it underwent a gradual decline; however, it did not drop below the detectability limit by the end of the test period. It is concluded that in bovine leukosis infected herds the performance of the AGID test is extremely impaired by the use of the tie-in housing system, as well as by a combined effect of tying in and parturition. Therefore, during the leukosis eradication and qualification of herds kept in a tie-in system, ELISA is the method that can be used, instead of AGID, with satisfactory efficacy even for the testing of sera.

Key words: Bovine leukosis, antibody level, blood serum, milk, housing system, parturition, serological tests

In the past ten years, the agar gel immunodiffusion (AGID) test and enzyme-linked immunosorbent assay (ELISA) have been used widely for the detection of bovine leukosis infection. The results of AGID tests show a considerable variation in virus-induced antibody titres in cattle infected with bovine leukaemia virus (Kono et al., 1982). In some cases that fluctuation caused the test to give a negative result. In other studies, the drop of antibody level related to calving also resulted in a transient negativity of some infected animals (Bause et al., 1978;

Bause and Schmidt, 1980; Burridge et al., 1982). Taking into consideration these statements, AGID is still an accepted test regarded as a standard method both in the eradication of bovine leukosis from cattle herds and in tests performed in connection with animal exports (Hoff-Jørgensen, 1989; Johnson and Kaneene, 1992).

Prompted by the fact that the results obtained by AGID testing of large herds during eradication procedures could not be explained by our earlier findings (Tekes, 1985; Tekes, 1986; Tekes, 1989), in this study I aimed at elucidating the following questions: (*i*) To what extent does the type of housing affect the antibody levels? (*ii*) How long does the periparturient decline of antibody level last in connection with the housing system? (*iii*) How much does the periodic antibody drop affect the results of ELISA, a method which is more sensitive than the AGID test and is suitable for the testing of not only blood but also milk samples (Behrens et al., 1979; Manz et al., 1981; Bauer et al., 1984; Mammerickx et al., 1985)?

Materials and methods

Herds

Herd I consisted of 97 Holstein-Friesian Red cows aged 2–8 years at the start of the study and kept in isolation in a loose housing system. At the first testing during leukosis eradication the herd had reacted positively in the AGID test. During the first three tests the cows were kept in the same barn under loose housing conditions. They were fed from troughs placed near the fence of pens and received drinking water from valve-type self-waterers located along the barn wall at 2 m distance from each other. The tests were performed at 90-day intervals, simultaneously with other herds of the farm. Blood serum samples taken from the animals were tested by AGID.

Before the 4th test, the herd was transferred to two cow-houses of another farm, where they were kept in the tie-in system together with 32 animals that had been kept isolated at other units of the farm and proved positive for bovine leukosis. With the decrease in the number of infected animals, from the 5th test the animals were kept in a single house. They were fed from a feeding alley running in the midline of the barn. Drinking water was provided from valve-type self-waterers fitted onto the side of the feeding troughs. Straw bedding was available in all barns: in loose housing it served as deep litter while in the tie-in system it was provided periodically, as needed. The number of cows in the herd changed because some animals were culled and slaughtered after the tests.

Herd II consisted of 100 Holstein-Friesian cows (age: 2–10 years) that had proved positive by the AGID test during the eradication of bovine leukosis from a large herd kept under loose housing conditions. These animals were accommo-

dated in a separate barn in the tie-in system. Their management and feeding were the same as in herd I.

In that isolated herd comprising 100 positive animals, the blood tests were carried out by AGID and ELISA, whereas the milk samples were tested by ELISA.

Blood samples were taken at 60 and 30 days before term, at calving, and 7, 14, 21, 28, 60 and 90 days after calving. Milk samples were obtained at the same times except prepartum day 30. During the one-year period of study, all the above samples could be obtained only from 28 of the 100 cows; therefore, only these data are evaluated in this paper.

Tests

AGID test. From the sera, a doubling dilution series extending from 1:2 to 1:32 was made in saline. The sera were tested with a Hungarian-made antigen (Harrach et al., 1984). A Hungarian reference serum obtained from an infected animal, corresponding to the European type 4 standard serum, was used as positive serum. The test was performed as described earlier (Tekes, 1985).

ELISA. The antigen was prepared from the supernatant of a fetal lamb kidney cell culture permanently infected with bovine leukaemia virus (Institut für Virologie der Tierärztlichen Hochschule, Hannover). The virus was propagated according to Ressang et al. (1981) and purified according to Todd et al. (1980). A non-infected fetal lamb kidney cell culture served as the source of control antigen production. The control antigen was prepared in the same way as the test antigen.

From the serum samples a doubling dilution series extending from 1:40 to 1:2,560, while from the milk samples a doubling dilution series extending from 1:2 to 1:256 was prepared with the solution used also for washing. A positive serum derived from an infected cow, corresponding to the European type 4 standard serum, was used as positive standard serum in the test.

The starting dilution applied in the system was 1:40 for the blood serum and 1:2 for the milk samples. For the blood tests, the positive control serum was diluted 1:400 in diluent (1:10 × 40), while for the milk tests it was diluted 1:500 with negative milk (1:10 × 25 × 2).

The test was carried out as described previously (Tekes et al., 1987), with the difference that 4 mM tetramethylene-benzidine (TMB, Serva) dissolved in acetate buffer of pH 5.5 was used as substrate.

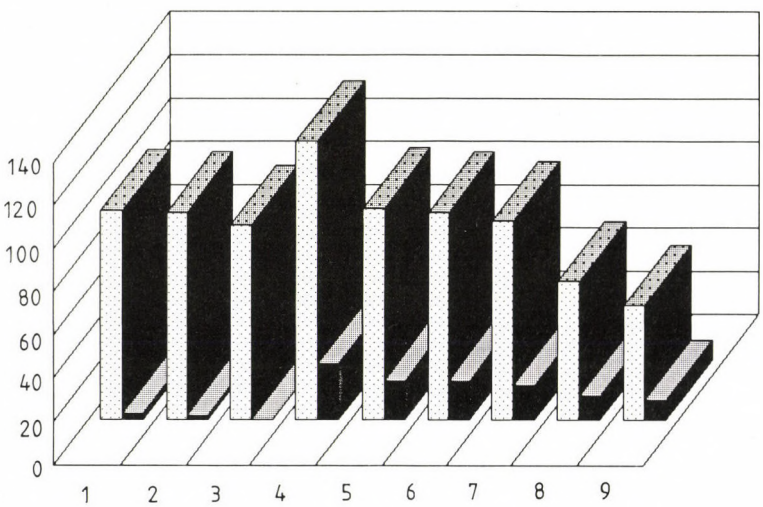
The obtained colour changes were read by a Titertek Multiscan R Plus photometer connected with a computer, and evaluated with the help of the following evaluating programme. Those blood sera were considered positive whose net optical density (OD) value ($OD_{antigen}-OD_{control\ antigen}$) value reached or exceeded the corresponding values obtained for the 1:10 dilution of the E4 standard. Milk samples were considered positive if their net OD ($OD_{antigen}-OD_{control\ anti-}$

gen) value reached or exceeded the corresponding values of the 1:250 dilution of the E4 standard. In all other cases the samples were regarded as negative.

The antibody titre of the blood serum or milk was expressed as the reciprocal of the dilution which still gave a positive result. In Table 2, only a + sign denotes the results of sera giving a positive reaction in the undiluted state by the AGID test, in the 1:40 dilution by the blood ELISA and in the 1:2 dilution by the milk ELISA.

Results

The results obtained by the AGID test in herd I kept first under loose housing conditions and then in the tie-in system are shown in Figs 1 and 2. Figure 1 illustrates the number of all animals tested and that of animals giving a negative reaction at the individual tests, while Fig. 2 shows the ratio of negative animals to the total number of animals, expressed in per cent. Table 1 shows the number of animals that gave a positive or a negative reaction by the AGID test in the periparturient period in herd II, for the 28 animals evaluated. Table 2 presents the blood serum and milk antibody titres of the 7 animals that were negative by AGID at calving time, for all the tests performed.



Total	97	96	90	129	98	96	92	64	53
Negative	3	2	0	26	18	18	16	11	9

Fig. 1. AGID testing of bovine leukaemia virus (BLV) infected animals under loose housing conditions (tests 1–3) and in the tie-in system (tests 4–9)

Table 1

Number of positive and negative cows during the test period (herd II)

AGID	Prepartum		Calving	Postpartum					
	day 60	day 30		day 7	day 14	day 21	day 28	day 60	day 90
	Number of animals								
Positive	28	24	21	22	22	22	23	25	27
Negative	0	4	7	6	6	6	5	3	1

Table 2

Peripartal variations in BLV antibody levels of cows that gave a negative reaction by AGID at calving (herd II)

Animal no.	Test	Prepartum		Calving	Postpartum					
		day 60	day 30		day 7	day 14	day 21	day 28	day 60	day 90
2379	Blood AGID	+	–	–	–	–	–	+	+	+
	Blood ELISA	+	+	+	+	+	+	+	+	80
	Milk ELISA	32*	nd	32	4	4	+	+	+	+
1036	Blood AGID	2	+	–	+	+	+	+	+	+
	Blood ELISA	80	+	+	80	80	80	80	+	80
	Milk ELISA	8	nd	16	+	+	+	+	+	+
2140	Blood AGID	2	+	–	–	–	–	–	–	–
	Blood ELISA	80	80	+	+	+	+	+	+	+
	Milk ELISA	4	nd	16	+	+	+	+	+	+
1929	Blood AGID	+	–	–	–	–	–	–	–	+
	Blood ELISA	80	+	+	+	+	+	+	160	320
	Milk ELISA	16	nd	128	8	2	+	+	4	2

Table 2 continued

Animal no.	Test	Prepartum		Calving	Postpartum					
		day 60	day 30		day 7	day 14	day 21	day 28	day 60	day 90
1432	Blood AGID	+	+	–	–	–	–	–	4	4
	Blood ELISA	+	+	+	+	+	+	+	+	80
	Milk ELISA	4	nd	8	+	+	+	+	4	+
2235	Blood AGID	+	–	–	–	–	–	–	2	2
	Blood ELISA	80	+	+	+	+	+	+	80	320
	Milk ELISA	8	nd	64	16	4	4	4	4	2
2363	Blood AGID	+	–	–	–	–	–	–	–	+
	Blood ELISA	80	+	+	+	+	+	+	+	80
	Milk ELISA	16	nd	32	+	+	+	+	+	+

+ = positive reaction in the basic dilution of the test (AGID: undiluted serum; blood ELISA: 1:40; milk ELISA: 1:2);
– = negative reaction in the basic dilution of the test; nd = not determined; * = reciprocals of the dilution applied

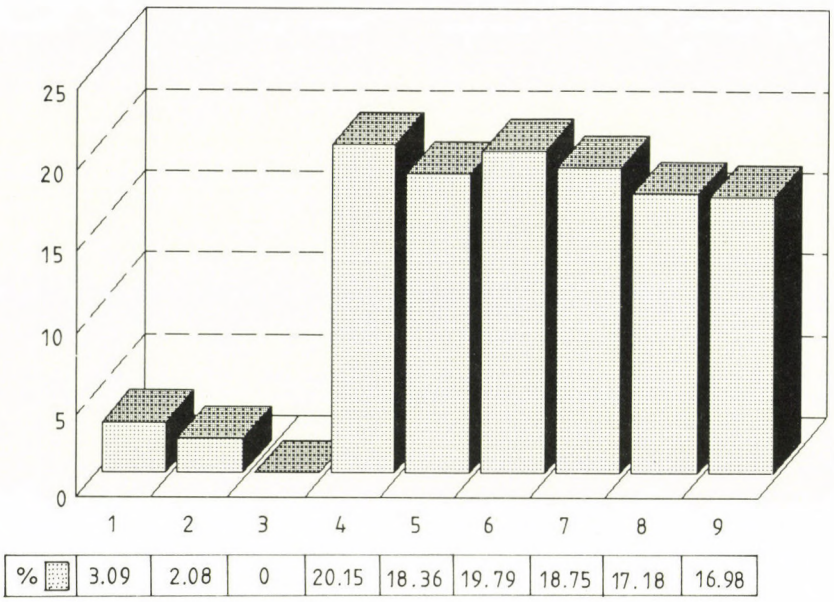


Fig. 2. Ratio of AGID-negative animals among BLV infected cows kept under loose housing conditions (tests 1–3) and in the tie-in system (tests 4–9)

Discussion

The reduction of serum antibody titres and the substantial increase of antibody levels in the colostrum have been reported by several authors. It has been established (Larson, 1958; Dixon et al., 1961; Williams and Millar, 1979) that this phenomenon is due to a transformation of serum antibodies to milk antibodies, as the drop of serum antibody titres is quantitatively identical with the antibody level rise found in the colostrum.

In some cases, by the time of calving the titre of antibodies to bovine leukaemia virus dropped below the detectability limit of the AGID test (Bause et al., 1978; Bause and Schmidt, 1980; Burridge et al., 1982; Popov, 1986; Ebertus et al., 1987). In a three-year period of observation of clinically symptomless animals, Kono et al. (1982) observed fourfold fluctuations in bovine leukaemia virus (BLV) antibody titres. In some cases, in animals with low antibody titres the AGID test even became negative. During earlier eradication attempts carried out in large herds we observed that, on the basis of the AGID test, bovine leukosis free status could be achieved seemingly earlier in herds kept under loose housing

conditions (by the 4th or 5th test) than in herds of the same size but kept in a tie-in system (by the 7th or 8th test; Tekes, unpublished data).

The 24-month long investigations conducted in herd I revealed that in the case of tie-in housing the ratio of negative reactions in the AGID test increased to 16.9–20.1% from the 0–3.1% ratio found in the loose housing system. This phenomenon may be attributed to the reduction of stress and the consequent resting period of the immunobiological processes. Stock and Ferrer (1972), as well as Wyatt et al. (1989) demonstrated that lymphocyte stimulation was necessary for the virus included in the lymphocytes to appear on the cell surface, as well as for its propagation. As a result of the "state of rest" of the immune system arising from the stress reduction due to loose housing, the virus is present in the lymphocytes in non-productive state and does not constitute a permanent antigenic stimulus for the organism which thus fails to produce antibodies against it. Because of the natural excretion of the circulating antibodies the animals may temporarily become negative.

At the same time, the question arises what influence the tie-in housing system exerts on the degree of the well-known periparturient antibody titre decrease and on the duration of negativity.

Authors studying the periparturient antibody titre drop found the ratio of animals becoming negative in the AGID test to be between 3.7% (Burridge et al., 1982) and 20% (Ebertus et al., 1987). However, all authors agreed in that the postpartal serum antibody rise is so considerable that by postpartum week 4 at the latest the antibody titre reaches the highest prepartal value and is easily detectable by the AGID test, too. In this study, we found that 25% of the animals became negative in the AGID test at the time of calving. Five animals were negative even on postpartum day 28, three animals on day 60, and 1 even on day 90, i.e. at the end of the period of observation (Table 1). The tendency of antibody titre fluctuation in the serum and milk samples was consistent with what had been described by other authors (Larson, 1958; Dixon et al., 1961; Williams and Millar, 1979; Burridge et al., 1982; Beier et al., 1985; Ebertus et al., 1987), i.e. the antibody titre of the blood serum decreased up to calving and afterwards it increased again, while in the milk it was the highest in the colostrum and declined very rapidly in the days after calving. The blood and milk antibody titres of animals becoming negative at calving (Table 2) also show that those animals became negative at calving which had a low antibody level also at the time of drying off.

In agreement with the findings of other authors (Behrens et al., 1979; Manz et al., 1981; Bauer et al., 1984), the results of this study show that the ELISA is more sensitive than the AGID test, as it gave a positive reaction for both the serum and the milk samples throughout, despite the antibody fluctuations. These findings explain why infected animals were recognized later in herds kept in the

tie-in housing system, and furnish an explanation also for the unexpected occurrence of positive reactions during the repeated testing of herds declared leukosis-free on the basis of the AGID test.

Based upon the above facts, instead of the AGID test ELISA, a method of satisfactory sensitivity, should be used in the future for the qualification of cattle herds and during the eradication of bovine leukosis.

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DETECTION OF *Mycoplasma gallisepticum* ANTIBODIES IN TURKEY BLOOD SAMPLES BY ELISA AND BY THE SLIDE AGGLUTINATION AND HAEMAGGLUTINATION INHIBITION TESTS

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Comparative examination of a total of 1,030 blood samples from six turkey flocks of three Eastern Hungarian turkey farms was performed by the conventional haemagglutination inhibition (HI) and slide agglutination (SA) tests and by a competitive ELISA visualizing the inhibition by a positive test serum of the reaction between a monoclonal antibody and the specific epitope of *Mycoplasma gallisepticum* recognized by it. All the three tests detected the flocks which were certainly infected. The highest rate of positivity (93% of the samples tested) was revealed by the ELISA. By SA and HI the positivity rate was 56% and 55%, respectively. Thirty-five per cent of the positive blood samples reacted in all three tests, 17% of them only by ELISA and HI, another 17% only by ELISA and SA, while 3% only by SA and HI. In the case of positive flocks first the SA test and ELISA, then the HI test and ELISA give parallel results.

Key words: *Mycoplasma gallisepticum*, antibody, turkey, detection, serology, ELISA, slide agglutination, haemagglutination inhibition

Because of the difficulty of isolating the pathogen, serologic methods play an important role in the diagnosis of poultry mycoplasmoses. Efforts to reduce losses from mycoplasmoses by keeping disease-free grandparent and parent flocks call for the use of sensitive, specific and rapid serologic tests.

The still widely used slide agglutination (SA) test is a simple, sensitive and rapid procedure; however, it is not specific enough and cross-reactions are common (Avakian et al., 1988).

The haemagglutination inhibition (HI) test is specific but less sensitive than SA, and requires higher technical skills and more sophisticated laboratory conditions.

Besides using the conventional serologic methods, in recent years several ELISA tests have also been elaborated (Ansari et al., 1983; Opitz et al., 1983; Patten et al., 1984; Opitz and Cyr, 1985; Talkington et al., 1985; Higgins and Whithear, 1986) for the detection of *Mycoplasma gallisepticum* specific antibod-

ies. The indirect ELISA tests described are sensitive enough but have proved to be of insufficient specificity. High background reactions and frequent cross-reactions were observed when testing sera from poultry infected by the antigenically related *M. synoviae* (Avakian and Kleven, 1990; Bradley et al., 1988; Higgins and Whithear, 1986). The test can be rendered more specific only by the use of adequately purified antigen or by treating the test sera with immunoadsorbents (Higgins and Whithear, 1986; Stipkovits et al., 1993).

The objective of this work was to compare the results of a competitive ELISA using *M. gallisepticum* monoclonal antibody (Czifra et al., 1993*a*) with those of the SA and HI tests on turkey sera.

Materials and methods

Test sera

The samples were obtained from six turkey flocks on three farms of Eastern Hungary, from turkeys of different age.

Methods

Slide agglutination (SA) test. The *M. gallisepticum* antigen nobilis, *M. synoviae* antigen nobilis, and *M. meleagridis* antigen nobilis (Intervet International B.V.) antigens were used.

Haemagglutination inhibition (HI) test. Fresh 16- to 24-hour culture of *M. gallisepticum* strain S_6 grown in medium B (Ernø and Stipkovits, 1973) was used (Stipkovits et al., 1993).

ELISA. The monoclonal antibody and the antigen were produced as described previously (Czifra et al., 1993*a, b*). In brief: BALB/c mice were immunized intraperitoneally with an ISCOM preparation of *M. gallisepticum* membrane proteins (Czifra et al., 1993*b*) twice, at an interval of 3 weeks. Three months after the first immunization a booster injection was administered on three consecutive days, and the spleen cells were fused with Sp 2/0 myeloma cells. For the fusion, 50% polyethylene glycol (Köhler and Milstein, 1967) was used.

The hybridoma supernatants were tested by indirect ELISA and the *M. gallisepticum* positive clones were subcloned three times.

The monoclonal antibody used in the competitive ELISA belongs to the IgG1 isotype. By immunoblotting it has been shown to bind to the 56 kD polypeptide of *M. gallisepticum* (Czifra et al., 1993*b*).

After purification, the monoclonal antibody was labeled with peroxidase, using the periodate method (Nakane and Kavaoi, 1991).

M. gallisepticum strain 1226 was grown in medium B (Ernø and Stipkovits, 1973), and the mycoplasmas were sedimented by centrifugation at

20,000 *g* for 45 min. After discarding the supernatant, the cell suspension was washed three times in PBS, the sediment was resuspended in PBS to give 1:100 of the original broth culture, then Sarcosyl (Sigma Chemical Co., St. Louis, U.S.A.) was added in a final concentration of 0.5% and dissolved during incubation at room temperature for one hour. After repeated centrifugation the protein content of the supernatant was determined by Bradford's method (Bradford, 1976). The antigen was stored at –20 °C until used.

From the antigen dissolved with Sarcosyl a solution of 200 mcg/ml protein concentration was prepared in PBS (pH 7.2) containing 0.02% sodium azide, and 50 µl aliquots of that solution were measured into the wells of the microtitre plates. The plates were incubated in a humid box at 4 °C overnight. Subsequently, they were washed three times in PBS containing 0.05% Tween. Fifty µl aliquots of undiluted serum samples were measured into the wells, and the plates were incubated in a humid box at room temperature for 1 h. After three additional cycles of washing, the peroxidase-labeled monoclonal antibody was added and the plate was incubated for 1 h. The optimal concentration of the labeled monoclonal antibody and antigen was determined by the checker-board method. After the last washing, 50 µl aliquots of a substrate solution containing orthophenylene-diamine (OPD, Reanal, Budapest), 0.1 M citric acid and 0.05% H_2O_2 were measured into the wells. Twenty min later the reaction was stopped by the addition of 50 µl 4 N H_2SO_4. The extinction values were measured at a wavelength of 492 nm in a Titertek Multiscan (Flow Laboratories, U.K.) spectrophotometer.

The principle of the monoclonal "blocking" ELISA is illustrated in Fig. 1. The test measures the inhibiting ("blocking") effect of antibodies present in the test serum sample. If, because of this inhibitory effect, the reaction between the monoclonal antibody and the only *M. gallisepticum* specific epitope recognized by it does not take place, this indicates that *M. gallisepticum* specific antibodies are present in the test serum sample, i.e. the test is positive. If nothing inhibits the binding of the monoclonal antibody, the test is negative.

This inhibition can be elicited by *M. gallisepticum* antibody containing serum from any species of animal. The test is suitable for the serologic testing of all poultry species producing antibodies against *M. gallisepticum*.

Results

The comparative examination of a total of 1,030 turkey blood samples from 6 flocks of 3 Eastern Hungarian farms for the presence of *M. gallisepticum* antibodies was carried out by the SA and HI test and by ELISA.

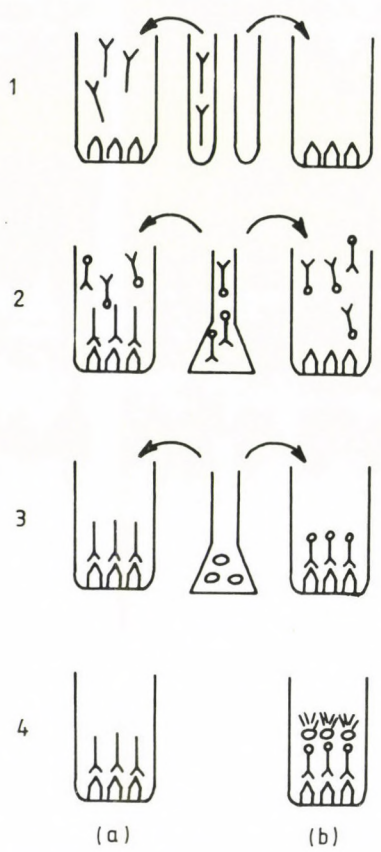

Fig. 1. Principle of the blocking ELISA. (a) positive serum; (b) negative serum; = *M. gallisepticum* antigens; = anti-*M. gallisepticum* antibodies; = HPRO-labeled anti-*M. gallisepticum* monoclonal antibody; = substrate

The results are summarized in Table 1. All sera derived from flock I/3 were negative in all the three tests. Only two samples from farm III proved positive by the SA test. The other four flocks gave positive results in all the three tests.

Table 2 shows how many of the samples derived from infected turkeys reacted positively in all the three tests, in two tests, or in one test only. A total of 167 samples (35% of the positive sera) were positive in all the three tests, 17% only by ELISA and HI, 17% only by ELISA and SA, 3% only by SA and HI, 25% only by ELISA, 2% only by SA, and 1% only by HI.

Table 1

Results obtained for sera from turkey flocks by the slide agglutination (SA), ELISA and haemagglutination inhibition (HI) tests

Farm	Unit	No. of samples	*M. gallisepticum* positive								*M. synoviae*	*M. meleagridis*
			SA	ELISA	HI	Titres						
						1:8	1:16	1:32	1:64	1:128		
I	1	200	61	67	27	16	7	4	–	–	–	–
	2	360	155	279	184	102	61	17	5	–	–	24
	3	180	–	–	–	–	–	–	–	–	–	–
	4	160	9	44	1	1	–	–	–	–	–	–
II		70	43	56	51	20	15	8	5	3	–	50
III		60	2	–	–	–	–	–	–	–	–	31
Total		1030	270	446	263	139	83	29	10	3	–	105
%			26	43	26							

Table 2

Results obtained for sera from infected turkey flocks by the slide agglutination (SA), ELISA and haemagglutination inhibition (HI) tests

Farm	Unit	No. of samples	*M. gallisepticum* positive							
			Total	ELISA SA HI	ELISA HI	SA HI	SA ELISA	SA	HI	ELISA
I	1	200	74	22	1	3	32	3	1	12
	2	360	294	110	67	7	37	2	3	68
	3	180	–	–	–	–	–	–	–	–
	4	160	46	1	–	–	7	2	–	36
II		70	61	34	14	4	4	1	–	4
III		60	2	–	–	–	–	2	–	–
Total		1030	478	167	82	14	80	8	4	120
%			46	35	17	3	17	2	1	25

Table 3

Results obtained for sera from an infected turkey flocks (from birds of different age) by the slide agglutination (SA), ELISA and haemagglutination inhibition (HI) tests

Age (weeks)	No. of samples	*M. gallisepticum* positive								*M. synoviae*	*M. meleagridis*
		SA	ELISA	HI	Titres						
					1:8	1:16	1:32	1:64	1:128		
25	80	61	67	27	16	7	4	–	–	–	–
31	60	50	56	33	16	14	3	–	–	–	–
33	60	33	53	48	25	12	9	2	–	–	–
41	60	12	56	29	17	11	1	–	–	–	–
45	60	29	57	35	24	10	1	–	–	–	–
50	60	31	57	40	20	14	3	3	–	–	–
Total	380	216	346	212	118	68	21	5	–	–	–
%		57	91	56							

Table 4

Performance of the slide agglutination (SA), ELISA and haemagglutination inhibition (HI) test on the basis of results obtained from an infected turkey flocks, from birds of different age

Age (weeks)	No. of samples	*M. gallisepticum* positive							
		Total	ELISA SA HI	ELISA HI	SA HI	SA ELISA	SA	HI	ELISA
25	80	74	22	1	3	32	3	1	12
31	60	60	31	2	–	19	–	–	8
33	60	58	28	18	4	–	1	–	7
41	60	59	7	20	1	3	1	1	26
45	60	58	21	12	–	8	–	2	15
50	60	59	23	15	2	7	–	–	12
Total	380	368	132	68	10	69	5	4	80
%		97	36	18	3	19	1	1	22

All the blood samples were negative for *M. synoviae* infection, while 105 sera gave a positive reaction with *M. meleagridis* antigen.

Tables 3 and 4 present the results obtained for blood samples submitted from one of the farms as a function of the birds' age. A total of 380 blood samples taken from turkeys between the age of 25 and 50 weeks were examined. A positivity rate between 92 and 100% was observed in all age groups.

Discussion

In this study, the monoclonal "blocking" ELISA reliably indicated *M. gallisepticum* infection in turkey flocks. Its sensitivity surpassed that of the SA and HI tests in all infected flocks. Only 5% of the 478 reacting samples were negative by ELISA; at the same time, 25% of them contained antibodies detectable only by ELISA.

One out of the six flocks surveyed was negative by all three tests, while in one flock only the SA test indicated a 3% positivity. This must have been the result of cross-reaction which is frequently observed in the SA test after the use of certain vaccines (Higgins and Whithear, 1986; Avakian et al., 1988; Bradley et al., 1988; Avakian and Kleven, 1990). By repeating the tests (ELISA after 1–2 weeks and HI after 3–4 weeks) such doubtful cases can be settled. The SA test is suitable primarily for detecting IgM-type antibodies while the HI test for detecting IgG-type antibodies appearing later. Birds experimentally infected with *M. gallisepticum* became ELISA-positive 7–10 days after the infection. Owing to the immunodominant character of the *M. gallisepticum* protein containing the epitope recognized by the monoclonal antibody, as well as due to the sensitivity of the ELISA, a long persistence of the blocking antibodies could be demonstrated in the infected birds (Czifra et al., 1993*a*).

Data from the case analyzed in Table 3 also indicate that, among the blood samples submitted, the largest number of positive samples was always detected by ELISA.

Thus, in seropositive flocks the three tests may give different results for a given sample in different periods. If we study the results for a specific blood sample, it appears (Table 4) that in infected flocks first the SA test and ELISA, then the HI test and ELISA yield parallel results.

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EFFECTS OF T-2 TOXIN TREATMENT ON THE EGG YIELD AND HATCHABILITY IN GEESE*

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Ten groups of one-year-old geese, each including 10 layers and 3 ganders, were treated with T-2 toxin at doses of 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0 and 3.0 mg/kg body weight/day for 18 days by injection into the stomach. At the toxin dose of 0.1 mg/kg/day, the egg yield remained unchanged while the hatching rate slightly decreased. T-2 toxin administered at a dose of 0.2 mg/kg/day decreased the hatching rate and the egg yield. The toxin dose of 0.3 mg/kg/day resulted in a 50% reduction of the hatching rate. The mortality rate was significant at toxin levels higher than 0.8 mg/bwkg/day; however, 30% of the birds survived even the 3.0 mg/kg/day toxin dose.

Key words: T-2 toxin, goose, *Fusarium*, mycotoxin, egg yield, hatching rate

Birds have been reported to sensitively react to mycotoxins of feed origin. The reactions usually include a reduction in the egg yield which, however, widely varies depending on the quality and quantity of the toxins present.

At levels occurring in the field, deoxynivalenol can hardly exert an influence on laying hens (Farnworth et al., 1983). However, other trichothecenes like T-2 toxin and diacetoxyscirpenol can severely impair the health status of animals even when ingested in small doses (Hoerr et al., 1982). Synergism between mycotoxins has also been described, e.g. between deoxynivalenol and T-2 toxin (Kubena et al., 1989*b*) and between deoxynivalenol and ochratoxin (Kubena et al., 1988). Ochratoxin A alone has proved very toxic, too (Huff et al., 1988*b*; Gibson et al., 1989; Rotter et al., 1989). When both ochratoxin A and T-2 toxin were present, a severe impairment of the health status of chickens was observed (Huff et al., 1988*a*; Kubena et al., 1989*a*).

In this work, the effects of T-2 toxin on the egg yield and hatching rate of geese were studied. To the best of our knowledge, such a study has not been published on geese. Goose breeding is an important branch of animal production in

* This study was carried out in the framework of project no. 1246 of the National Research Fund

several European countries (Hungary, France, Poland, etc.). Observations made in the field suggest that the goose may be more sensitive to T-2 toxin that it has been thought before.

Materials and methods

T-2 toxin was produced by the fermentation of strain NRRL 3299. The fermented product was purified by chemical methods. The active ingredient content was determined by chromatography. An extract with 80% active ingredient content was used in the feeding trials. The toxin was dissolved in 4 cm^3 of propylene glycol and administered through a stomach tube.

Ten groups of one-year-old geese, each consisting of 10 layers and 3 ganders, were formed. Each group contained birds of similar body weight. The average body weight of each group was determined and the toxin quantities were adjusted to these mean values. The groups, identified by colour wing markings, were housed in the same shed, separated by metal fences. The experimental design is shown in Table 1.

For the sake of homogeneity, the control geese were treated also with propylene glycol. The control group contained twice as many geese as the experimental groups.

The feeds were assayed for their content of eight different mycotoxins including zearalenone, T-2 toxin, HT-2 toxin, deoxynivalenol, diacetoxyscirpenol, fusarenone-X, nivalenol and ochratoxin A (Bata et al., 1983). None of these mycotoxins was detectable in the experimental feed.

The toxin was administered for 18 days. Toxin treatment was started after the plateau of egg production had been reached, at week 4–6 of the laying period. The geese were kept under veterinary control throughout the experiment. The following parameters were recorded: deaths, daily egg yield, and the hatching rate of eggs on a weekly basis.

Results

The deaths that occurred in the groups are shown in Table 2. The first deaths took place on day 3 of the treatment period. After the end of treatment no more deaths were detected. Egg production was finished in the 11th week after the beginning of toxin treatment.

Table 1
Design of the experiment

Group	No. of layers	No. of ganders	Toxin level (mg/kg body weight/day)
1	20	6	0.0
2	10	3	0.1
3	10	3	0.2
4	10	3	0.3
5	10	3	0.4
6	10	3	0.5
7	10	3	0.6
8	10	3	0.8
9	10	3	1.0
10	10	3	3.0

Table 2
Number of deaths in the different groups

Group	Number of deaths		
	Layers	Ganders	Total
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	1	-	1
8	3	1	4
9	2	1	3
10	6	1	7

The changes in egg yield as a function of the toxin dose are shown in Fig. 1. Figure 2 illustrates the hatching rate versus the toxin doses administered, while Fig. 3 shows the number of geese hatched as a function of the toxin dose. The hatching rate versus the toxin doses administered in the last week of T-2 toxin treatment is presented in Fig. 4.

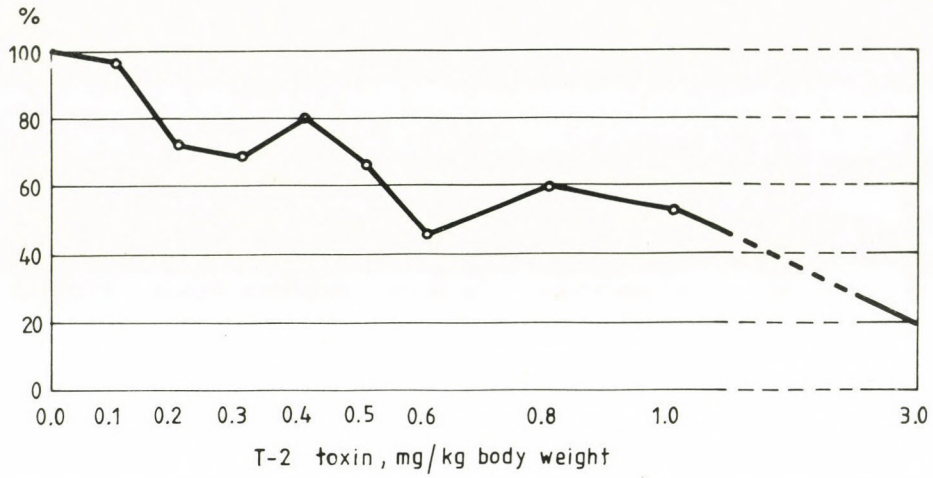

Fig. 1. Egg yield in the different treatment groups, expressed as per cent of the control

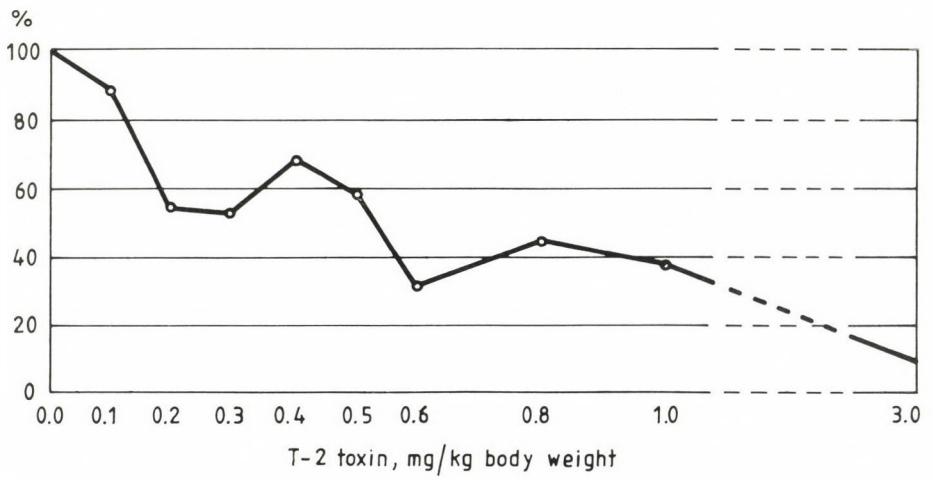

Fig. 2. Hatchability of eggs in the different treatment groups, expressed as per cent of the control

Fig. 3. Hatchability of eggs in the different treatment groups, expressed as per cent of the eggs placed into the incubator

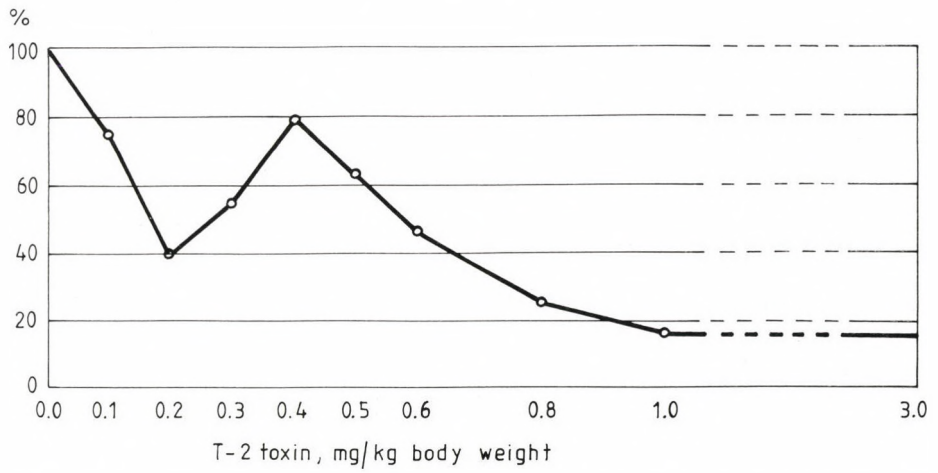

Fig. 4. Hatchability of eggs in the different treatment groups in the last week of toxin administration (expressed as per cent of the control)

Discussion

The hatching rate decreased by 10% even when the smallest dose of T-2 toxin (0.1 mg/kg body weight/day) was administered to geese (Fig. 3). In the last week of toxin treatment the decrease was 25% (Fig. 4). A significant reduction in

the egg yield could be observed at a toxin level of 0.2 mg/kg body weight/day or higher. In the last week of toxin treatment, however, the hatching rate dropped below half the control value (Fig. 4).

At a toxin level of 0.3 mg/kg body weight/day the hatching rate was only 50% of that of the control group (Fig. 3).

The hatchability curve showed the local maximum at the toxin dose of 0.4 mg/kg body weight/day. The egg yield and hatching rate curves showed the same extreme values (Figs 1 and 2). Deaths occurred at dose levels of 0.8–1.0 mg/kg body weight/day. Only 30% of the geese survived the toxin dose of 3.0 mg/kg body weight/day. This also reflected the different sensitivity of birds to T-2 toxin intake.

Self-compensation of geese to the toxin dose of 0.1 mg/kg body weight/day was achieved two weeks after termination of the experiment. However, at the 0.2 mg/kg/day dose self-compensation, i.e. the return of the body weight to a value similar to the control, required 5–6 weeks after the end of the experiment.

In groups treated with toxin levels of 0.3, 0.4 and 0.5 mg/kg body weight/day, respectively, the toxic effects were partly eliminated within 2–3 weeks after the end of the experiment. The hatching rates were close to those of the control group; later, however, a decrease occurred again.

Groups treated with toxin doses of 0.6 mg/kg body weight/day or higher showed consequent decreases in the egg yield after 3–5 days of treatment.

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SURVEY OF THE PARATENIC HOSTS OF *Anguillicola crassus* IN LAKE VELENCE, HUNGARY

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Anguillicola crassus infection of paratenic hosts was investigated in Lake Velence, Hungary, in October 1992. A total of 155 specimens of 8 species (*Gymnocephalus cernuus, Lepomis gibbosus, Stizostedion lucioperca, Abramis brama, Alburnus alburnus, Cyprinus carpio, Pseudorasbora parva* and *Rutilus rutilus*) were examined: all species proved to be infected. There were large differences in the prevalence of infection among fishes, but the prevalence was generally higher in Perciform fishes and it was the highest in *G. cernuus* (100%). The occurrence and physical stage of larvae did not depend on the size of the fish. In *S. lucioperca* the size of third stage larvae was bigger than in other fishes, and only live larvae were found. The comparison of Lake Velence and Lake Balaton indicates that the rate of larval infection in paratenic hosts of *A. crassus* is higher in Lake Velence. This might be explained by the significant ecological differences between the two lakes (population of intermediate hosts, water depth, salinity, pH).

Key words: *Anguillicola crassus,* paratenic hosts, Lake Velence

Anguillicola crassus is a parasitic nematode of eels (*Anguilla* spp.), which originates from East Asia (Kuwahara et al., 1974). It was introduced to Europe in the early 'eighties via the import of infected eels for consumption or restocking, and spread quickly through most European countries (Peters and Hartmann, 1986). In Hungary *A. crassus* was first recorded in Lake Balaton in 1990 by Székely et al. (1991), who reported heavy infections in eels. They did not observe dead fish in the lake, but found mortality among collected, transported and stored (stressed) specimens.

This species is widely distributed in the Japanese eel (*Anguilla japonica)* living in natural waters and eel farms of Japan but causes no, or very little damage. However, the European eel (*Anguilla anguilla)* introduced to Japan develops much heavier infection by this nematode which produces

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pathological changes in the swimbladder and causes the eels to lose their appetite and become emaciated (Egusa, 1979).

By now *A. crassus* infection of the European eel (*A. anguilla*) has become widespread in natural basins and in intensive eel cultures (Moravec and Taraschewski, 1988). Some reports indicate that in intensive culture this infection can cause economic losses (Mellergaard, 1988). Repeated mortality caused by *A. crassus* in eels living in natural waters has been reported by Molnár et al. (1991, 1993).

The intermediate hosts involved in the life cycle of *A. crassus* are copepods in which second stage larvae (L_2) develop to the third stage (L_3) infective to eels. As copepods are not considered a major food item for eels, because eels of larger size feed almost exclusively on fish and on chironomids (Paulovits and Biró, 1987), it was reasonable to assume that other fish species might act as paratenic hosts (Haenen and Van Banning, 1990).

These paratenic hosts are facultatively involved in the life cycle of *A. crassus*. The parasites do not develop within them; they only accumulate there and remain infective to the definitive host only (Thomas and Ollevier, 1992).

In Europe there have been only a few reports on the occurrence of *A. crassus* larvae in various fish species other than eels (De Charleroy et al., 1990; Haenen and Van Banning, 1990; Thomas and Ollevier, 1992). These authors have reported that the transfer of L_3 from experimentally infected *Leucisus idus* (ide), *Cyprinus carpio* (common carp), *Osmerus eperlanus* (smelt) and *Gymnocephalus cernuus* (ruffe) is possible. They mentioned that these fish species are able to act as paratenic host, and they are facultative in the life cycle of the parasite, which means that the parasites do not develop within them, only accumulate and remain infective only to the final host. The most comprehensive investigations on the paratenic hosts of *A. crassus* were conducted by Thomas and Ollevier (1992) on fishes of Belgian channels and by Székely (1994) on fishes of Lake Balaton, Hungary.

Data obtained from a survey of *A. crassus* larval infection of different freshwater fish species of Lake Velence are presented in this paper.

Materials and methods

Materials

A total of 155 fish were caught by net from Lake Velence (Hungary) on 15 October 1992. These fish belonged to the following 8 species: bleak (*Alburnus alburnus*), bream (*Abramis brama*), common carp (*Cyprinus carpio*), ruffe (*Gymnocephalus cernuus*), pumpkinseed (*Lepomis gibbosus*), Chinese rasbora

(*Pseudorasbora parva*), roach (*Rutilus rutilus*), and pike perch (*Stizostedion lucioperca*). The fish were immediately sent to the laboratory alive, where they were kept in aquaria until dissected for detailed light microscopic examination.

Characteristics of Lake Velence

The water surface of the lake is 25 km^2. The lake is shallow, with an average water depth of 1–1.6 m. Annual average water temperature of the lake is 10.8 °C. The pH of the water is high (8–9.5) and its salinity is not the same in different parts of the lake: in the average season it varies between 1,500 and 2,000 $\mu S\ cm^{-1}$ and in dry periods it is 2,200–2,700 $\mu S\ cm^{-1}$. Water is supplied by rainfall, Brook Császár and small springs. The fish population mostly comprises cyprinids and percids. Stocking of eel into the lake was started 25 years ago and finished 10 years ago. The eel population of the lake comprises only large specimens (with a length exceeding 50 cm).

Methods

The length of the fish was measured and they were dissected at the laboratory. The presence of *A. crassus* was checked in squash preparations made between two slides. Preparations from swimbladder (including the surrounding mesenteric tissues), stomach, intestine, liver, and kidney were examined under a light microscope. The total number of live and dead L_3 larvae was determined by moving the microscopic field step by step in a zigzag line at 32- to 400-fold magnification.

Statistical analysis of the data of relevant fish species was performed and "prevalence" and "mean intensity" were calculated to provide an index of the degree of dispersion of *A. crassus*.

Results

Prevalence and mean intensity

A total of 155 fish specimens of 8 species, belonging to 4 different families and 2 orders, were examined. With the exception of 2 *Alburnus alburnus*, 1 *P. parva* and 8 *S. lucioperca*, all fishes (144) were found infected with L_3 larvae, at least with one larva (Table 1). The highest prevalence (100%; Fig. 1) and mean intensity (184 larvae per infected fish; Fig. 2) were found in *G. cernuus*, followed by *Alburnus alburnus* and *R. rutilus* (Table 2). In some cases only 1 to 3 individuals (*Abramis brama, C. carpio, L. gibbosus* and *P. parva*) were examined, but they were also infected. Species belonging to Perciformes showed a higher prevalence of live larvae than most Cypriniformes.

Table 1

Anguillicola crassus larval infection of paratenic host fishes in Lake Velence

Fish species	Mean length cm	No. of non-infected fish	No. of fish infected only by dead larvae	Mean intensity	No. of fish infected by live and dead larvae	Mean intensity (live larvae)	Mean intensity (dead larvae)	No. of fish infected only by live larvae	Mean intensity	Total no. of fish
Alburnus alburnus	7.7	2	22	8	18	1	7	3	2	45
Abramis brama	8.8	0	1	16	0	0	0	0	0	1
Cyprinus carpio	4.4	0	1	4	0	0	0	0	0	1
Gymnocephalus cernuus	6.8	0	0	0	77	96	88	0	0	77
Lepomis gibbosus	4.5	0	1	10	0	0	0	2	1	3
Pseudorasbora parva	8.5	1	1	1	1	3	5	0	0	3
Rutilus rutilus	7.5	0	2	14	7	11	8	0	0	9
Stizostedion lucioperca	7.5	8	0	0	0	0	0	5	3	13

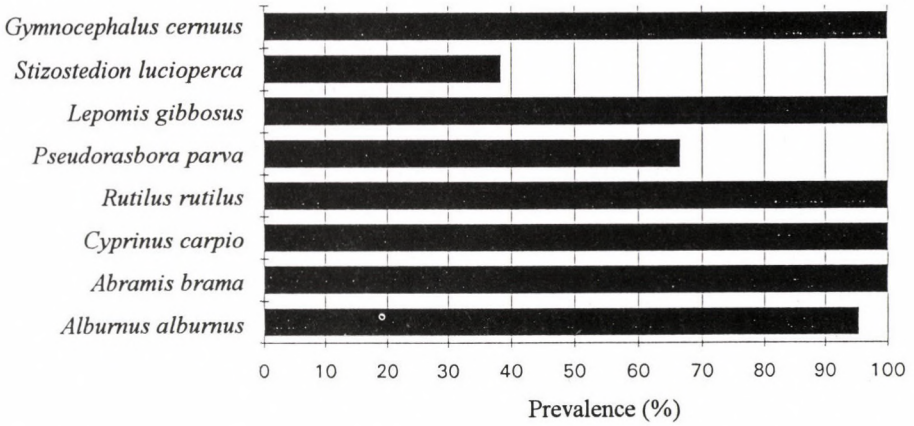

Fig. 1. Prevalence of *Anguillicola* larval infection in different fish species in Lake Velence, 1992

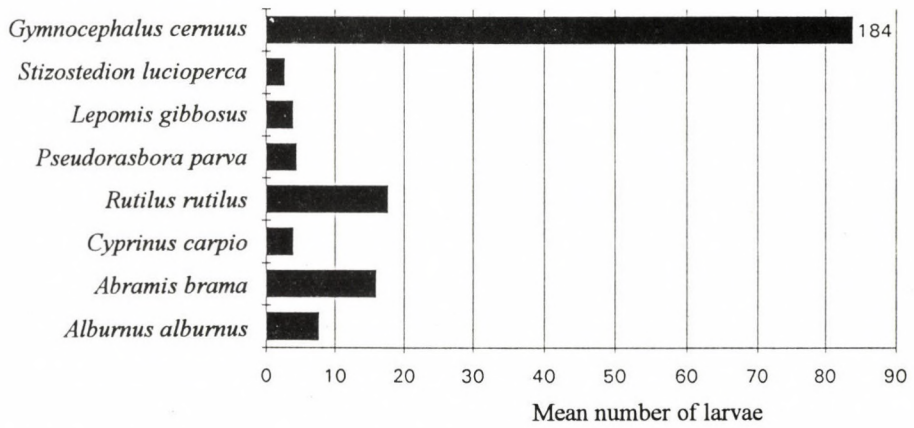

Fig. 2. Mean intensity of *Anguillicola crassus* infection in different fish species in Lake Velence, 1992

The overall mean intensity and range of L_3 larvae differed by fish species. Mean intensity was the highest in species showing the highest prevalence and was lower for other species (Table 2).

Table 2

Prevalence and mean intensity of infection by 3rd stage *Anguillicola* larvae in the fish examined

Fish species	No. of fish examined	Mean lenght (cm)	Prevalence (%)	Mean intensity	Range min.– max.
Alburnus alburnus	45	7.7	95.5	7.74	1–37
Abramis brama	1	8.8	100*	16	–
Cyprinus carpio	1	4.4	100*	4	–
Gymnocephalus cernuus	77	6.8	100	184	103–290
Lepomis gibbosus	3	4.5	100*	4	1–10
Pseudorasbora parva	3	8.5	66.6	4.5	1–8
Rutilus rutilus	9	7.5	100	17.7	7–36
Stizostedion lucioperca	13	7.5	38.4	2.8	1–4

*Only a few fish were examined or only one fish was infected

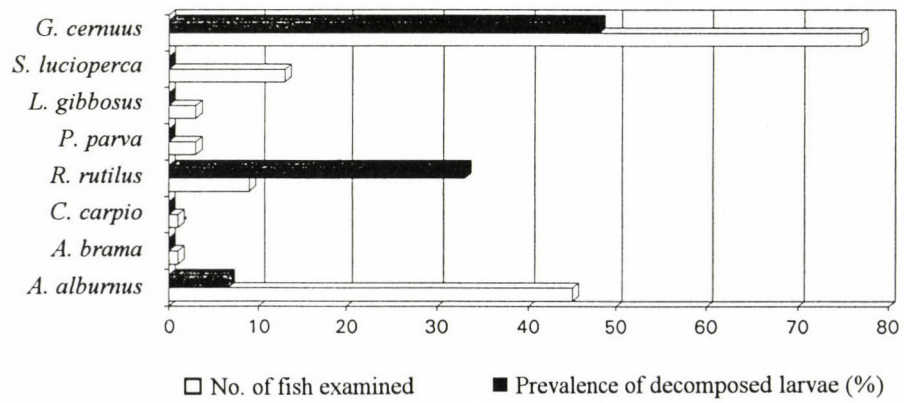

Fig. 3. Ratio of decomposed third-stage larvae of *Anguillicola crassus* in different fish species of Lake Velence, 1992

Table 3

Prevalence and mean intensity of decomposed larvae in different fish species

Fish species	No. of fish examined	Mean length (cm)	No. of fish infected by decomposed larvae	No. of decomposed larvae	Prevalence (%)	Mean intensity	Range min.– max.
Alburnus alburnus	45	7.7	3	4	6.6	1.3	1–2
Abramis brama	1	8.8	0	0	0	0	0
Cyprinus carpio	1	4.4	0	0	0	0	0
Gymnocephalus cernuus	77	6.8	37	45	48	1.2	1–2
Lepomis gibbosus	3	4.5	0	0	0	0	0
Pseudorasbora parva	3	8.5	0	0	0	0	0
Rutilus rutilus	9	7.5	3	11	33	3.6	1–7
Stizostedion lucioperca	13	7.5	0	0	0	0	0

Appearance and identification of Anguillicola crassus larvae

According to the description of Moravec and Taraschewski (1988), all larvae found in or around the swimbladder and in fresh preparations from other inner organs of the different fish species were determined as L_3 larvae of *A. crassus*. A rounded head end with 4 papillae, 2 amphids and 2 vertically oriented labia which surround a split-shaped mouth opening, the darkly coloured body and the conical, short tail could be distinguished in the light microscope.

In the majority of cases freely located live L_3 larvae were found, whose viability could easily be detected by their movements. Less frequently dead larvae covered only by a thin layer of connective tissue were detected. However, encapsulated live and dead larvae in granules were also found.

In fish species with a high prevalence and mean intensity of infection encapsulated wormlike structures of different shape and size were found which could not be positively identified as dead L_3 larvae, but which we assumed to be degenerated larvae. In *G. cernuus* these degenerated larvae were very common and were found in 48% of all infected *G. cernuus*. In one *R. rutilus* 7 such larvae were counted while only 3 out of 45 *Alburnus alburnus* contained degenerated larvae (1–2 larvae per fish). In other fish species we could not detect degenerated larvae (Table 3, Fig. 3).

The reproductive organs of female fish were more infected than those of males. In the ovaries of females the number of unencapsulated live larvae was high, but in other organs the rate of infection was approximately the same in both sexes.

Only 5 out of the 13 *S. lucioperca* specimens examined were infected, and only by live larvae. These larvae were bigger than those found in other fishes.

Discussion

The present study shows that a great variety of freshwater fish species can carry *A. crassus* larvae. This part of the life cycle has not been noticed in Asia, the original region of the nematode, and has been studied in Europe only for four years.

This is the first attempt to survey the prevalence of *Anguillicola* larval infection in paratenic fish hosts species in Lake Velence, Hungary.

In comparison with the study of Haenen and van Banning (1990), in this study more species were found infected and the numbers of larvae found in the various species were in a broader range.

Considering only the more frequently infected species, infection was not restricted to a certain size class. Already young fry (length: 2.9 cm) were infected

by *A. crassus* larvae, but the number of larvae in older fishes was higher. One of the reasons for dispersion in older fish might be the individual variability in prey selection.

Larval survival rate in the various fish species is still not known, but the high prevalence of encapsulated dead larvae in comparison with that of live larvae in Cypriniformes, similar to what was observed for fishes of Lake Balaton (Székely, 1994), may indicate a special host reaction which decreases the survival rate of larvae. On the other hand, *S. lucioperca* harboured only live larvae, suggesting that the longevity of *A. crassus* larvae might differ in different species of fishes. The high intensity of live larvae in the reproductive organs of females may also indicate that the longevity of larvae might differ in the various organs.

The bigger (overgrown) larvae seen in the swimbladder wall of *S. lucioperca* indicate that in some fish species L_3 larvae may reach a larger size than usual: they grow bigger and their intestine becomes darker with or without reaching the L_4 stage (less motile, dark-coloured intestine). In the study of Thomas and Ollevier (1992) these grown larvae were seen in the swimbladder of *P. fluviatilis*, *S. lucioperca* and *G. cernuus*. All these fishes infected by grown larvae belong to Perciform fishes. The high prevalence of live and overgrown larvae might indicate that the survival rate of larvae in these fishes is higher than in Cypriniformes.

Fishes belonging to physoclist (closed swimbladder) and physostome (open swimbladder) species were equally represented in this study. There were large differences in prevalence and mean intensity among the fish species, and the prevalence of infection was generally higher in physoclist fishes. The swimbladder wall of *G. cernuus* showed the highest rate of, in agreement with what was reported by Thomas and Ollevier (1992). It is not clear whether the difference in swimbladder structure or some other physiological or ecological difference plays a role in these findings.

Other aspects in which the fishes differed were feeding habit, although most fish feed on plankton during at least some period of their life (Lazzaro, 1987). The most heavily infected species in Lake Velence, the ruffe (*G. cernuus*), is a benthic fish. This is consistent with the results of a Swedish study on a benthic paratenic host of *Anguillicola,* the black goby (*Gobius niger*; Höglund and Thomas, 1992). Both the ruffe and the black goby feed on worms, insect larvae and zooplankton, and look for food in the mud. On the other hand, free-living L_2 larvae sink to the bottom, and the prevalence of infection in copepod intermediate hosts is the highest in the bottom region. These data indicate that a benthic mode of life (in deeper water layers) exposes the fish to a higher risk of becoming infected with *A. crassus* larvae. Most Perciformes are benthic fishes but the Cypriniformes involved in this study are mostly planktivorous species. This might be a reason why the rate of infection is lower in these fishes.

Due to their bottom-dwelling mode of life and the restricting effect of mouth size, eels prefer rather small fish inhabiting the bottom of lakes (Sinha and Jones, 1967; De Nie, 1988). A benthic mode of life enhances the probability of infection and of transferring L_3 larvae to the eel.

Paratenic hosts accumulate *A. crassus* larvae and contribute to their transfer to the definitive host but are not essential for the completion of the life cycle (Ryzhikov, 1964). Thomas and Ollevier (1992) suggested in their study that some species like *Alburnus alburnus* and *R. rutilus* were only occasionally infected and could therefore be considered accidental hosts. Our study has proved that these species are not accidental hosts, because the prevalence and intensity of infection in these fishes are considerable. Our findings are supported by the results of Székely (1994) who, calculating with the feeding habits of eels in Lake Balaton, found that the bleak (*Alburnus alburnus*) was the most important paratenic host for *A. crassus* in that lake.

There is a wide range of possible paratenic hosts with high infection rates in Lake Velence and Lake Balaton. A comparison between the two lakes revealed that the rate of infection is higher in Lake Velence than in Lake Balaton (Székely, 1994). This result is rather surprising because Lake Balaton has a significantly bigger eel population than does Lake Velence, though the rate of *A. crassus* infection of eels is the same in both lakes. It seems that in Lake Velence the larvae can find more intermediate hosts and have better chances of further development. This contradictory phenomenon might be explained by the significant ecological differences between the two lakes, such as shallow and eutrophic water, temperature or salinity.

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Theileria annulata INFECTION IN NEONATAL BOVINE CALVES

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On an organized farm in India, 70 out of 79 cows expected to calve within 30 days revealed the presence of *Theileria annulata* schizonts and/or piroplasms in their peripheral blood. Similarly, 33 out of 59 calves borne to the above cows also exhibited *T. annulata* schizonts on day less than 1 to day 19 post partum. The cows and calves were free from *Hyalomma anatolicum*, an established vector of *T. annulata*, during the course of study in the winter months, when the activity of ticks is reduced to the minimum. The infection appears to have taken place antenatally.

Key words: *Theileria annulata*, calf, intrauterine infection

Theileria annulata, the causative agent of bovine tropical theileriosis, is transmitted in nature by an ixodid tick vector, *Hyalomma anatolicum anatolicum* (Uilenberg, 1981). In the past few years neonatal male calves, procured for experimental purpose from this farm, have been found to reveal *T. annulata* schizonts in the peripheral blood even at the age of 5–7 days. This prompted the authors to undertake this study.

Materials and methods

A total of 79 crossbred cows at an organized farm in India, expected to calve within a period of 30 days, were included in the present study. Blood smears from all the cows were prepared once a week till the day of parturition, stained with Giemsa stain and examined microscopically under oil. The cows, calving pens, sheds and calves borne to these cows were also regularly screened for the presence of ticks. The clinical signs, if observed at any time during the study, were also recorded.

Fifty-nine neonatal calves borne to the above-mentioned cows also formed part of the present study. Blood smears from these calves were prepared daily till postpartum day 21 or the day they revealed the presence of *T. annulata*, whichever was earlier.

Results

None of the cows under study revealed any clinical sign of theileriosis. All of them were normal and free from *H. (a) anatolicum*. However, the cows had a low-grade infestation (5–10 ticks) with *Boophilus microplus*, and a history of low infestation with *Hyalomma anatolicum* also. The farm had been regularly following tick control measures using acaricides.

Blood smears from 70 out of the 79 cows examined revealed the presence of *T. annulata* in the peripheral blood. Four cows had intra-erythrocytic forms of the parasite, while the remaining 66 had only the schizont stage. Parasitaemia did not exceed 4–5% in any of the cases.

Table 1

Calves showing *Theileria annulata* schizonts in the peripheral blood

Age of calves (days)	Number of calves showing *T. annulata*
>1	6
1	2
2	6
4	1
5	1
6	2
9	1
11	1
12	2
13	3
14	3
16	2
17	1
19	2

Blood smears from 59 male and female calves were screened for the presence of *T. annulata* by microscopy. Thirty-three revealed the presence of *T. annulata* schizonts in the peripheral blood till the end of the observation period.

All calves were free from ticks till the time of detection of *T. annulata* schizonts or the end of the 21-day observation period.

Discussion

Twenty to 80% of pregnant bovine uteri have been observed to contain some bacteria, e.g. streptococci, staphylococci, coliform bacilli, etc., between the maternal and the fetal placenta (Nalbandov, 1970). Besides these, other bacteria, moulds and viruses have also been reported, and they may come from no place other than the dam's blood stream (Robert, 1961). Hafez (1975) reported the transfer of whole cells through the placenta by way of pinocytosis or leakage through pores in the placental membrane. At a livestock research station in Rajasthan (India), two neonatal female bovine calves revealed clinical signs of theileriosis just after birth, and the examination of their blood showed *T. annulata* ring forms in the erythrocytes (Banerjee, 1987). The presence of *T. annulata* schizonts in various organs of fetuses aborted at 3 to 7 months of pregnancy has been recorded (cited by Banerjee, 1987). The intrauterine transmission of *Babesia bigemina*, though rare, has also been reported (Soulsby, 1968). In a recent report, a foal revealed the presence of *B. equi* with Maltese cross in a blood smear immediately after birth, and excreted urine of red colour (Singh et al., 1992). Babesiosis has also been described in a 2-day-old pup (Jardine and Collett, 1992). Besides haemoprotozoan parasites, induced transplacental transmission of *Neospora caninum* in cattle (Dubey et al., 1992*a*) and in a stillborn pygmy goat (Dubey et al., 1992*b*) has been observed.

In the present study 70 out of 79 cows (87.59%) revealed the presence of *T. annulata*. The remaining 9 were found to be negative on microscopical examination. However, in 9 cows only 1 or 2 blood smears could be examined and, hence, the possibility of their being an asymptomatic carrier cannot be ruled out.

In the absence of the specific vector of bovine tropical theileriosis in the animal sheds, such a high incidence of theilerial infection, although of low grade, suggests that these dams had acquired the infection during their calfhood or during the season of vector activity and manifested parasitaemia due to the stress represented by pregnancy. Since the cows at this farm had not been vaccinated against theileriosis before, the possibility of infection by experimental inoculation of attenuated schizonts (vaccine) can be ruled out. The normal health status of these asymptomatic carriers indicates the existence of enzootic stability when all the cows appear to be naturally immunized and well protected against the clinical disease.

The salient findings of the present study indicated that 6 out of 33 infected calves from a total of 59 examined revealed the presence of *T. annulata* schizonts in the peripheral circulation within 10–15 h after birth (Table 1). The present findings are in line with those of Banerjee (1987). Since during the first 10–15 hours of life these neonatal calves were neither vaccinated nor exposed to tick

vectors, the possibility of infection during the antenatal phase can be entertained. Similarly, 12 calves revealed the presence of theilerial parasites from day 1–6 post partum (Table 1), which supports the passage of these parasites from dam to calf.

Thus, 33 out of the 59 calves examined revealed the presence of *T. annulata* schizonts, which strongly suggests that all these animals might have acquired the infection from their dams antenatally.

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COMPARATIVE ANTHELMINTHIC EFFICACY AND SAFETY OF *Caesalpinia crista* SEED AND PIPERAZINE ADIPATE IN CHICKENS WITH ARTIFICIALLY INDUCED *Ascaridia galli* INFECTION

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The antiascarid activity of *Caesalpinia crista* Linn. seeds, popularly known as Karanjwa, was evaluated in chickens of the Fumi breed, suffering from artificially induced *Ascaridia galli* infection. Eggs per gram (EPG) counts were determined in the droppings of chickens prior and after treatment with powdered *C. crista* at doses of 30, 40 and 50 mg/kg of body weight along with its extracts in water and methanol in amounts representing 50 mg/kg of crude powder. The crude drug at the dose rates of 40 and 50 mg/kg and its methanol extract induced a significant ($P<0.001$) effect on post-treatment days 10 and 15 while the 30 mg/kg dose was efficacious ($P<0.05$) on day 15 only. However, the aqueous extract did not show significant results. These results suggest that a 50 mg/kg dose of *C. crista* seed powder, its equivalent methanolic extract and piperazine (200 mg/kg) are equieffective in treating the ascarid infection of poultry. The crude *C. crista* powder appears to be potent and safer than its methanol extract on the basis of the side effects observed.

Key words: *Caesalpinia crista*, piperazine adipate, *Ascaridia galli*, anthelminthics

Synthetic anthelminthics are well known to possess several adverse and/or side effects. Thus, the research for alternative therapeutic agents for the treatment and control of helminth infection in animals has increased in importance (Akhtar and Ahmad, 1992).

Caesalpinia crista, Linn. (Leguminosae) seeds locally known as Karanjwa have been considered in the folk medicine as anthelminthic, antiperiodic and antipyretic (Anonymous, 1956; Chopra et al., 1956; Awan, 1981). Studies conducted by Akhtar et al. (1985) on the antiascarid activity of *C. crista* in buffalo calves have shown that *C. crista* seed powder, its equivalent extract in methanol, and morantel tartrate (0.01 g/kg) are equieffective against *Neoascaris vitulorum*. Glycosides isolated from the seeds of *C. crista* have also been reported to exert an anthelminthic effect against nematodal infection of sheep (Akhtar and Aslam, 1989). Ascaridosis is also a serious problem in poultry and has been observed to

cause serious losses (Hussain, 1966; Sheikh et al., 1968; Ikeme, 1971). Therefore, the present study was conducted to evaluate the anthelminthic efficacy and safety of powdered *C. crista* seed and its extracts in water and methanol against artificially induced *Ascaridia galli* infection in chickens.

Materials and methods

During the months of April to July, guts from 740 poultry of mixed sex, breed and age were collected from local poultry meat shops. The small intestines were incised to find mature ascarids. The female ascarids were identified on the basis of their large size and absence of spicules. They were crushed, put in 1% formaline and sieved through a fine mesh to obtain ascarid eggs (Alicata, 1985). The filtrate obtained was incubated at 27 °C in 1% formaline. Infective stage larvae appeared in the eggs after 10–14 days.

A total of 150 day-old chicks of the Fumi breed were obtained from the University Poultry Farm. The chickens were reared in an infection-free environment and fed a balanced chick starter ration prepared and supplied by the Department of Nutrition, University of Agriculture, Faisalabad.

At the age of 8 weeks, all the chicks were inoculated with ascarid eggs containing infective larvae. Each bird received 55–60 eggs/day. The procedure was repeated for four consecutive days as done by Alicata (1985). After three weeks of inoculation the chick droppings were tested for ascarid eggs by the direct smear method as described by Soulsby (1982). After 6 weeks the ascarid eggs started to appear in the droppings. At this stage the birds were randomly divided into 7 groups each comprising 16 chicks. The droppings of 16 individuals of each group were further examined to determine the pre-treatment EPG (eggs per gram) counts by Stoll and Hauscheer's technique using the method described by Thienpont et al. (1963).

Seeds of *Caesalpinia crista*, Linn. were purchased from the local herbal market of Faisalabad. These were dried in the shade and finely powdered with an electric grinder. The powder was administered orally to the individual chicks in capsules at the rate of 30, 40 and 50 mg/kg body weight. Water extract was prepared by soaking 2.5 g of powdered seed in 100 ml distilled water, kept overnight and then sieved through muslin cloth to obtain the water extract (25 mg = 1 ml). This extract was administered orally to individual chicks at the rate of 2 ml/kg, representing 50 mg of crude drug/kg body weight. The methanol extract was prepared using a Soxhlet apparatus. The evaporated extract representing 50 mg/kg of powder was administered orally, in capsules, individually to the chicks. Piperazine adipate was administered at the dosage of 200 mg/kg of body weight to another

group (treated control) while a group was left untreated (infected control). The post-treatment EPG counts were again determined on days 3, 10 and 15. The data were expressed as mean EPG counts ± standard error of the mean (SEM), and their percentage reductions were calculated.

Student's *t* test was used to check the significance (Snedecor and Cochran, 1967). Safety of the drug was determined by carefully observing the chicks for side effects like dullness, staggering gait, convulsions and rejection of feed on days 3, 10 and 15 post treatment. However, these side effects disappeared in 12–48 hours.

Results and discussion

Out of a total of 740 poultry guts collected from poultry meat shops of Faisalabad, 355 were found heavily infested with mature *Ascaridia galli* worms. Thus, the incidence of *A. galli* was 48% in the birds checked. This percentage reflected that the prevalence of *A. galli* infection is quite high in this area of Punjab province. An almost similar incidence has been reported by Awan (1963) and Akhtar and Riffat (1985). Therefore, this parasitic infection of poultry needs immediate thorough investigation and appropriate steps must be taken towards its therapy and control.

Table 1 shows that the EPG counts in the droppings of the untreated infected group were not significantly altered ($P>0.05$) at day 3, 10 and 15. In the group treated with 30 mg/kg of *Caesalpinia crista* powder, pretreatment EPG counts changed from 1133 ± 126 to 1083 ± 90, 833 ± 82 and 550 ± 62 on post-treatment days 3, 10 and 15 respectively. The effect of that dosage level was significant ($P<0.05$) only on day 15 after drug administration, causing a 51 ± 3% EPG reduction. However, administration of 40 mg/kg of the powder highly significantly ($P<0.001$) reduced the EPG counts from 1083 ± 117 to 317 ± 61 and 100 ±56 on day 10 and 15, respectively. The respective percentage EPG reductions were 71 ± 3 and 91 ± 2. Similarly, in birds treated with 50 mg/kg body weight of powder, the pretreatment EPG count of 1133 ± 123 was reduced to 283 ± 68 and 67 ± 53 on post-treatment days 10 and 15. These EPG counts were highly significantly ($P<0.001$) lower than those obtained before the treatment, and corresponded to percentage EPG reductions of 75 ± 4 and 94 ± 3, respectively. Both dosage levels of 40 and 50 mg/kg of powder nonsignificantly ($P>0.05$) reduced the EPG counts in the droppings of treated birds by day 3 after the administration of drug.

Table 1

Mean ± SEM EPG counts and percentage reductions (n=16) in droppings of chickens suffering from *Ascaridia galli* infection before and after treatment with *Caesalpinia crista* Linn. seed, its water and methanol extracts, and piperazine adipate (200 mg/kg)

Medication	Dosage (oral)	Mean EPG ± SEM on post-inoculation day 45	Mean EPG reduction ± SEM on post-treatment day			Mean percentage reduction ± SEM in EPG on post-treatment day		
			3	10	15	3	10	15
Untreated (infected)		1117±100	1167±129	1583±133	1917±133	0±0	0±0	0±0
Piperazine adipate	200 mg/kg	1050±97	83±34**	17±16**	0±0	92±3	99±1	100±0
Caesalpinia crista seed powder	30 mg/kg	1133±126	1083±90	833±82	550±62*	15±2	27±2	51±3
Caesalpinia crista seed powder	40 mg/kg	1083±117	867±80	317±61**	100±56**	20±1	71±3	91±2
Caesalpinia crista seed powder	50 mg/kg	1133±123	867±82	283±68**	67±53**	23±3	75±4	@94±3
C. crista water extract	Equivalent to 50 mg/kg of powder	1550±131	1317±99	1256±96	1182±93	15±2	19±5	24±15
C. crista methanol extract	Equivalent to 50 mg/kg of powder	1683±146	1304±95	267±62**	33±21**	23±2	87±3	@98±1

EPG = Eggs per gram of dropping; * = Significantly less ($P<0.05$) than the pretreatment value; ** = Highly significantly less ($P<0.001$) than the pretreatment value; @ = Nonsignificantly ($P>0.05$) different from respective values obtained with piperazine adipate; n = Number of birds in each group

Table 2

Side effects observed in chickens treated with *C. crista* seed powder (30, 40, 50 mg/kg), its extracts and piperazine adipate (200 mg/kg)*

Side effects	*C. crista* powder/kg			*C. crista* powder extracts/kg		Piperazine adipate/kg (200 mg)
	30 mg	40 mg	50 mg	Water 50 mg	Methanol 50 mg	
Dullness	0	1	2	1	3	0
Staggering gait	0	0	1	0	0	0
Convulsions	0	1	0	1	0	0
Rejection of feed	0	0	1	0	2	0
Percentage side effects	0	12	25	12	31	0

* Total number of chickens treated with each dosage level: n = 16

The water extract of *C. crista* equivalent to 50 mg/kg body weight was administered to another group of chickens. The pretreatment EPG count was 1550 ± 131, which was reduced to 1317 ± 99, 1256 ± 96 and 1182 ± 93 on days 3, 10 and 15, respectively. Nonsignificant ($P>0.05$) reductions in EPG counts were noted on all post-treatment days. In chickens treated with the ethanol extract in amounts representing 50 mg/kg of powder, a highly significant ($P<0.001$) effect was produced on days 10 and 15 after treatment. This effect was statistically similar to that produced by 40 and 50 mg/kg of the crude drug. However, the percentage reductions found in EPG counts on days 10 and 15 were more pronounced, i.e. 87 ± 3 and 98 ± 1. A single treatment of another group of chickens with piperazine adipate (200 mg/kg) reflected highly significant ($P<0.001$) results on all post-treatment days. The EPG count was 1050 ± 97 which was reduced to 83 ± 34, 17 ± 16 and 0 ± 0 on days 3, 10 and 15, respectively. The respective percentage reductions in EPG counts were 92 ± 3, 99 ± 1 and 100 ± 0. The data obtained in the present study clearly show that powdered *Caesalpinia crista*, Linn. seed and its methanol extract exerted a significant and potent antiascarid effect in chickens. Furthermore, these results suggest that 50 mg/kg of *C. crista* seed powder, its equivalent amount of methanol extract, and piperazine adipate (200 mg/kg) are equieffective for the treatment of artificially induced *Ascaridia galli* infection. However, the water extract of powdered *C. crista* produced a non-

significant effect on all post treatment days. This observation is contrary to the prior evaluations of powdered *C. crista* against *Neoascaris vitulorum* infection in buffalo calves in which a significant ($P<0.05$) EPG count reduction was noted on post-treatment day 3 (Akhtar et al., 1985). In view of the present findings it may be assumed that the *Ascaridia galli* strain was resistant to the water-soluble active ingredient(s) of the *C. crista* seed. On the other hand, the efficacy of the methanol extract of this indigenous powdered plant clearly showed that its active ingredient was soluble in alcohol. Thirty-one percent of the chickens treated with the methanol extract of *C. crista* showed side effects (dullness, feed rejection, etc.) which were more pronounced than those noted in 25% of the birds treated with the 50 mg/kg dose of powder dose (Table 2).

This suggested the presence of some corrective ingredients in powdered *C. crista* seeds which prevent the appearance of more side effects. However, this observation is contrary to what was found earlier in goats with a significant antinematodal efficacy of the alkaloid tetrahydroharmine, isolated from *Peganum harmala* seeds (Akhtar and Ahmad, 1991). Further pharmacological and chemical studies are still needed for evaluating the exact mechanism of action and safety of the *C. crista* Linn. seed. However, it can safely be said that the results presented here do encourage the appropriate use of this cheap indigenous anthelminthic medicinal plant against *Ascaridia galli* infection in poultry.

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ORAL ADMINISTRATION OF MONENSIN AND LEAD TO BROILER CHICKS: EFFECTS ON HAEMATOLOGICAL AND BIOCHEMICAL PARAMETERS*

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Monensin and lead were administered separately or concurrently at different toxic doses to broiler chicks. Administration of lead alone did not result in a significant depression of haematological parameters. Administration of higher levels of monensin caused a reduction in haematocrit and an increase in blood serum levels of alanine aminotransferase, aspartate aminotransferase and cholesterol. Concurrent administration of monensin and lead caused a severe depression of haematological profiles which indicated the existence of an interaction between the two substances. It was concluded that concurrent administration of monensin and lead potentiated the toxic effects of each other.

Key words: Chicken, monensin, lead, blood parameters

Monensin, an ionophore antibiotic used as an anticoccidial agent in poultry, selectively facilitates the transmembrane exchange of sodium and potassium for protons (Mollenhauer et al., 1990). Toxicity of monensin is potentiated when administered together with certain other drugs like tiamulin (Weisman et al., 1980; Hilbrich and Trautwein, 1980; Koncicki et al., 1987), oleandomycin (Umemura et al., 1984) and chloramphenicol (Dorn et al., 1983; Tipold et al., 1988). It also enhances selenium accumulation in the body tissues of sheep (Smyth et al., 1990) and chicks (Khan et al., 1993*a*).

Lead, an environmental pollutant particularly widespread in industrial areas, decreases the vitality and production of domestic animals (Milhaud et al., 1979; Kwatra et al., 1986). Lead toxicity is significantly affected by variation in levels of certain food components like protein, vitamins and minerals (Levander,

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1979). High concentrations of lead in polluted areas may also be suspected to interact with monensin present in the poultry feeds. We have reported that concurrent administration of toxic levels of lead and monensin enhanced the accumulation of lead and iron in liver tissue (Khan et al., 1993*b*). The effects of concurrent oral administration of lead and monensin upon some haematological and biochemical parameters of boiler chickens are described in this paper.

Materials and methods

A total of 105 broiler chicks (Astra B breed) were kept on basal feed (BK-2 broiler grower mash) for two weeks. Subsequently, the birds were divided into seven equal groups and fed basal feed amended by the addition of monensin and lead as follow: Groups M0 and ML = monensin (240 mg/kg) as monensin sodium; Groups L0 and LM = lead (1,200 mg/kg) as lead acetate ($C_2H_3O_2.3H_2O$); Groups 0M, 0L and 00 = control fed basal feed without any addition. Birds were kept on these feeds throughout the course of experiment.

After four weeks of adaptation on these amended diets, birds in groups ML and 0L were orally administered lead acetate to provide 400 mg lead/kg body weight on alternate days for four weeks. Similarly, birds in groups LM and 0M were given 40 mg monensin/kg body weight on alternate days for four weeks. In this way, birds in groups LM and ML were concurrently intoxicated with lead and monensin at different proportions. Birds in groups L0 and 0L received lead only, while those in groups M0 and 0M received monensin only at different dose levels. An overview of the experiment is presented in Table 1. Three or four birds from each group were sacrificed at weeks 1–4 post exposure (PE) to monensin or lead, and blood and serum samples were collected.

Haematological and biochemical methods

Red blood cell (RBC) and white blood cell (WBC) counts (using a haemocytometer), haemoglobin (Drabkin method) and haematocrit (by microhaematocrit technique) were determined as described by Coles (1986). Serum was used to determine alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by the method of Reitman and Frankle (Lachma, N. P. Brno Chempol, Czech Republic), alkaline phosphatase (AP) by the method of Bassey and Lowery (Biochem. POCH, Gliwice, Poland), total protein by the Biuret method (Biochem. POCH, Gliwice, Poland), and cholesterol by a colorimetric method (Biochem. POCH, Gliwice, Poland).

Table 1

Overview of the experiment

Groups[1]	1st toxic substance in feed (mg/kg)	2nd toxic substance[2] (mg/kg body weight)	Combination
M0	monensin (240)	nil	monensin-nil
ML	monensin (240)	lead (400)	monensin-lead
L0	lead (1200)	nil	lead-nil
LM	lead (1200)	monensin (40)	lead-monensin
0M	nil	monensin (40)	nil-monensin
0L	nil	lead (400)	nil-lead
00	nil	nil	control

[1] Each group consisted of 15 chickens;
[2] Given through the oral route on alternate days for four weeks following a four-week adaptation period on amended feeds

Comparisons were made between the groups which received the same chemical as a second toxic substance (lead or monensin exposure) and their counterparts which received a single or no toxic substance. The data were subjected to an analysis of variance and the group means were compared by Duncan's multiple range test at the probability level of 0.05 (Steel and Torrie, 1980).

Results

Lead exposure

Haematological parameters of the different groups during lead exposure are presented in Table 2. These parameters in birds given either monensin (Group M0) or lead (Group 0L) remained nonsignificantly different from control (Group 00). A concurrent administration of both monensin and lead (Group ML), however, resulted in a significant decrease in haematocrit, haemoglobin and RBC count compared with the control birds at all stages of the experiment.

Biochemical parameters in birds exposed to lead varied nonsignificantly among the different groups (Table 3).

Table 2

Haematological parameters in different groups of broiler chicks following lead exposure (mean ± SD)

Groups (dietary addition)	Lead* mg	Haematocrit %	Haemoglobin g/l	RBC count 10^{12} /l	WBC count 10^{9} /l
		At one week PE			
M0 (mon)*	0	24.3 ± 2.1b	74.6 ± 9.0bc	1.99 ± 0.1ab	16.1 ± 1.3a
ML (mon)	400	21.0 ± 2.0a	54.0 ± 5.0a	1.73 ± 0.1a	21.7 ± 1.6ab
0L (–)	400	27.7 ± 1.5c	86.3 ± 8.0c	2.48 ± 0.2c	29.2 ± 8.5c
00 (–)	0	29.0 ± 0.0c	88.3 ± 0.2bc	2.36 ± 0.0bc	20.8 ± 1.9ab
		At two weeks PE			
M0 (mon)	0	30.7 ± 1.5b	90.9 ± 0.7b	2.52 ± 0.3b	17.3 ± 7.8
ML (mon)	400	22.0 ± 1.0a	57.0 ± 5.0a	1.86 ± 0.2a	20.0 ± 1.8
0L (–)	400	28.7 ± 1.5b	85.0 ± 16.0b	2.11 ± 0.2ab	16.3 ± 3.5
00 (–)	0	29.7 ± 0.6b	88.3 ± 0.2b	2.46 ± 0.2b	20.7 ± 3.8
		At four weeks PE			
M0 (mon)	0	27.3 ± 0.6b	91.0 ± 3.0b	2.06 ± 0.2b	21.1 ± 2.4
ML (mon)	400	20.3 ± 3.1a	52.4 ± 4.0a	1.39 ± 0.5a	22.4 ± 2.6
0L (–)	400	27.3 ± 0.6b	83.3 ± 2.0b	2.17 ± 0.5b	18.8 ± 5.5
00 (–)	0	28.3 ± 0.6b	87.6 ± 6.0b	2.31 ± 0.1b	22.8 ± 3.7

* oral doses given per kg body weight orally on alternate days; ** monensin

Values in each column followed by different letters are statistically significant ($P < 0.05$)

Table 3

Serum biochemical parameters in different groups of broiler chicks following lead exposure (mean ± SD)

Groups (dietary addition)	Lead* mg	ALT IU/l	AST IU/l	Total protein g/l	Cholesterol mmol/l	AP IU/l
			At one week PE			
M0 (mon)**	0	8.2 ± 1.0b	243 ± 67	35.6 ± 1.5a	3.1 ± 0.3	249 ± 096a
ML (mon)	400	9.5 ± 3.2b	278 ± 24	40.5 ± 1.0ab	3.0 ± 0.3	261 ± 033a
0L (–)	400	5.7 ± 3.2ab	306 ± 43	43.3 ± 4.2b	3.2 ± 0.8	579 ± 055b
00 (–)	0	3.5 ± 0.6a	184 ± 26	35.6 ± 1.5a	2.2 ± 0.2	280 ± 018a
			At two weeks PE			
M0 (mon)**	0	2.8 ± 0.0a	230 ± 21	33.1 ± 0.6a	3.1 ± 0.3	165 ± 042a
ML (mon)	400	7.2 ± 4.8b	240 ± 63	32.9 ± 4.0a	2.8 ± 0.4	410 ± 082b
0L (–)	400	1.3 ± 0.5a	245 ± 49	39.6 ± 3.0b	2.4 ± 0.5	356 ± 126ab
00 (–)	0	3.5 ± 0.6a	164 ± 41	37.5 ± 1.3b	2.4 ± 0.1	247 ± 018a
			At four weeks PE			
M0 (mon)**	0	4.4 ± 3.0	327 ± 67b	34.3 ± 4.6	2.7 ± 0.6	311 ± 045
ML (mon)	400	4.7 ± 1.9	283 ± 11b	33.3 ± 1.7	3.1 ± 0.4	198 ± 015
0L (–)	400	6.0 ± 2.1	278 ± 32b	32.6 ± 2.2	3.1 ± 0.3	318 ± 098
00 (–)	0	2.5 ± 1.1	146 ± 10a	36.9 ± 2.6	2.6 ± 1.1	259 ± 031

*oral doses given per kg body weight orally on alternate days; ** monensin;
Values in each column followed by different letters are statistically significant ($P < 0.05$)

Table 4

Haematological parameters in different groups of broiler chicks following monensin exposure (mean ± SD)

Groups (dietary addition)	Monensin* mg	Haematocrit %	Haemoglobin g/l	RBC count 10^{12} /l	WBC count 10^{9} /l
			At one week PE		
L0 (lead)	0	28.7 ± 1.5	93.0 ± 3.6	2.62 ± 0.3	18.6 ± 2.2
LM (lead)	40	28.3 ± 3.2	88.7 ± 9.8	2.30 ± 0.4	21.4 ± 4.2
0M (–)	40	32.3 ± 6.5	84.3 ± 7.0	2.27 ± 0.1	17.6 ± 1.4
00 (–)	0	29.0 ± 0.0	88.3 ± 2.3	2.36 ± 0.0	20.8 ± 1.9
			At two weeks PE		
L0 (lead)	0	27.3 ± 1.5b	83.3 ± 3.2b	2.47 ± 0.5	23.7 ± 2.1b
LM (lead)	40	22.7 ± 2.1a	67.0 ± 3.4a	1.98 ± 0.3	14.3 ± 1.1a
0M (–)	40	28.3 ± 0.3b	81.3 ± 0.6b	2.25 ± 0.1	22.4 ± 5.4b
00 (–)	0	29.7 ± 0.6b	88.3 ± 2.3b	2.46 ± 0.2	20.7 ± 3.8b
			At four weeks PE		
L0 (lead)	0	25.0 ± 1.0b	78.7 ± 4.9ab	2.03 ± 0.2	17.8 ± 1.1b
LM (lead)	40	22.3 ± 1.5a	62.7 ± 12.4a	2.01 ± 0.5	13.3 ± 2.4a
0M (–)	40	25.3 ± 1.2b	79.0 ± 5.2ab	1.98 ± 0.0	11.6 ± 2.7a
00 (–)	0	28.3 ± 0.6c	87.7 ± 6.0b	2.31 ± 0.1	22.8 ± 3.7c

*oral doses given per kg body weight orally on alternate days;
Values in each column followed by different letters are statistically significant ($P < 0.05$)

Table 5

Serum biochemical parameters in different groups of broiler chicks following monensin exposure (mean ± SD)

Groups (dietary addition)	Monensin* mg	ALT IU/l	AST IU/l	Total protein g/l	Cholesterol mmol/l	AP IU/l
			At one week PE			
L0 (lead)	0	5.2 ± 3.6	369 ± 134ab	44.8 ± 4.5c	3.48 ± 0.3b	560 ± 114b
LM (lead)	40	5.7 ± 3.3	320 ± 135b	42.5 ± 2.3bc	3.13 ± 0.4b	499 ± 031b
0M (–)	40	4.8 ± 3.3	472 ± 149b	43.9 ± 7.4c	2.29 ± 0.1b	214 ± 018a
00 (–)	0	3.5 ± 0.6	184 ± 026a	35.6 ± 1.5ab	2.17 ± 0.2a	280 ± 018a
			At two weeks PE			
L0 (lead)	0	3.5 ± 0.6	189 ± 130ab	37.6 ± 2.4a	2.73 ± 0.4ab	419 ± 101b
LM (lead)	40	6.6 ± 1.7	556 ± 099c	38.0 ± 1.7a	3.57 ± 0.4c	338 ± 067ab
0M (–)	40	4.7 ± 2.8	376 ± 075bc	41.1 ± 6.6b	3.15 ± 0.2bc	120 ± 058a
00 (–)	0	3.5 ± 0.6	164 ± 041a	37.5 ± 1.3a	2.41 ± 0.1a	247 ± 018a
			At four weeks PE			
L0 (lead)	0	6.5 ± 0.8c	247 ± 059ab	39.6 ± 0.3a	3.24 ± 0.2b	1024 ± 133b
LM (lead)	40	3.0 ± 1.0ab	292 ± 063b	37.7 ± 2.8a	3.81 ± 0.4b	231 ± 055a
0M (–)	40	5.0 ± 2.0bc	465 ± 140c	37.8 ± 1.4a	4.90 ± 1.4c	281 ± 113a
00 (–)	0	2.5 ± 1.1a	146 ± 010a	37.0 ± 2.7a	2.12 ± 0.3a	259 ± 031a

*oral doses given per kg body weight orally on alternate days;
Values in each column followed by different letters are statistically significant ($P < 0.05$)

Monensin exposure

Haematological parameters (Table 4) showed a significant decrease in birds given monensin and lead concurrently (Group LM), compared with those given either lead (Group L0) or monensin (Group 0M) as well as with the control (Group 00).

The biochemical parameters are presented in Table 5. An increase in ALT, AST and cholesterol levels was observed in birds given lead (Group L0), monensin (Group 0M) or both substances concurrently (Group LM) when compared with the control birds (Group 00) at the 2nd and 4th week PE.

Discussion

The ingestion of lead is reported to induce anaemia and alterations in different biochemical parameters in cockerels (Frarson and Custer, 1982), Japanese quails (Morgan et al., 1975), pigeons (Anders et al., 1982) and waterfowl (O' Halloran et al., 1988; Pain and Rattner, 1988). In the present study, administration of lead acetate to broiler chicks caused no significant alterations in haematological and biochemical parameters. This could be due to either the lower doses or the shorter duration of administration of lead acetate. It has been reported that chicks have an unusually high resistance to plumbism and individual birds may even seem not to respond to toxic doses (Knowles and Donaldson, 1990). Higher doses of monensin in the present study when administered alone resulted in decreased haematocrit value and increased ALT, AST, and cholesterol levels, indicating the harmful effects of the compound on various systems of the body. Increases in ALT and AST have been observed in chicks (Horovitz et al., 1988) and turkeys (Koncicki et al., 1987), but no effect of monensin upon haematocrit and serum cholesterol has been reported in chicken.

Concurrent administration of lead and monensin has caused a remarkable depression of haematocrit, haemoglobin levels and RBC counts. These depressions were significantly more severe when compared with birds administered either lead or monensin alone at the same doses. These observations indicate the presence of an interaction between monensin and lead, leading to the enhancement of toxic reaction. Lead is known to produce anaemia in birds (Morgan et al., 1975; Frarson and Custer, 1982; Pain and Rattner, 1988). It can be stated that the presence of monensin in feed potentiated the toxic effects of lead which at the same dose levels otherwise failed to produce any significant depression in the haematological parameters. Similarly, depression was more severe in birds kept on a lead-containing feed and exposed to monensin. In a previous report we also reported an interaction between lead and monensin, as evidenced by the increased

accumulation of lead and iron in the liver of broiler chicks (Khan et al., 1993*b*). The present results further support our previous findings.

In conclusion, the results of the present study demonstrated the potentiation of the toxic reaction when monensin and lead were administered concurrently. Whether such an interaction is also present between the recommended levels of monensin and the toxic levels of lead present in polluted areas is not known. Further experiments using lead levels similar to those present in polluted areas may be helpful for demonstrating whether these results are valid in the field.

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EFFECT OF HIGH- AND LOW-NITROGEN DIETS ON CELL ULTRASTRUCTURE IN THE CORTICAL PART OF THE OVINE KIDNEY

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The ultrastructure of cells of the renal cortex was studied in two groups of sheep fed a low- and a high-nitrogen diet, respectively. In the low-nitrogen group alterations were detected on the mitochondriae. In the cells of the proximal and distal tubules, dilatation of the endoplasmic reticulum as well as vacuolation of the cytoplasm could be observed.

Key words: Low- and high-nitrogen diet, sheep, kidney, ultrastructure

Ruminants are able to use the single non-proteinous components of the diet to produce proteins of high value. In their organism, the kidneys play an important role in the mechanism of nitrogen preservation. In contrast to those of other species, ruminant kidneys have the power to retain urea and return it into the blood circulation.

Materials and methods

Two groups of sheep (age: 1–2 years) were fed a high-nitrogen (7 animals) and low-nitrogen (8 animals) diet for 9 weeks, respectively. A third group was kept under normal conditions as control. After the experimental period, the animals' kidneys were perfused with 3% glutaraldehyde. After primary fixation, the sections were postfixed in osmium tetroxide, dehydrated with ethanol, saturated in embedding media and finally embedded in Durcupan. Ultrathin sections were prepared, contrasted with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) and examined in a JEM 1200 EX electron microscope.

Results

Ultrastructure of cells in the renal cortex of sheep fed a high-nitrogen diet

The cells of the proximal tubule are of a high slender cylindrical shape. In their apical part a typical brush border can be observed (Fig. 1), made of a number of high, slender, parallel-running villi. Long, rod-like mitochondriae (Fig. 2),

running perpendicularly to the basal membrane, are a typical finding. The basal membrane appears to be stratified and creates deep and narrow folds that encroach deep into the cells, thus increasing their surface. Beneath the brush border, pinocytotic sacs can be found (Fig. 1). The nuclei of the cells of the proximal canaliculus are large and circular.

In comparison with the above cells, the cells of the distal canaliculus are lower, with the brush border missing from the apical surface. The microvilli are short, sparsely located and unevenly distributed. The borders between the cells are more distinct. The mitochondriae are arranged into long columns. The renal body is made of a glomerulus of capillaries and is covered with a bilayered capsule. The visceral lamina closely adjoins the capillary walls. The endothelial lining of the glomerular capillaries creates a filtrating-crevicular membrane with longer cytoplasmic processes, the trabecules. Flat cells termed podocytes pool on the outer side of the blood capillaries and develop long primary cytoplasmic processes; from the latter, parallel, thinner secondary processes called pedicles protrude in perpendicular direction. The continuous basal membrane, the endothelial lining of the glomerular capillaries and the layer of podocytes constitute the filtrating-crevicular membrane.

Ultrastructure of cells in the renal cortex of sheep fed a low-nitrogen diet

The apical part of the cells of the proximal tubules was characterized by a brush border made of slender microvilli (Fig. 3). Below the brush border pinocytotic sacs were observed (Fig. 3). On the cell surface of the distal tubules small numbers of short microvilli were found. The cell cytoplasm of both tubules contained large numbers of mitochondriae; however, the structure of the latter appeared to be disturbed (Fig. 4), revealing matrix vacuolation and a reduced number of cristae. In addition to mitochondriae, numerous vacuoles (Fig. 4), lysosomes (Figs 3 and 4) and dilated cisterns of endoplasmic reticulum were found in the cytoplasm. The stratified basal membrane developed deep invaginations into the cell cytoplasm, creating together with the endothelial lining of the glomerular capillaries and the podocytes the filtrating-crevicular membrane (Fig. 5).

Discussion

The ovine kidney is characterized by the ability to retain nitrogen and use it in protein synthesis. The nitrogen of the endogenous urea is of great importance for ruminal protein synthesis. A close correlation has been documented between nitrogen metabolism in the rumen and the excretion of urea by the kidneys. Decreased excretion of urea via the urine facilitates and accompanies the increased secretion of endogenous urea into the rumen. The kidneys play a remarkably

important role in the conversion of nitrogenous substances in the ruminant organism. In clearance experiments Leng et al. (1985) found that sheep are able to significantly increase the tubular transport capacity for urea during a low nitrogen intake. According to Schmidt-Nielsen et al. (1984), under certain conditions

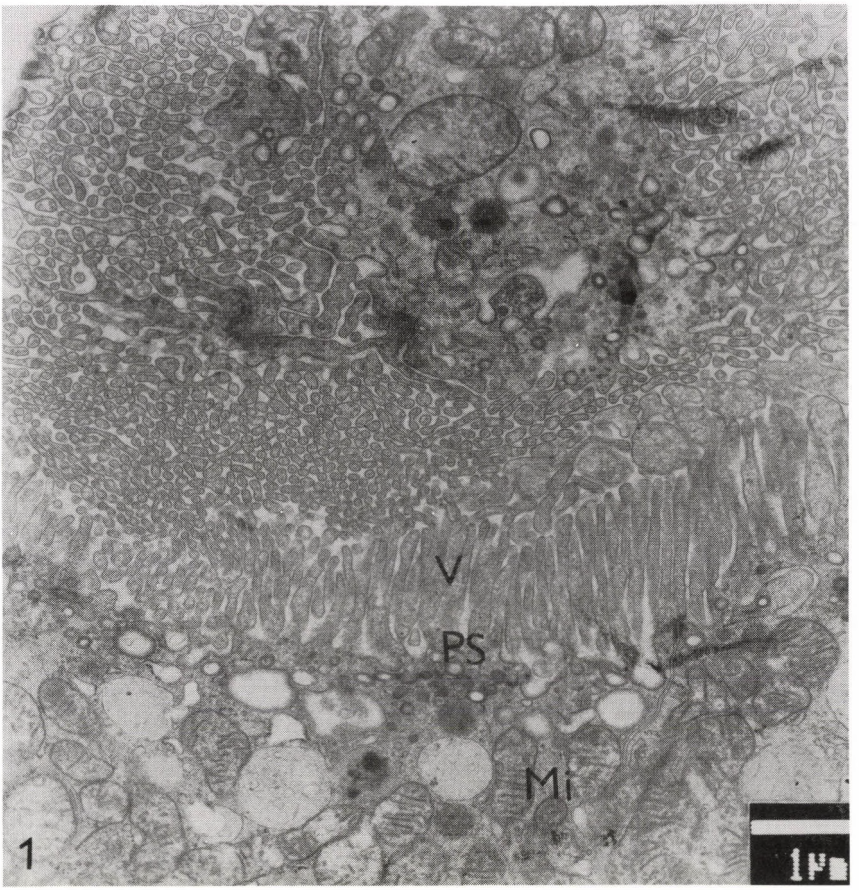

Fig. 1. Electron micrograph of a part of the proximal tubule in sheep fed a high-nitrogen diet. On the apical cell surface a typical brush border can be observed, which is made of a number of high and parallel-running villi (V). PS: pinocytotic slender sacs, Mi: mitochondria

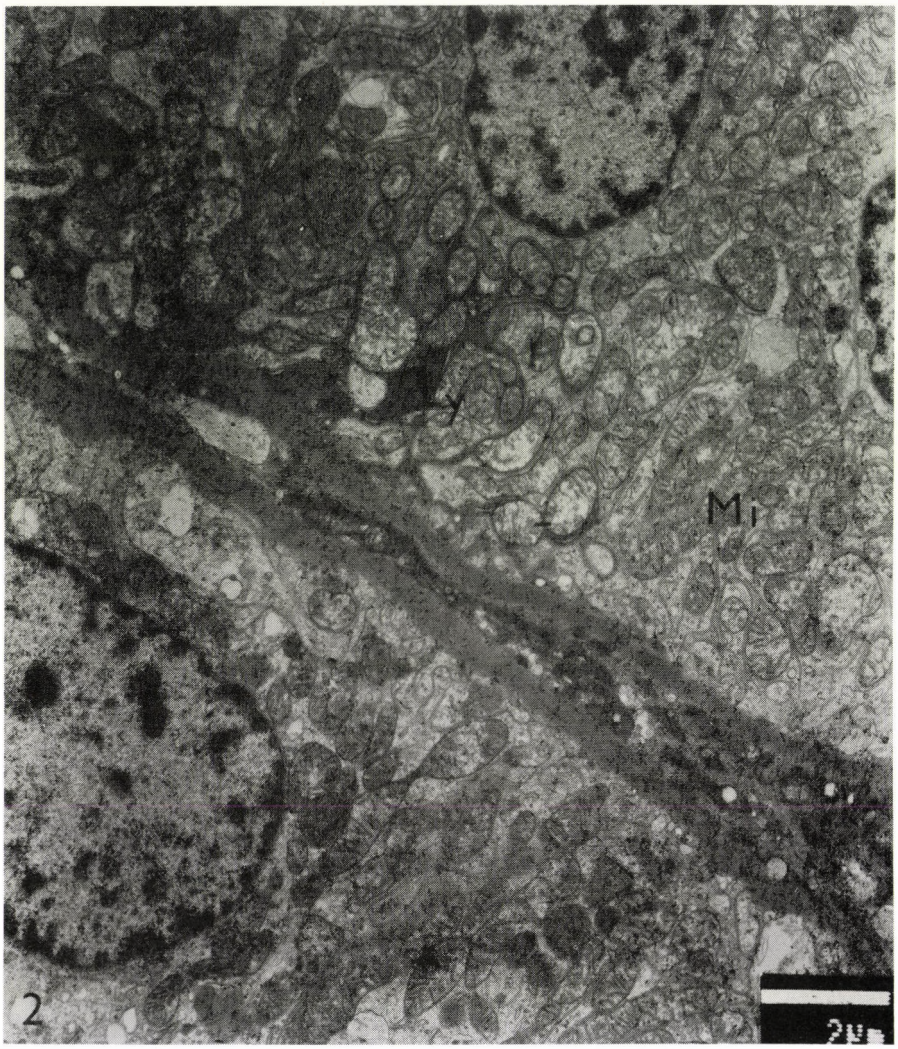

Fig. 2. Electron micrograph of a part of the proximal tubule in sheep fed a high-nitrogen diet. Long, rod-like mitochondriae located perpendicularly to the basal membrane are a typical finding. Mi: mitochondria, Ly: lysosome

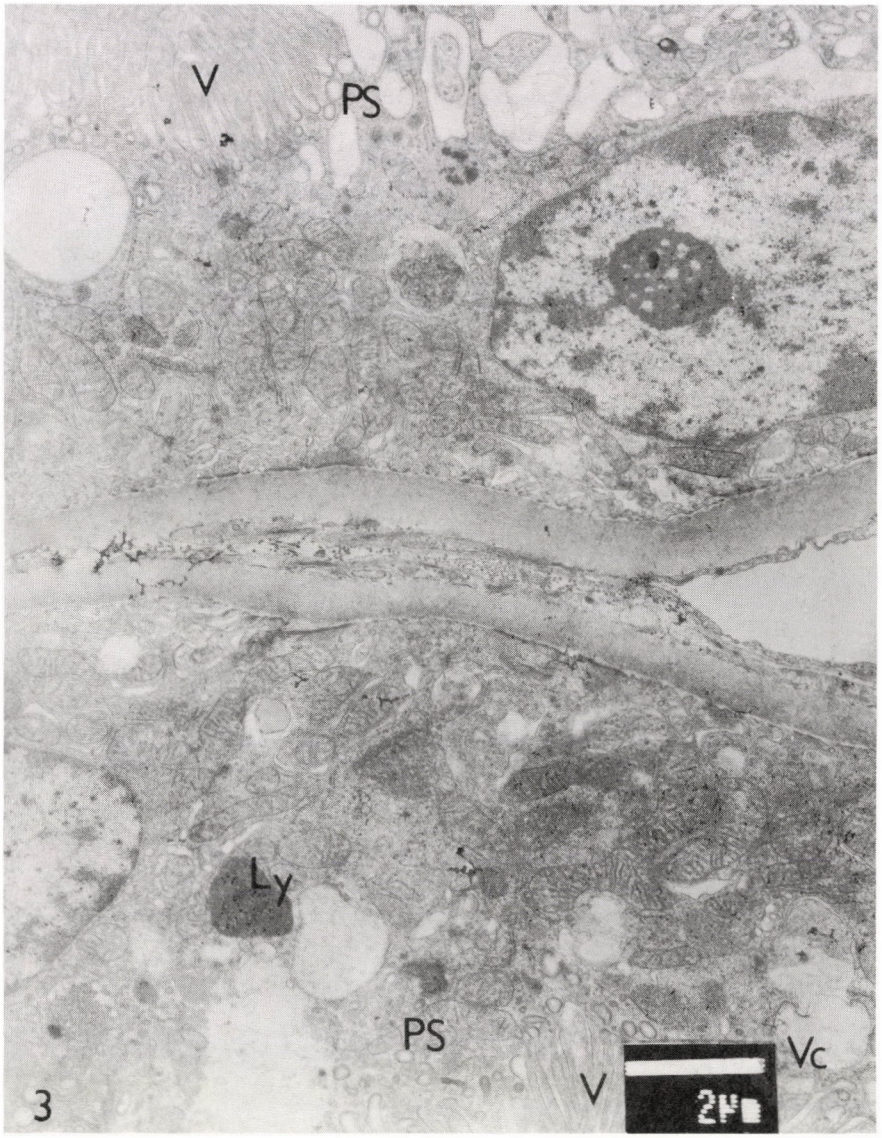

Fig. 3. Electron micrograph of the apical part of the cells of the proximal tubule in sheep fed a low-nitrogen diet. Note a brush border made of slender microvilli. Below the brush border pinocytotic sacs can be observed. Vc: vacuoles, Ly: lysosomes

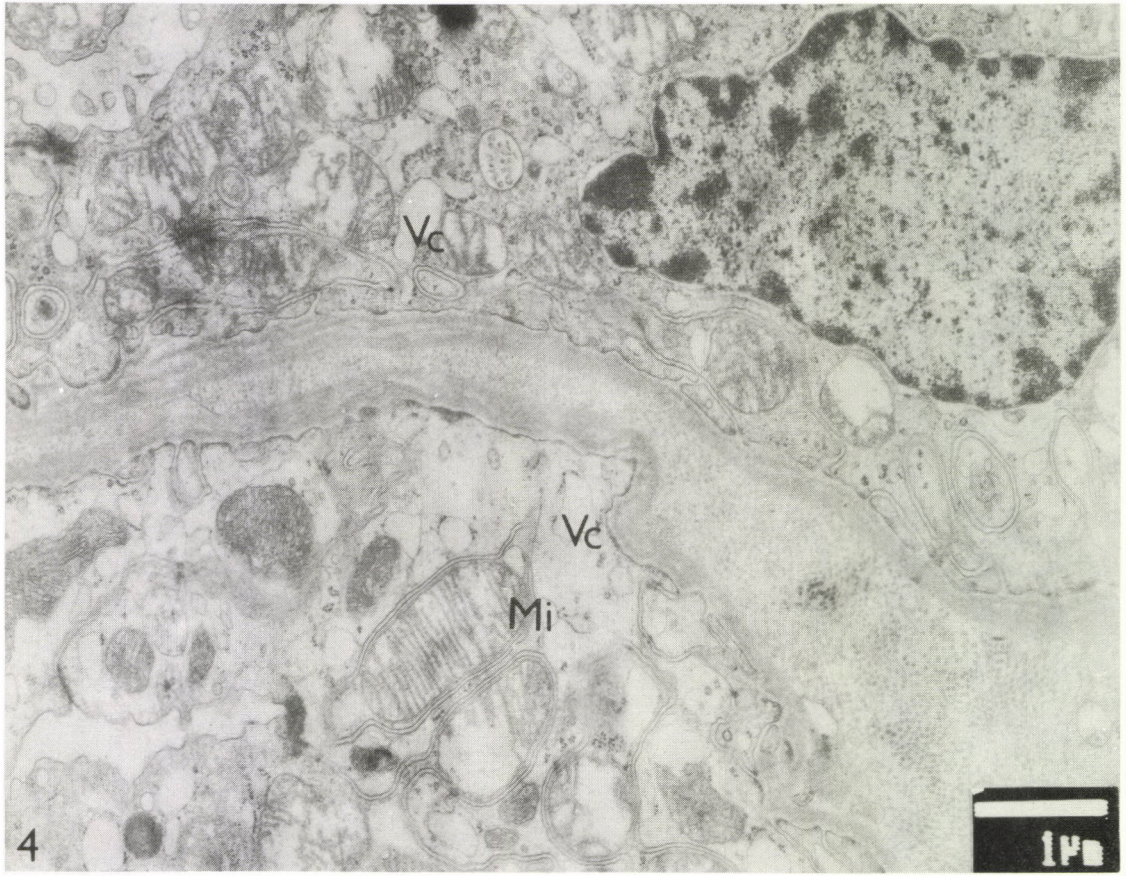

Fig. 4. Electron micrograph of a part of the proximal tubule in sheep fed a low-nitrogen diet. The cell cytoplasm contains large numbers of mitochondriae, revealing matrix vacuolation and reduced numbers of cristae. Mi: mitochondria; Vc: vacuoles

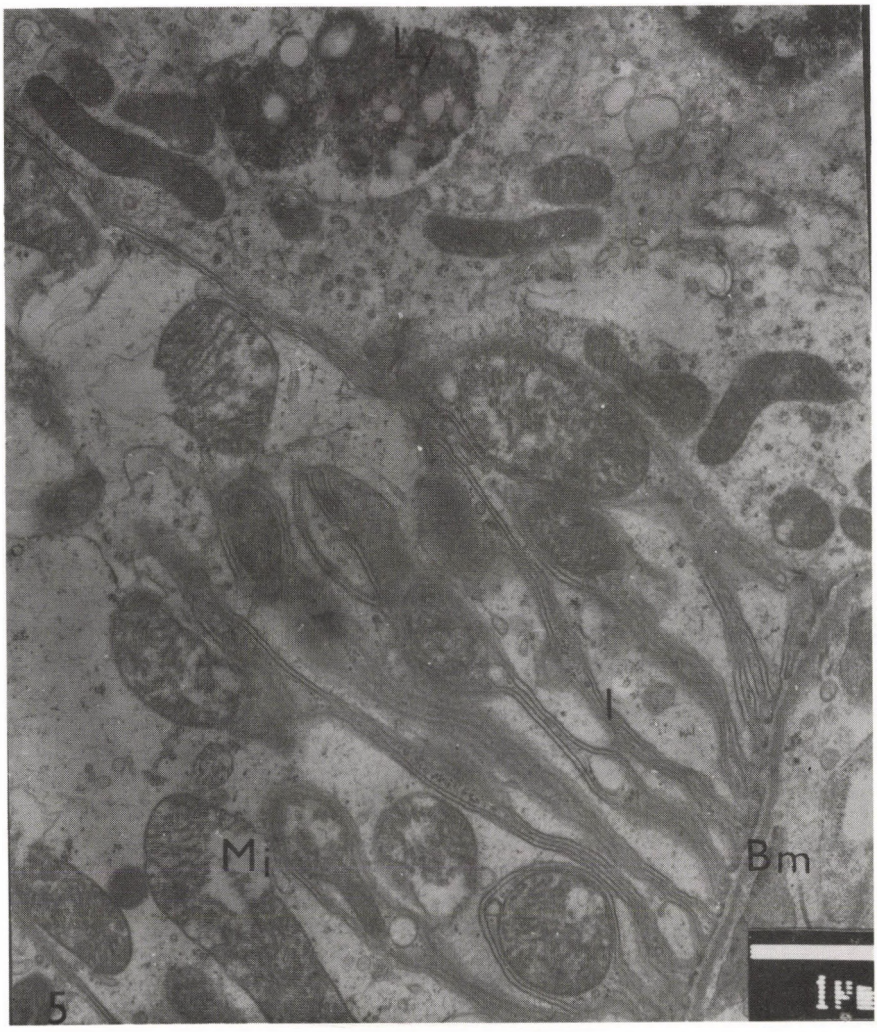

Fig. 5. Electron micrograph of a part of the proximal tubule in sheep fed a low-nitrogen diet. The stratified basal membrane developed deep invaginations into the cell cytoplasm. Bm: basal membrane, I: invaginations, Mi: mitochondria; Ly: lysosome

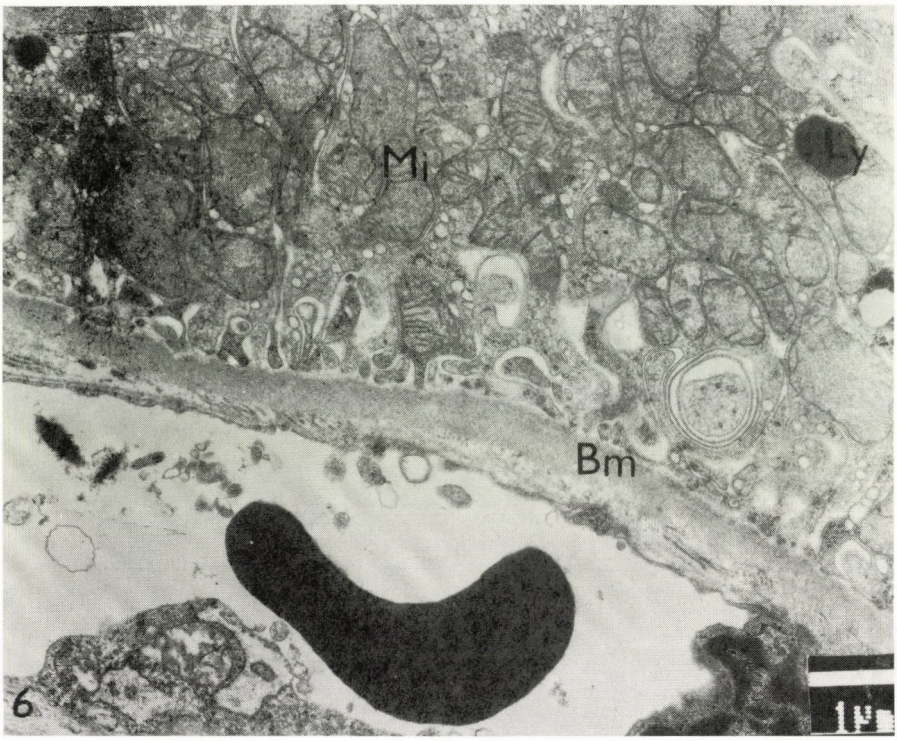

Fig. 6. Electron micrograph of a part of the proximal tubule of the control group. The cell cytoplasm contains lysosomes and a large number of mitochondriae. The basal membrane is stratified and lies to the blood capillary. Mi: mitochondria, Ly: lysosome, Bm: basal membrane, Bc: blood capillary

the epithelium of the renal tubules reveals a considerable capacity to actively reabsorb almost all filtrated urea. Morphologically, this claim was supported by the different thickness of the wall and width of the lumen of canaliculi in sheep on low- and high-nitrogen diets. Data on the role of the rumen wall and the kidneys in nitrogen metabolism were published by Leng et al. (1991). Our work supplies additional morphological data.

The apical part of the cells of the proximal tubules revealed a brush border. This finding suggested that the increased resorption surface of the cells was not disturbed. The alterations observed in the cytoplasm and on the mitochondriae pointed to metabolic physiological conditions. Similarly, dilatation of the endoplasmic reticulum and vacuolation of the cytoplasm were the outcome of altered metabolic processes in the cells. The above changes were found in experimental sheep of the low-nitrogen group. The increased tubular reabsorption of urea in

sheep fed a low-nitrogen diet (Leng et al., 1985), however, suggests the activation of some form of facilitated urea transport in the more distal segments of the nephron. The accumulation of urea in the inner strip of the outer medulla in these animals supports this conclusion.

According to physiological observations the kidneys play a fundamental role in the mechanism of nitrogen conservation during nitrogen metabolism thus, the above capacity also manifests itself in the morphological changes occurring in the cells of the proximal and distal tubules of the ovine kidney. This assumption requires further evidence to be obtained by studying the cells in the medullar zone of the kidneys.

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VARIATIONS IN MILK SOMATIC CELL COUNT AND HAEMATOLOGIC VALUES OF DAIRY COWS DURING LACTATION

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Variations in milk somatic cell count (SCC) and haematologic values were studied in a dairy cow colony of the Holstein-Friesian and Hungarian Red-Spotted breeds (n=23) from May 1992 to July 1993. Milk and blood samples were taken approximately at monthly intervals and data were assigned into ten lunar months of lactation. After a maximum in month I, SCC dropped abruptly in month II and continued to decline through the subsequent four months, then it again tended to increase through months VII-X. The SCCs varied within the physiological range throughout the lactation period parallel with red blood cells and white blood cells constituting a major segment of the somatic cell population. Positive correlations were recorded between SCC and the blood variables (packed cell volume, red blood cell count, white blood cell count). The lactation pattern of SCC was comparable to previous observations.

Key words: Milk, somatic cell count, haematologic values, lactation, dairy cows

The somatic cell count (SCC) of raw milk is a useful index of mastitis and milk secretion disturbances. In milk produced by healthy cows, SCC varies from 100 to 200 x 10^3 cells per ml, whilst 300 to 500 x 10^3 cells per ml may indicate subclinical mastitis and a count over 500 x 10^3 cells per ml suggests clinical mastitis (Miller, 1973).

In the last decade increased attention has been focused on milk quality, especially on SCC throughout Europe (Christensen, 1983; Gehring, 1988; Kennedy, 1990). According to the Hungarian Raw Milk Standard enacted in 1991, milk of extra and first quality category, resp., must contain less than 400 to 500 x 10^3 somatic cells per ml with a total germ count of 100 to 300 x 10^3 per ml (MSZ 3698 N 17, 1991). The price of raw milk also varies with SCC. Lowering SCC in raw milk is therefore important for the dairy farmers.

SCC is known to vary by the speed of milking (Moore et al., 1993), parity, stage of lactation and major changes in physiological status (e.g. heat, galactosta-

sis, injuries to udder) as well as by abrupt dietary changes (Csiszár, 1954; Merényi and Wagner, 1989).

This paper presents variations in milk SCC parallel with the haematologic values of a dairy cow colony during the lactation period.

Materials and methods

Animals. A colony of 23 first- and/or second-lactation cows of the Holstein-Friesian and Hungarian Red-Spotted breeds were examined from May 1992 to July 1993. Animals were kept in a cow-house of the Dairy Farm of the Gödöllő University of Agricultural Sciences. They were provided a cow ration supplemented with corn silage, alfalfa or meadow hay, green alfalfa, green rye and corn cutting, as well as drinking water.

Samples. Milk and blood samples were taken approximately at monthly intervals.

Milk was collected from afternoon (16:00) and morning (5:00) milkings using a Tru Test milk sampler. The combined individual milk samples were analyzed for SCC with a FOSSOMATIC 360 cell counter.

Blood was taken from the jugular vein between 8:00 and 9:00 a.m. Heparinized blood was used for the quantitative blood parameters and native blood served for blood films.

Packed cell volume (PCV) was determined by the micromethod (Frankel et al., 1970). Haemoglobin (Hb) concentration was measured by the cyanhaemoglobin method (Richterich, 1969). Red blood cell (RBC) and white blood cell (WBC) counts were determined according to the method of Hegedűs (in: Ormay, 1972). A differential leucocyte count was appraised from 100 cells on May-Grünwald and Giemsa stained blood films.

Biometry. Data were assigned into ten lunar months of lactation. Means and standard deviations were computed for all ten months and compared by Student's *t*-test (Sváb, 1973). Values for SCC and the blood variables (RBC, WBC, PCV) were analyzed by Pearson's bivariate correlation analysis as outlined in SPSS (SPSS Inc., 1990).

Results

Variations in milk SCC, as well as in PCV, RBC count, haemoglobin and WBC count during lactation are presented in Table 1.

Table 1

Variations in milk somatic cell count (SCC) and quantitative haematologic values of dairy cows during lactation (mean ± SD)

Lactation month	(n)	PCV l/l	RBC 10^6/ml	Hb mval/l	WBC 10^3/ml	SCC 10^3/ml
Weeks 1–2	(9)	0.30±0.03	6.66±0.68	6.26±0.79	5.59±1.69	747.00±676.32
Weeks 3–4	(8)	0.32±0.03	6.74±0.66	6.17±0.79	5.40±1.61	339.88±309.19
I	(17)	0.31±0.03	6.70±0.68	6.22±0.79	5.50±1.66	555.41±573.10
II	(17)	0.27±0.03	5.99±0.80	5.56±0.66	5.38±1.75	277.41±367.12
III	(15)	0.25±0.02	5.58±0.70	5.23±0.45	4.32±1.26	252.73±360.66
IV	(15)	0.25±0.03	5.42±0.64	5.05±0.58	4.46±0.95	191.73±215.55
V	(17)	0.24±0.02	5.28±0.84	4.94±0.70	4.60±1.34	213.47±231.64
VI	(16)	0.23±0.03	5.77±0.74	5.04±0.49	4.02±0.92	176.19±173.89
VII	(17)	0.24±0.02	5.71±0.90	5.04±0.71	4.20±0.84	267.59±255.20
VIII	(18)	0.255±0.024	6.14±0.88	5.24±0.71	4.61±1.18	301.39±263.53
IX	(17)	0.26±0.03	6.28±0.74	5.39±0.66	5.28±1.85	326.94±279.49
X	(18)	0.25±0.02	6.17±0.70	5.26±0.56	5.15±1.28	334.28±246.28

Mean SCC of milk was the highest (555.41 x 10^3 cells/ml) in month I, with a peak (747.00 10^3 cells/ml) during the colostral period. It dropped to ca. 50% in month II and continued to decline significantly ($P<0.01$; $P<0.05$) through the subsequent four months with a minimum in month VI (176.19 x 10^3 cells/ml). After that, SCC showed a consistent increase, significant in months IX and X ($P<0.1$; $P<0.05$), and the means (ca. 330 x 10^3 cells/ml) approximated that recorded for weeks 3–4.

The quantitative haematologic parameters showed similar variations. Commencing with maximums in month I, values kept declining during the subsequent four months, then tended to increase through months VII–X. Means for PCV, RBC and haemoglobin were significantly lower ($P<0.001$; $P<0.01$; $P<0.05$; $P<0.1$) through nine months. The difference in WBC count was significant ($P<0.01$; $P<0.1$; $P<0.05$) between months III and VIII, compared to month I.

A Pearson bivariate correlation analysis of the monthly data and the total sample revealed slight, mainly nonsignificant, positive correlations between SCC

Table 2

Variations in the differential blood count of dairy cows during lactation (mean ± SD)

Lactation month	(n)	Relative proportion (%)					Ly : Ne
		Lymphocyte	Monocyte	Neutrophil	Eosinophil	Basophil	
Weeks 1–2	(9)	0.637±0.100	0.053±0.017	0.267±0.110	0.037±0.041	0.006±0.007	2.4:1
Weeks 3–4	(8)	0.640±0.103	0.054±0.021	0.240±0.081	0.060±0.058	0.006±0.008	2.7:1
I	(17)	0.640±0.10	0.054±0.019	0.250±0.100	0.050±0.050	0.006±0.008	2.6:1
II	(17)	0.65±0.12	0.035±0.021	0.260±0.110	0.050±0.030	0.005±0.010	2.5:1
III	(15)	0.70±0.08	0.040±0.021	0.200±0.070	0.050±0.040	0.010±0.010	3.5:1
IV	(15)	0.66±0.10	0.050±0.022	0.240±0.110	0.050±0.040	0.010±0.014	2.8:1
V	(17)	0.67±0.10	0.038±0.020	0.220±0.110	0.060±0.030	0.012±0.011	3.0:1
VI	(16)	0.670±0.140	0.037±0.020	0.240±0.120	0.040±0.020	0.013±0.010	2.8:1
VII	(17)	0.650±0.120	0.044±0.027	0.230±0.050	0.063±0.050	0.013±0.011	2.8:1
VIII	(18)	0.630±0.110	0.051±0.040	0.240±0.110	0.067±0.035	0.012±0.010	2.6:1
IX	(17)	0.630±0.100	0.050±0.020	0.240±0.110	0.064±0.450	0.012±0.011	2.6:1
X	(18)	0.610±0.120	0.034±0.021	0.290±0.120	0.060±0.036	0.006±0.009	2.1:1

and the blood variables (Table 3). Analyzing the monthly means definite and highly significant correlations were found between SCC and PCV as well as between SCC and RBC, and a modest and nearly significant correlation between SCC and WBC throughout the lactation period (Figs 1–3).

The differential blood count (Table 2) was characterized by a 2.6–2.5:1 ratio of lymphocytes to neutrophils in months I–II. In months III through VI the ratios varied between 3.5:1 and 2.8:1 due to increases in the relative proportions of lymphocytes, significant in month III ($P<0.1$). Subsequently, ratios returned to 2.6–2.1:1 in months VIII through X. Proportions of monocytes, eosinophils and basophils showed neither consistent nor significant variations during lactation.

Table 3

Variations in correlations between milk somatic cell count and blood cellular elements of dairy cows during lactation

Month	(n)	SCC – RBC		SCC – WBC		SCC –PCV	
		r	P	r	P	r	P
I	(17)	0.05	0.84	0.40	0.11	0.02	0.93
II	(17)	0.16	0.54	0.07	0.78	0.17	0.52
III	(15)	0.21	0.45	0.67	0.01	0.29	0.30
IV	(15)	0.04	0.88	0.09	0.73	0.21	0.46
V	(17)	0.05	0.85	0.24	0.34	0.04	0.86
VI	(16)	0.04	0.89	0.07	0.79	0.41	0.12
VII	(17)	0.21	0.41	0.06	0.83	0.34	0.19
VIII	(18)	0.14	0.59	0.02	0.95	0.21	0.40
IX	(17)	0.01	0.97	0.12	0.65	0.12	0.64
X	(18)	0.59	0.82	0.09	0.97	0.12	0.65
Total	(167)	0.08	0.31	0.20	0.01	0.11	0.17

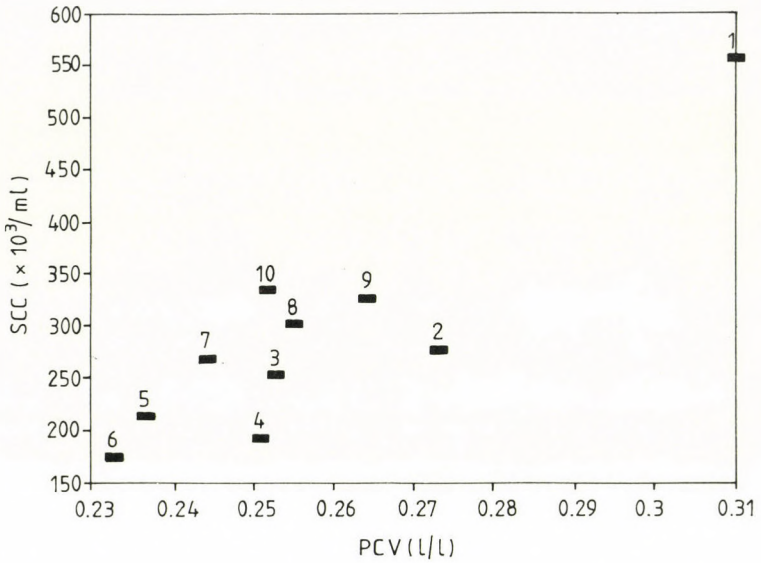

Fig. 1. Correlation between packed cell volume (PCV) and milk somatic cell count (SCC) during lactation. The correlation is statistically significant ($r=0.81$; $P<0.01$). Figures indicate the months of lactation

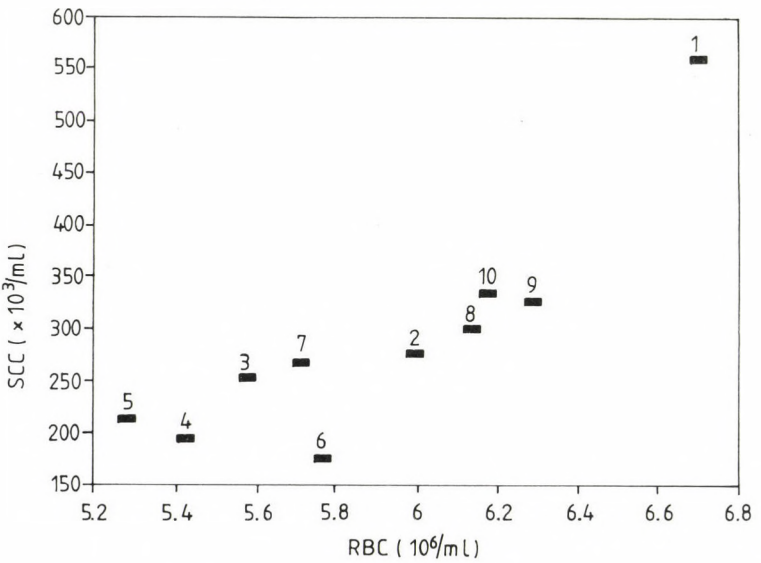

Fig. 2. Correlation between red blood cell (RBC) count and milk somatic cell count (SCC) during lactation. The correlation is statistically significant ($r=0.76$; $P<0.02$). Figures indicate the months of lactation

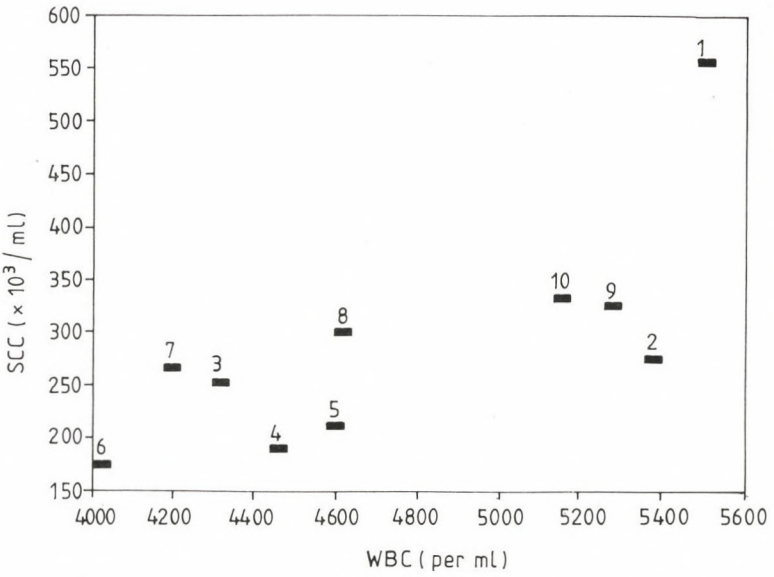

Fig. 3. Correlation between white blood cell (WBC) count and milk somatic cell count (SCC) during lactation. The correlation is statistically non-significant (r=0.54; P>0.10). Figures indicate the months of lactation

Discussion

The variation in milk SCC observed in a dairy cow colony of the Holstein-Friesian and Hungarian Red-Spotted breeds throughout ten months of lactation confirms previous observations. Namely, after an initial peak the SCC is the lowest between months II and V, then it again tends to increase (Bicsérdy and Süle, 1991; Székely, 1993).

In this study, cows were sampled during the first and/or second lactation. However, these periods show SCC variations of similar tendency as reported for 75 cows of the Hungarian Red-Spotted x Holstein-Friesian breed (Székely, 1993).

The SCCs recorded during the lactation period vary within the physiological range, parallel with red blood cells and white blood cells constituting a major segment of the somatic cell population. Despite of high individual variations resulting from small sample size, means for SCC well correlated with those for PCV (r=0.81), RBC (r=0.76) and WBC (r=0.54) during lactation (Figs 1–3).

The initial peak (747 x 10^3 cells/ml) for days 1–13 and the still high mean (ca. 340 x 10^3 cells/ml) recorded for weeks 3–4 correspond to the values given for clinical and subclinical mastitis (Miller, 1973). The fact may be attributed to puerperial involution and commencement of milk secretion (colostral period) asso-

ciated with mobilization of blood cells, as well as to the onset of (mechanical) milking with possible irritation of the udder epithelium. Postpartum oestrus may be another factor. Coincidences of maximal RBC and WBC counts, together with a slight relative neutrophilia (accompanying oestrus) and a modest but nearly significant correlation (r=0.40; P=011) between SCC and WBC in month I (Table 3), also support the phenomenon.

Stressful conditions (corticotropin injection and environmental heat stress, resp.) resulting in moderate blood leucocytosis were also associated with modest increases in somatic cell counts of milk in mastitis-free cows (Wegner et al., 1976).

Increases in SCC as well as in PCV, haemoglobin, RBC and WBC counts in the last months of lactation, together with a slight left shift in differential blood count, may be related to the cows' drying off due to late stages of gestation. SCC is known to increase with the degree of drying-off (Bicsérdy and Süle, 1991). In this study, dry cows (n=13) showed a similar blood count (PCV=0.28 ± 0.03 l/l; RBC=6.22 ± 1.32 x 10^6/ml; haemoglobin=5.95 ± 0.81 mval/l; WBC=4.86 ± 1.28 x 10^3 /ml; ratio of lymphocytes to neutrophils=2.1:1).

We hope that the variation observed in the SCC of milk, supported by the haematological values, may contribute to a better understanding of the lactation pattern of dairy cows.

Acknowledgement

We thank Mr. S. Csányi for correlation analysis of the data.

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BOOK REVIEW

Horst SCHEBITZ, Wilhelm BRASS and Hans-Jürgen WINTZER: *General Surgery for Veterinarians and Students.* Verlag Paul Parey, Berlin und Hamburg, 1993. 2nd completely revised edition. 406 pages, 210 illustrations, 20 of them colour, 20 charts. Price: DM 158.

The list of contributors is extremely honourable: Wilhelm Brass (Hannover), Hans Georg Buschmann (München), Lakus Dammrich (Berlin), Keesj. Dick (Utrecht), Erich Eisenmenger (Wien), Werner Giese (Hannover), Ulrike Matis (München), Urs Schatzmann (Bern), Johann Schaffer (Hannover), Theodor Schliesser (Giessen), Leo-Clemens Schultz (Hannover), Peter Tschudi (Bern), Reinhard Weiss (Giessen), and Hans-Jürgen Wintzer (Berlin).

A total of 210 illustrations, 20 of them colour, as well as 20 charts complete the 406-page text. With its well-arranged structure and the enormous body of knowledge it contains about the science of surgery, the book reveals the authors' basic intention to help veterinary students attain a detailed knowledge of surgery. In its 26 chapters the volume systematically discusses the essential questions and methods of this discipline.

The titles of the chapters reflect the structure and logic of the book. Below the titles are listed in the same order as the authors discuss them. "From the farrier to the veterinary surgeon — introduction to the new age history of veterinary surgery", "Traumatology", "Shock", "Water and electrolyte balance", "Remarkable diseases of the skin, subdermal connective tissue and mucous membranes", "Remarkable diseases of the lymphatic and blood vessels", "Remarkable diseases of the fasciae and muscles", "Remarkable diseases of the tendons, tendon sheaths and bursae", "Congenital diseases of the passive motive organs", "Acquired diseases of the passive motive organs", "Diseases of the joints", "Remarkable diseases of the body cavities", "Remarkable diseases of the nervous system", "Hernias", "Antisepsis and asepsis", "Infections in surgery", "Chemotherapy of bacterial infections", "Tissue transplantation", "The neoplasms", "The biological effect of ionizing radiation", "Instrumental diagnostic tools (X-ray, ultrasound) in surgery", "The use of laser in surgery", "The laboratory diagnostic work in surgery", "Pain-relieving methods", "Bandage study".

The titles listed above may cause the readers to sense a lack of certain details. As the book includes a vast volume of knowledge, the scope of the present summary does not allow us to write a more detailed review.

One may conclude that this book serves as an excellent tool which helps veterinary students attain a fundamental knowledge of surgery. At the same time, it also provides a brilliant opportunity for members of the older generations to expand or perhaps revive the knowledge they once mastered.

The book is too detailed rather than incomplete, but no doubt that both students and veterinary practitioners can use it very well.

József TÓTH

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INSTRUCTIONS TO AUTHORS

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Materials and methods. Describe materials, methods, apparatus, experimental procedure and statistical methods in sufficient detail to allow other authors to reproduce the results. This part may have subheadings.

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Discussion should be focussed on the interpretation of experimental findings. Do not repeat literary data presented in the Introduction or information given in Results. References should be cited as follows: e.g. ... as observed by Faith and Trump (1976); or in parentheses: ... were found (Starr et al., 1978; Manson and Starr, 1979).

Acknowledgement of grants and technical help.

References. Cite only essential references. They should be arranged in alphabetical order of the authors' surnames, without serial numbers. The reference list at the end of the paper should contain

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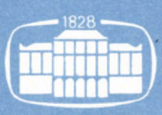

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Proceedings of the International Symposium on Aujeszky's Disease Virus

Budapest, Hungary, 29th–31st August 1993

A. BARTHA
S. EDWARDS
B. LOMNICZI
P.-P. PASTORET
B. LIESS
(Editors)

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Epidemiology and control of ADV

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INTRODUCTION TO THE MEETING

Dear Colleagues, Ladies and Gentlemen,

On behalf of the European Society for Veterinary Virology, it is a great pleasure for me to introduce this meeting in the city of Budapest.

In 1902, in this very city, *Aladár Aujeszky* described for the first time the disease which was to be known under his name. This disease was later also called pseudorabies since Aladár Aujeszky was already able with very simple but accurate tools to discriminate between rabies and this newly recognized neurotropic disease.

From that time on, the study of Aujeszky's disease and its causal virus has been pursued at the Veterinary Medical Research Institute of Budapest, and the tradition of this research of the highest quality has been maintained by our hosts, namely *Adorján Bartha* and *Béla Lomniczi*.

The Bartha strain of Aujeszky's disease virus has played a key role as a reference strain, and for the development of new control measures on a worldwide basis.

The European Society for Veterinary Virology is particularly pleased to sponsor this symposium on Aujeszky's disease virus. It is the first time that the Society has organized a meeting in the Eastern part of Europe; by this means we want to emphasize that our activities relate to the whole of Europe.

We also warmly welcome the participation of our colleagues from other continents.

At this stage, it is noteworthy that Aladár Aujeszky, born in Pest in 1869, graduated 23 years later not as a veterinarian but as a medical doctor.

It is well within the spirit of our Society which aims not just to gather veterinarians interested in virology, but also virologists interested in viruses of veterinary concern.

We consider this Symposium as a major scientific event. It is organized at a turning point in the evolution of the field. In the last few years we have amassed an enormous body of information on Aujeszky's disease virus and its molecular biology; Aujeszky's disease virus is presently the best known of all herpesviruses of veterinary interest. We are now in a position to apply these basic understandings for practical purposes in the control or eradication of the infection.

The importance of this meeting has been recognized by the scientific community, as demonstrated by the participation of so many distinguished scientists from this field.

Akadémiai Kiadó, Budapest

The European Society for Veterinary Virology would like to express our gratitude, firstly to our hosts, *Adorján Bartha, Béla Lomniczi,* the *Hungarian Academy of Sciences,* the *University of Veterinary Science in Budapest;* to the members of the scientific committee for their support and advice; to the Editor of *Acta Veterinaria Hungarica* for agreeing to publish the proceedings and, last but not least, to the local and international sponsors, who have made the whole event possible.

I wish you a very fruitful meeting and a very pleasant stay in Budapest.

Paul-Pierre Pastoret
President,
European Society for Veterinary Virology

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AUJESZKY MEMORIAL LECTURE

A. BARTHA

Veterinary Medical Research Institute, Hungarian Academy of Sciences, H–1143 Budapest, Hungária krt. 21, Hungary

This year we commemorate the 60th anniversary of the death of *Dr. Aladár Aujeszky,* one of the well-known figures of veterinary microbiology. None of us had the possibility to know him personally. We have organized this symposium in honour of him. The disease named after him is known worldwide by veterinarians, but very few of them know Professor Aujeszky's career, scientific activity, and the circumstances of his discovery. In the next minutes, I would like to take the opportunity for presenting the scientific career of this modest, nice man, loved by everyone.

Aladár Aujeszky (born in Budapest, 11th of January, 1869) graduated from the Hungarian Royal Medical University, Budapest in 1892. After gaining his degree, he worked in the practice for three years, first at the surgical department of a hospital, later as an assistant at the clinic for internal diseases of the Medical University. During his practice he got acquainted with the work of clinical laboratories, and became interested in it. In 1895, he joined the Pasteur Institute led by Professor Hőgyes. Throughout his life, he successfully utilized the bacteriological knowledge gained there. His further scientific career was influenced by the time spent in the General Pathological Institute of the Medical University from 1896 as an assistant professor. Here he mainly studied the pathogenesis, pathology and aetiology of human infectious diseases.

In the Medical Pathological Institute he had the opportunity to study the aetiology, clinical signs, pathogenesis, and diagnostic possibilities of human rabies. He writes the following about that time, during his inaugural lecture at the Szent István Academy in 1926: "As a young physician, I worked for 5 years as assistant at the side of Professor Hőgyes, one of the greatest scientists of Hungarian medicine. Several times I was an eyewitness of the terrible pain that rabies-infected people suffered from, whom we could not help in spite of our efforts. Then I decided, with the enthusiasm of a young physician, that I would take my share in the struggle necessary to fight against this terrible illness. One of the tools of this struggle was to introduce the vaccination of domestic animals against rabies in Hungary."

Aujeszky, as an assistant professor, joined the Hungarian Royal Bacteriological Institute led by Professor Preisz in 1901. Professor Preisz was well known in Europe as an expert on rabies. The Hungarian Royal Bacteriological

Institute was part of the Veterinary College, supervised by the Ministry of Agriculture. The building you saw in Hungária körút yesterday was built at the beginning of the century, had the latest equipment, and the internationally known bacteriological experts guaranteed a good performance in education, diagnostics, research, and vaccine production.

At the Institute, medical and veterinary microbiologists worked together and studied similarities and differences in infectious diseases of humans and animals with unified conception, methods and points of view.

Besides his degree in medicine, Aujeszky got a second degree at the Veterinary College in 1903 and was qualified as lecturer of bacteriological diagnostics. He became director of the Bacteriological Institute in 1906, when Professor Preisz, the former director, got a professorship at the Medical University. The Bacteriological Institute had more and more tasks, they successfully studied tuberculosis, paratuberculosis, brucellosis and malleus of humans and animals. The Institute produced more and more medical and veterinary vaccines, diagnostics for the detection of several infectious diseases of humans and animals, and serum against human diphtheria. From 1907 on, Aujeszky was responsible for checking vaccines produced by private companies in the whole country.

His name has been preserved in the international scientific literature by the disease which was named after him. Aujeszky was the first to describe the illness in 1902, and he differentiated it from rabies. The bacteriological and pathological knowledge gained at the Institute led by Professors Hőgyes and Preisz made the discovery possible. Its importance could be assessed only more than half a century later. Aujeszky's work was promoted by the fact that, at the Medical University, he studied the clinical symptoms, pathology and pathogenesis of human and animal (dog, cat, rabbit, mouse) rabies not only in the practice, but also in his own scientific experiments.

Few microbiological experts know the exact circumstances of the discovery of the illness named after him. His detailed description in 1902 in German and Hungarian revealed that he discovered a new infectious disease with unknown aetiology. I presume that it is worthwhile to make you acquainted with the circumstances of this significant discovery.

As I have already mentioned, one of the main tasks of Aujeszky, as an excellent expert in rabies at the Hungarian Royal Bacteriological Institute was to diagnose the rabies of animals, especially of animals that may have been connected to human cases.

In 1902, the brain of a two and a half year old ox was sent to the Institute from a Northern Hungarian farm. Forty-seven oxen were kept in a stable: one of

them showed symptoms similar to rabies, and died in hours. The other 46 oxen remained healthy.

Like in rabies cases, Aujeszky could not detect any bacterium from the brain by different staining and culturing methods. On the other hand, 48 hours after subcutaneous, intramuscular and intraconjunctival inoculation of rabbits with 10% brain suspension, the animals showed symptoms of a disorder of the nervous system, and died hours later. The incubation period was 2–3 times shorter than in rabies cases. At that time he made the same experiments and got the same results with the brain of a dog that had died with symptoms of the nervous system.

On the basis of his experiments with the infectious agent, he described several properties of the virus. He writes in his paper: "The infectious material consists of a living microbe, but it is impossible to culture and to detect it with methods of bacteriological technique known at present. The nervous material containing the infectious *virus* maintains its infectious properties for quite a long time."

By continuous animal experiments, he determined the order of susceptibility of different species. This order is the following: rabbit, dog, guinea pig, mouse. Hen and pigeon were not susceptible. He precluded the possibility of susceptibility of humans, because if humans were susceptible, it would have already been evident from the comprehensive studies carried out.

About the differential diagnosis of the new illness he mentioned that the disease might surely be separated from rabies by experimental infections of animals. Properties of the illness, which are different from rabies, are the shorter incubation period, the faster development of symptoms, the lack of gradual paralysis characteristic of rabies, and the fact that the blood is infectious.

A few years before Aujeszky's paper, Professor Marek observed similar symptoms in cats, and later these symptoms were identifiable with the disease described by Aujeszky. Professor Marek called this illness "paralysis bulbaris infectiosa".

In 1975, Tolnay (in Hungary) described such symptoms in cattle kept on pasture, but he thought that the heat and the lack of water and shade caused the illness. The case described by Tolnay might have been rabies, but the possibility cannot be excluded that it was the disease determined later by Aujeszky. The nervous symptoms observed by Zschokke in Switzerland in 1896 in three cattle were not cleared up.

During his diagnostic studies on several species, Aujeszky did not write about symptoms or illness in pigs. He never carried out experimental infections in pigs, so we might suppose that he had never thought of the possibility that pigs were susceptible to the new illness. On the basis of our present knowledge it is easy to understand why there is no described occurrence of the illness in pigs for more than a decade. Nervous symptoms are very rare in pigs after the weaning

period, but quite frequent among sucking piglets. At that time, veterinarians regarded the nervous illness seen in sucking piglets as symptoms of toxicosis, and general symptoms with fever were explained by other causes.

The occurrence of Aujeszky's disease in pigs was first described in 1914 by Dr. Rátz, professor of pathology. In the case observed by Rátz, older pigs showed nervous symptoms. Aujeszky's disease in pigs was described more and more frequently after proving the fact that the illness had a completely different epidemiology and symptoms in pigs. In the second half of this century Aujeszky's disease became one of the illnesses which caused the biggest economic losses.

It is necessary to mention here that the first successful virus isolation was made in 1910 by Aujeszky's co-worker, Dr. Schmiedhoffer.

Aujeszky's name has been preserved by the illness named after him, and made him well known worldwide. Later, he worked on solving other questions of bacteriology, immunology and vaccine production. As the very first universal Hungarian bacteriologist, his activity covered almost all branches of microbiology, such as the bacterial flora of the seas, the hygienic problems of schools, and questions of practical vaccine production. In his native country Aujeszky got the greatest appreciation for his successful work on rabies. He did a pioneering work in producing a vaccine and organizing vaccinations against rabies of domestic animals. As a result of his work with planned vaccination programmes, Hungary had got rid of rabies in dogs by the end of the 'thirties.

He actively took part in the leadership and work of several medical, veterinary and biological scientific societies. In appreciation of his scientific merits he became a member of the Szent István Academy, and the Wiener Gesellschaft für Mikrobiologie elected him corresponding member. He was awarded several honours.

His fine behaviour, unselfishness, sense of duty, and puritan character elicited warm love and appreciation. Aladár Aujeszky is one of the acknowledged figures of not only the Hungarian but the international veterinary science, whom we are proud of, whose spirit inspires us for further work.

For his memory the Veterinary Research Institute of the Hungarian Academy of Sciences has founded a medal, which occasionally will be awarded to those scientists who have internationally recognised results in studying the pathogenic virus and the illness. The scientists first decorated with the medal include Dr. Tamar Ben-Porat (USA), Dr. Th. C. Mettenleiter (Germany), Dr. A. Gielkens (The Netherlands), Dr. J. T. Van Oirschot (The Netherlands), Dr. János Kojnok (Hungary) and Dr. Béla Lomniczi (Hungary). We hope that the medal, named after Aladár Aujeszky, will help in preserving the memory of this highly respected scientist.

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REVIEW
PSEUDORABIES (AUJESZKY'S DISEASE) VIRUS: STATE OF THE ART
August 1993

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The analysis of Pseudorabies (Aujeszky's disease) virus (PrV) at the molecular level has not only considerably increased the knowledge of PrV genes and gene products but has also had a major impact on the development of new vaccines and eradication strategies. So far, ca. 40 PrV genes have been sequenced which constitute approx. 60% of the PrV genome. Analysis of attenuated live vaccine viruses resulted in the identification of gene products that determine viral virulence and neurotropism which include virus-encoded enzymes, membrane glycoproteins, and protein(s) involved in virus particle formation. Based on these results the first genetically engineered live viral vaccine licensed for use is represented by an attenuated PrV mutant. Studies on viral surface glycoproteins led to the elucidation of some of their functions during the infectious cycle proving their role in attachment, penetration, and release of virions. The finding that some of the glycoproteins are nonessential for viral replication and can be deleted from the virus provided the basis for new eradication programs based on differentiation between infected and vaccinated animals. The recent isolation of PrV mutants unable to spread between animals opens up new ways for construction of safe PrV-based vector vaccines. Taken together, PrV can serve as an excellent example where molecular biological research has a direct impact on the practical aspects of controlling, and eventually eradicating an important animal disease.

Key words: Pseudorabies (Aujeszky's disease) virus, genome, viral glycoproteins, virulence, latency, vaccines

Pseudorabies Virus (PrV), also designated as Aujeszky's disease virus (ADV), represents the causative agent of Aujeszky's disease (AD). Clinical symptoms typical for AD have first been described in the USA in the first half of the 19th century. However, only in 1902 did Aladár Aujeszky in Hungary recognize the disease as a separate illness of viral origin. Pigs represent the only natural reservoir for PrV and are, therefore, regarded as the natural host species. However, most mammals can be infected by PrV with a mortality approaching 100%. As exceptions, horses appear to be highly resistant and single reports of suspected infection of humans remain highly questionable. Generally, after PrV

infection of pigs two different disease pictures may be seen dependent on the age of the animal and the virulence of the virus strain. In piglets, the central nervous system is heavily afflicted, whereas in older pigs mostly respiratory symptoms are seen (reviewed in Pensaert and Kluge, 1989; Wittmann and Rziha, 1989; Wittmann, 1991).

Due to its biological properties PrV has been classified as a member of the subfamily Alphaherpesvirinae of the family Herpesviridae. Other members of this subfamily include the human pathogens herpes simplex virus (HSV) and varicella–zoster virus (VZV), as well as other important animal pathogens such as bovine herpesvirus 1 (BHV-1), equine herpesvirus 1 (EHV-1), and infectious laryngotracheitis virus of poultry (ILTV) (Roizman et al., 1992). The elucidation of DNA sequences of either complete genomes or single genes allowed a reassessment of the classification based on deduced protein sequences. These results confirmed the original grouping of PrV into a genus Varicellovirus encompassing VZV, PrV, BHV-1, and EHV-1 (Roizman et al., 1992). Surprisingly, this approach led to the finding that Marek's disease virus (MDV) is closely related to the alphaherpesviruses (Buckmaster et al., 1988).

Besides its importance as an animal pathogen, PrV infection has become more and more attractive as a model for studying a herpesvirus infection at the molecular level with the availability of an experimentally accessible natural virus–host system. Therefore, our knowledge on the molecular biology of PrV has increased dramatically. This brief review will focus on several aspects where the most significant progress has been made. They include characterization of the viral genome and identification of PrV genes, analysis of viral envelope glycoproteins, identification of viral proteins involved in PrV (neuro)virulence, and the impact of these advances on the construction of novel PrV vaccines.

The Genome

The genome of PrV consists of a linear double-stranded DNA of approx. 150 kbp with a mol. wt. of ca. 95×10^6 d. It is divided into a unique long (U_L) and a unique short (U_S) part with the U_S being bracketed by inverted repeats. This arrangement is classified as a class 2 herpesviral genome (Ben-Porat and Kaplan, 1981). Presence of one set of inverted repeat sequences leads to inversion of the short component and concomitant appearance of PrV DNA in two isomeric forms. Both forms appear to be biologically identical. Close examination has revealed that the U_L region of wildtype PrV can also undergo isomerization which is not dependent on sequence homology between both ends of the U_L. However, normally less than 5% of the genomes of wildtype PrV virions are found with an inverted U_L region (Rall et al., 1991). After passaging in chicken cells, it has sur-

prisingly been shown that the standard genome evolves into a typical class 3 DNA in which the U_L region is bracketed by repeated sequences (Lomniczi et al., 1984*a*). These repeats arise from the translocation and duplication of sequences normally present at the extreme left terminus to the right terminus of the U_L (Lu et al., 1989). The resulting virus mutants gain a growth advantage over wildtype PrV in chicken cells (Reilly et al., 1991).

Whereas complete genomic sequences are known for the human alphaherpesviruses HSV-1 (McGeoch et al., 1985, 1988) and VZV (Davison and Scott, 1986), as well as for EHV-1 (Telford et al., 1992), only partial sequence information is available for PrV. Extrapolation from the known alphaherpesvirus sequences leads to an estimated number of approx. 70 PrV genes. Sequence determination of PrV DNA is greatly hindered by its high content of G+C nucleotides of 73%. Despite these difficulties, up to now ca. 40 PrV genes have been sequenced which constitute approx. 60% of the viral genome (see Table 1). However, only 24 (approx. 34% of the total genome) of these sequences have been published so far. With sequencing continuing in several laboratories, the complete genomic DNA sequence of PrV can be expected to be determined in the not too distant future. Generally, alphaherpesvirus genomes including PrV are arranged in a colinear fashion. It has, however, to be kept in mind that the prototype orientation of the U_L region of HSV runs antiparallel to that of VZV, EHV-1, and PrV (Table 1). In addition, a peculiar feature of PrV DNA is the presence of an internal inversion of a large conserved gene block within the U_L region as compared to HSV, VZV, and EHV-1 (Ben-Porat et al., 1983). Within the gene blocks, however, colinearity is strictly maintained. From the known sequence data one can conclude that for the majority of the genes homologs can be found in all alphaherpesviruses. Therefore, and not to confuse the nomenclature further, newly identified PrV genes are proposed to be named after their HSV homologs according to the nomenclature of McGeoch et al. (1988). This practice is followed throughout this review.

After infection of target cells, transcription of herpesviral genomes proceeds in a coordinated cascade-like fashion (Feldman et al., 1979). The first genes transcribed, even when cellular protein synthesis is blocked by cycloheximide, have been designated as immediate–early (ie) genes. They encode potent transcriptional regulatory proteins. Only one ie-regulated gene has so far been identified in the PrV genome encoding the immediate early protein IE180 (Cheung, 1989*a*; Vlcek et al., 1990; Cheung et al., 1990). Its transcriptional regulatory function has been studied in detail. IE180, after binding to recognition sequences in appropriate viral promotors (Martin et al., 1990; Wu and Wilcox, 1991), recruits the cellular transcription apparatus to these sites leading to efficient repeated initiation of transcription (Ahlers and Feldman, 1987; Workman et al., 1988). A similar mecha-

nism has been found for its HSV-homolog, ICP4. In HSV, other IE-proteins have been identified. Homologous genes found in PrV include EP0, a homolog of HSV-ICP0, that is regulated as an early gene in PrV (Cheung, 1991); RSp40, a homolog of HSV ICP22, probably also regulated as an early gene in PrV (Zhang and Leader, 1990a); and UL54, encoding a protein homologous to HSV ICP27 (Baumeister and Mettenleiter, unpublished). How the latter is regulated remains to be analyzed.

"Early" (e) genes are characterized by their dependence on IE-proteins for transactivation and their transcription before the onset of viral DNA replication. Experimentally, they can be identified after inhibition of DNA replication by phosphonoacetic acid. Several enzyme-encoding genes have been assigned to this group in HSV which is probably also true for PrV. "Early–late" genes begin to be transcribed before viral DNA replication but reach their maximum expression level after replication has started. Finally, "late" genes, mostly encoding viral structural components, are expressed. In recent years it has become clear, however, that not all genes can be divided into these classes and that a more complex regulatory cascade might indeed be present (reviewed in Ben-Porat and Kaplan, 1985).

After circularization of the linear molecule replication of herpesviral DNA proceeds by a rolling circle mechanism. For HSV, seven virus-encoded proteins necessary for viral origin dependent DNA replication have been identified. Homologs for several of them have also been found in PrV implying a similar mechanism. They include UL5 (helicase; Theisen-Kugler and Rziha, unpublished), UL9 (ORI-binding protein; Klupp and Mettenleiter, unpublished), UL29 (major DNA binding protein; Pedersen and Enquist, 1989), UL30 (DNA-polymerase; Berthommé et al., 1993*b*), UL42 (DNA-polymerase associated factor; Berthommé et al., 1992), and UL52 (primase; Baumeister and Mettenleiter, unpublished). A protein homologous to the UL8 product, which is essential for replication of HSV DNA and together with the UL5 and UL52 products forms the helicase–primase complex, is very likely also present in PrV. The amino acid sequences of these proteins show very high homology to those in other herpesviruses indicating strong evolutionary pressure upon conservation of these genes. Two origins of replication have been localized in the PrV genome, both located within the U_L region. One ORI_L is located at the extreme left terminus of the viral genome (Kupershmidt et al., 1991), whereas the other can be found in the middle of the U_L region (Klupp et al., 1992*b*; Wu et al., 1986). Presence of an origin in the short segment (ORI_S) could not be confirmed. The strong conservation of DNA sequences within alphaherpesviral origins of replication, as well as the high homology found among replication-associated proteins suggest similar, if not identical mechanisms for DNA replication in all alphaherpesviruses, including PrV.

Table 1

PrV genes and homologs in VZV and HSV-1

VZV	HSV-1	PrV	Function	Literature
1	–			
2	–			
–	UL56		VIR	
3	UL55			
4	UL54 (ICP27/ IE63)	UL54	Gene reg.	
5	UL53	UL53	gK	Baumeister and Mettenleiter, unpublished
6	UL52	UL52	Hel./Prim.	
7	UL51	UL51		
8	UL50		dUTPase	
9A	UL49A		Membrane	
9	UL49 (VP22)		TEG	
10	UL48 (VP16)		TEG (Transact.)	
11	UL47 (VP13/14)		TEG	
12	UL46 (VP11/12)		TEG	
–	UL45 (18K)		Membrane ?	
13	–		Thym. Synth.	
14	UL44	gIII	gC	Robbins et al., 1986
15	UL43	UL43	Membrane	Ryan, pers. comm.
16	UL42	UL42	Pol. Assoc.	Berthommé et al., 1992
17	UL41	UL41	VHS	Berthommé et al., 1993*a*
18	UL40	UL40	small RR	de Wind et al., 1993
19	UL39	UL39	large RR	
20	UL38 (VP19c)		CAPSID	
21	UL37		DNA-bdg.?	
22	UL36 (ICP1/2)		TEG	
23	UL35 (VP26)		CAPSID	
24	UL34		Membrane	

Table 1 continued

VZV	HSV-1	PrV	Function	Literature
25	UL33		CLE/ENC	
26	UL32			
27	UL31			
28	UL30	UL30	DNA-POL	Berthommé et al., 1993*b*
29	UL29 (ICP8)	+	DNA-bdg.	Pedersen and Enquist, 1989
30	UL28 (ICP18.5)	ICP18.5	CLE/ENC	
31	UL27	gII	gB	Robbins et al., 1987
32	–			
33	UL26 (VP24)		Protease	
33.5	UL26.5 (VP22a)		ASSEM-BLY	
34	UL25		VP	
35	UL24	+		de Wind, 1992*c*
36	UL23	TK	TK	Kit, 1985
37	UL22	gH	gH	Klupp and Mettenleiter, 1991
38	UL21	UL21	CLE/ENC	de Wind et al., 1992*b*; Klupp et al., 1992*b*
39	UL20	UL20	Membrane	
40	UL19 (VP5)	UL19	MCP	Yamada et al., 1991
41	UL18 (VP23)	UL18	CAPSID	Klupp et al., 1992*b*
42/45	UL15	UL15	(Spliced)	
43	UL17			
44	UL16			
45	UL15			
46	UL14	UL14		de Wind et al., 1992*a*
47	UL13 (VP18.8)	UL13	Prot.Kin./ TEG	
48	UL12	UL12	DNase	
49	UL11		Membrane	
50	UL10	UL10	gM	Klupp and Mettenleiter, unpublished
51	UL9	+	ORI-Bdg.	
52	UL8		Hel./Prim.	
53	UL7			
54	UL6			

Table 1 continued

VZV	HSV-1	PrV	Function	Literature
55	UL5	UL5	Hel./Prim.	Theisen-Kugler and Rziha, pers. commun.; Dean and Cheung, 1993
56	UL4	UL4		
57	–	UL3.5		
58	UL3	UL3	Membrane ?	
59	UL2	UL2	Ura-Glyc.	Dean and Cheung, 1993; Klupp and Mettenleiter, 1993
60	UL1	UL1	gL	
61	IE110 (ICP0)	EP0	Gene reg.	Cheung, 1991
	γ34.5		Virulence	
62	IE175 (ICP4)	IE180	Gene reg.	Cheung, 1989
63	US1 (IE68 /ICP22)	RSp40	Gene reg.	Zhang and Leader, 1990
64	US10	–	VP	
–	–	–		
–	US2	28K		van Zijl et al., 1990
66	US3	PK	Prot. Kin.	van Zijl. et al., 1990; Zhang et al., 1990
–	US4	gX	gG	Rea et al., 1985
–	US5	–	gJ	
–	US6	gp50	gD	Petrovskis et al., 1986*a*
67	US7	gp63	gI	Petrovskis et al., 1986*b*
68	US8	gI	gE	Petrovskis et al., 1986*b*
–	US8.5	–		
65	US9	11K	TEG	Petrovskis and Post, 1987
–	US11	–	Nucl. Ac. bdg.	
–	US12 (IE12 /ICP47)	–		

Genes in PrV are indicated compared to their counterparts in VZV and HSV-1. Designation of VZV genes is according to Davison et al., that of HSV-1 genes according to McGeoch et al. PrV genes that have been completely sequenced are named, mostly according to their HSV-1 counterparts. "+" indicates that partial sequence information is available. Any information about function of the gene products in any of the three viruses is also indicated. Abbreviations for protein functions not described in the text: VIR = virulence function; TEG = tegument protein; VP = virion protein

After replication, concatemeric head-to-tail fused linear genomes accumulate in the nucleus in high molecular weight structures (Ben-Porat and Kaplan, 1981). Fusion of the genome ends, however, leads to the formation of a cleavage/encapsidation signal that directs correct cleavage into linear unit size molecules and their incorporation into newly formed capsids (Wu et al., 1986). So far, the processes of cleavage and encapsidation have not been separated from each other. PrV proteins involved in this process include the products of genes homologous to UL28 (ICP18.5) of HSV (Mettenleiter et al., 1993; Pedersen and Enquist, 1989), which is essential for formation of mature nucleocapsids, and UL21, a nonessential capsid protein which increases efficiency of capsid maturation (de Wind et al., 1992*b*; Klupp et al., 1992*b*). Genes encoding other capsid proteins identified in PrV include those homologous to HSV UL19 (the major capsid protein; Yamada et al., 1991; Klupp et al., 1992*b*), and HSV UL18 (Klupp et al., 1992*b*).

Viral Glycoproteins

PrV glycoproteins have for some time been the focus of attention. As constituents of the viral envelope they represent structural virion components recognized by the immune system (Ben-Porat et al., 1986). They also mediate important interactions between virus and target cell during virus infection (reviewed in Mettenleiter, 1991). In contrast to most other viruses, herpesviruses contain a large number of envelope glycoproteins. In HSV-1 eleven glycoproteins have been described to date (Spear, 1993). In PrV, nine glycoproteins have so far been identified, with sequence information for one more putative glycoprotein available. All PrV glycoproteins exhibit homologies to respective glycoproteins in other herpesviruses. Therefore, a new nomenclature for alphaherpesvirus glycoproteins has been agreed upon, based on the alphabetic designation of HSV glycoproteins. However, for this review the original PrV nomenclature will be used once more. A summary is presented in Table 2. Of the PrV glycoproteins, four have been found to be dispensable for viral replication in tissue culture and have, therefore, been designated as nonessential. One nonessential glycoprotein, gX, is not associated with virions but is secreted by PrV infected cells into the supernatant. Its function is still a mystery (Rea et al., 1985; Thomsen et al., 1987; Mettenleiter et al., 1990*b*). It should be emphasized, however, that PrV field strains normally express the whole complement of glycoproteins which indicates that the "dispensable" glycoproteins do exert a beneficial effect on virus growth, at least in the animal. Some of these effects will be discussed below.

Isolation of PrV mutants deficient in expression of specific viral glycopro-

teins allowed the elucidation of some of their functions during virus infection *in vitro* and *in vivo*. Whereas isolation of mutants lacking dispensable proteins is now commonplace, construction of mutants deficient in essential proteins is dependent on the prior genetic engineering of cell lines expressing the respective protein and therefore complementing in *trans* the corresponding virus mutant. Up to now PrV mutants lacking the nonessential glycoproteins gI, gp63, gIII, and gX, as well as mutants deficient in expression of gII, gp50, and gH have been constructed and analyzed.

PrV glycoprotein gII (gB) is a major constituent of the viral envelope and it elicits antibodies that neutralize both complement-dependently and -independently (Hampl et al., 1984; Lukács et al., 1985). Homologs to gII of PrV (Robbins et al., 1987) can be found in all herpesviruses analyzed so far, except in the fish herpesviruses. The gB-homologs represent the most highly conserved group of herpesviral glycoproteins. They are represented by homodimeric molecules that in most cases are posttranslationally cleaved by a cellular protease residing in the trans-Golgi into two subunits per monomer that remain linked via disulfide bonds (Whealy et al., 1990; Wölfer et al., 1990). One exception is HSV gB which is not proteolytically processed (reviewed in Spear, 1993). Up to now the importance of this processing event is unclear, although recent data indicate that cleavage of the gB-homologous glycoprotein of BHV-1 is not necessary for its function (Blewett and Misra, 1991; Kopp and Mettenleiter, unpublished). All gB-homologs analyzed so far play essential roles in penetration of free virions into target cells, i.e. in membrane fusion between virion envelope and cellular cytoplasmic membrane (Cai et al., 1988; Rauh and Mettenleiter, 1991; Peeters et al., 1992*a*). In addition, they are necessary for direct cell-to-cell spread of the virus, i.e. direct transfer of infectivity from infected primary target cells to noninfected adjacent cells. This process is also thought to require membrane fusion events. However, a direct fusogenic activity of gII has not yet been observed.

Trans-complementation studies showed that in certain cases heterologous gB-homologs can substitute for the homologous protein. It has e.g. been shown that BHV-1 gB can substitute for gII in PrV (Rauh et al., 1991), and gII(PrV) can substitute for gB in HSV (Mettenleiter and Spear, 1993). These results clearly indicate a close functional similarity between these glycoproteins. However, in no case analyzed so far was reciprocal two-way complementation observed indicating intrinsic functional or structural differences in these glycoproteins which are most likely located in the nonconserved regions.

Glycoprotein gp50, homologous to HSV gD (Wathen and Wathen, 1984; Petrovskis et al., 1986*a*), has also been shown to represent a virion component essential for infectivity. In contrast to gII, however, gp50 is only required for viral penetration, but is dispensable for cell-to-cell spread (Rauh and Mettenleiter,

1991; Peeters et al., 1992*a*). This means that after primary infection of target cells by phenotypically complemented gp50-PrV the virus can spread in cell culture (and in the animal) in the absence of gp50 by direct cell-to-cell transfer only (Heffner et al., 1993; Peeters et al., 1993). Virions released from these cells are noninfectious due to lack of gp50. These results demonstrated that membrane fusion during infectious entry of free virions (penetration) and that mediating direct transfer of infectivity from infected to uninfected neighbouring cells (cell-to-cell spread) are clearly distinct. It is notable that gp50-PrV is also able to cross the synaptic cleft after intraneuronal infection, although the mechanism for this transneuronal spread is unclear (Babic et al., 1993). The impact of these findings on the construction of a new generation of live vaccines will be discussed below. gp50 also plays a prominent role in the stable attachment of PrV which is discussed below. In addition to its functional role, gp50 is regarded as a major immunogen of PrV based on findings that anti-gp50 monoclonal antibodies are potent in neutralizing the virus both with and without complement (Wathen and Wathen, 1984; Eloit et al., 1988; Marchioli et al., 1988; Coe and Mengeling, 1990). In addition, purified gp50 has been shown to protect animals against challenge infection (Marchioli et al., 1987; Mukamoto et al., 1991).

Glycoprotein H represents the second most highly conserved glycoprotein among herpesviruses. It is also found in all herpesviruses analyzed (except the fish herpesviruses). Homologies, however, are lower than those of the gB-family (reviewed in Spear, 1993). Glycoprotein H of PrV has recently been identified as a virion structural component (Klupp and Mettenleiter, 1991; Klupp et al., 1992*a*). The phenotype of gH-mutants resembles that of gII-mutants in that gH proved to be necessary for both, viral penetration and cell-to-cell spread (Peeters et al., 1992*b*). Expression of gH(PrV) in the absence of other viral gene products showed incorrect processing of the carbohydrate moieties of the protein (Klupp and Mettenleiter, unpublished). Similar results had been obtained with gH-homologs in other virus systems. Based on studies in HSV, recently a novel glycoprotein, gL(PrV), has been identified (Klupp et al., 1993). Coprecipitation of gH by anti-gL antibodies indicated presence of a gH/gL complex and coexpression of gH and gL indeed demonstrated restoration of correct processing. In mutants lacking gH, gL is also missing from the virion envelope and restoration of gH-expression leads to reappearance of gL in the virion (Klupp et al., 1993). It is therefore assumed that the functional entity is represented by a noncovalently linked complex between gH and gL.

The genes for two other putative PrV glycoproteins have recently been characterized with deduced protein products exhibiting homology to HSV glycoproteins gK (Klupp et al., 1993) and gM (Klupp and Mettenleiter, unpublished). After *in vitro* translation and processing, the gK precursor protein has been ob-

served to become glycosylated (Klupp et al., 1993). However, no functional analysis of either protein has been performed yet.

One of the first glycoproteins described in PrV has been designated gI (Hampl et al., 1984; Lukács et al., 1985; Petrovskis et al., 1986*b*). Analysis of several avirulent PrV vaccine strains showed lack of gI in these viruses representing the first demonstration of a nonessential glycoprotein in PrV (Mettenleiter et al., 1985). Up to now, gI has been a major focus of attention. It was first shown that gI influences release of infectious virions from infected cells in a cell-type specific manner (Mettenleiter et al., 1987*b*; Zsák et al., 1989). Recent studies indicate that cell-to-cell spread (as measured by plaque size) is also affected by absence of gI (Zsák et al., 1992; Jacobs et al., 1993). In the animal, gI appears to be important for efficient infection of the central nervous system (CNS), as has been shown for certain neuronal pathways in rats (Card et al., 1991, 1992) and neurovirulence in pigs (Mettenleiter et al., 1987*c*; Kimman et al., 1992). Besides being a structural virion component, gI can therefore also be regarded as a virulence determinant, although the effect of deletion of gI on the virulent phenotype of the virus appears to be virus strain-dependent. Glycoprotein gI forms a complex with gp63, which is thought to represent the functional entity (Zuckermann et al., 1988). However, new results point to a difference in the animal between gI- and gp63-deleted PrV mutants (Kimman, personal communication) which might indicate a function for either gI or gp63 independent of complex formation. The importance of gI for vaccine construction will be discussed below.

Glycoprotein gIII (Robbins et al., 1986), although nonessential, has proven to be an important mediator of PrV attachment (Schreurs et al., 1988; Zuckermann et al., 1989). Primary attachment of wildtype PrV to target cells is mediated by interaction of gIII with a heparansulfate proteoglycan on the cell surface (Mettenleiter et al., 1990; Sawitzky et al., 1990). A major heparin-binding domain appears to be located in the amino-terminal third of the protein (Flynn et al., 1993). This mechanism of primary attachment has been identified in a number of other herpesviruses from all three subfamilies (reviewed in Spear, 1993). However, since gIII is strictly speaking "nonessential" (although gIII-negative virions exhibit a 10- to 100-fold decrease in specific infectivity reflecting their attachment defect; Whealy et al., 1988*a*), a gIII-independent pathway of viral attachment has to occur. Whereas in HSV, in the absence of the homologous glycoprotein gC, heparansulfate binding is still mediated by gB (Herold et al., 1991; 1993), the PrV gB-homolog gII is incapable of binding to heparansulfate by itself (Mettenleiter et al., 1990). In addition, infection of mutant cell lines lacking heparansulfate is indistinguishable from that of wildtype cells when gIII-negative PrV mutants are being used (Karger and Mettenleiter, unpublished). Taken together, this demonstrates that a second, heparansulfate- and gIII-independent

pathway for infection of cells by PrV exists. Recent data indeed indicate that after primary attachment of wildtype PrV mediated by gIII a second, more stable attachment step follows which is dependent on gp50 (Karger and Mettenleiter, 1993). In addition, using an assay involving attachment of PrV infected cells to noninfected cells it has been shown that in the absence of gIII antibodies against gp50 are able to block this association (Hanssens et al., 1993). Taken together, adsorption of PrV (and PrV-infected cells?) can be divided into two distinct phases: (*i*) primary attachment by interaction between gIII and heparansulfate; (*ii*) secondary binding involving gp50 and a different cellular virus receptor. It has also been shown that PrV and HSV-1 use overlapping, if not identical receptors for attachment which might explain their common broad *in vitro* host range (Lee and Fuller, 1993). Work to identify these receptors is in progress. In addition to its role in attachment, gIII has been shown to be involved in virus release from infected cells (Schreurs et al., 1988).

Glycoprotein gIII also appears to play an important role as immunogen. Anti-gIII antibodies neutralize PrV complement-independently (Hampl et al., 1984), and gIII is presently the only PrV antigen shown to elicit a cellular immune response (Zuckermann et al., 1990).

A diagram indicating glycoprotein functions is depicted in Fig. 1. Table 2 summarizes information on PrV glycoproteins.

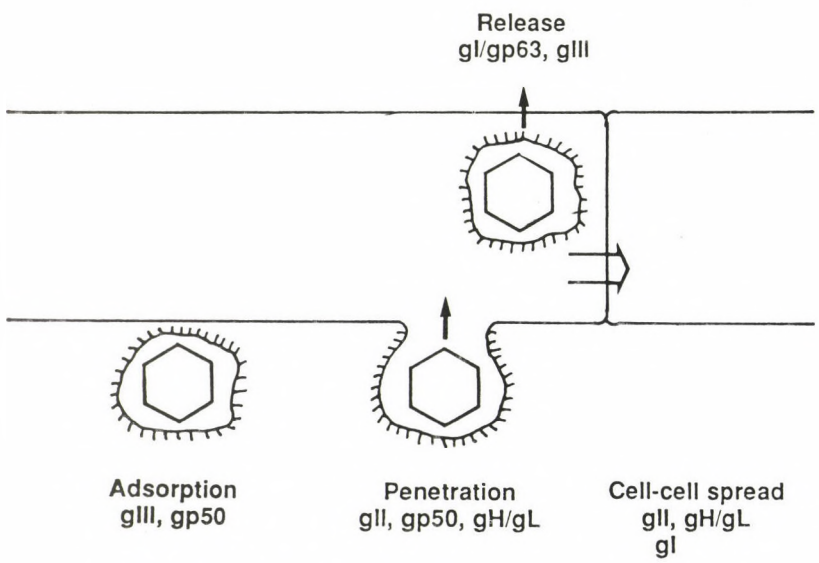

Fig. 1. Functions of PrV glycoproteins in infection

Table 2

PrV glycoproteins

Designation[a]		Essential[b]	Attachment[c]	Penetration[c]	Cell-cell spread[c]	Homology[d]	Conserved[e]		
PrV	HSV						α	β	γ
gII	gB	+	–	+	+	h	+	+	+
gIII	gC	–	+	–	–	l	+		
gp50	gD	+	+	+	–	l			
gI	gE	–	–	–	[+]	l	+		
gX	gG	–	–	–	–	l			
gH	gH	+	–	+	+	m	+	+	+
gp63	gI	–	–	–		l	+		
gK	gK					m	+		
gL	gL	+	–	+	+	l	+	+	+
UL10	gM					m	+	+	+

a) Homologous glycoproteins of PrV and HSV are shown in the same line. The original PrV nomenclature is about to be changed into a new nomenclature based on the alphabetical designation of homologous HSV glycoproteins

b) "+" denotes essential PrV glycoproteins, "–" denotes PrV glycoproteins nonessential in tissue culture. No entry means that data are not available yet

c) "+" indicates essential function of this glycoprotein in attachment, penetration or cell-cell spread, "(+)" indicates nonessential function, "–" indicates no involvement in these processes

d) Homology is indicated as high (h), medium (m), or low (l)

e) "+" indicates that homologous genes are found in all herpesviruses of this subfamily analyzed so far. No entry indicates lack of conservation in all members of the subfamily analyzed

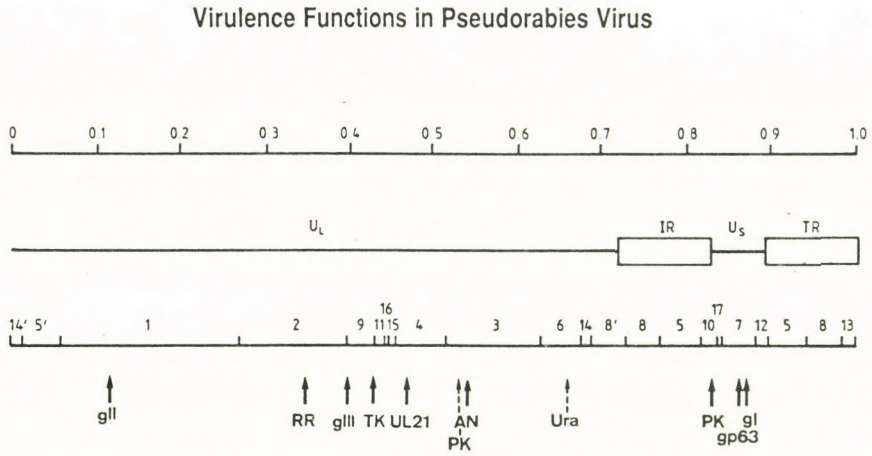

Fig. 2. Virulence functions recognized in PrV. Below a diagram of the PrV genome a Bam HI restriction fragment map is shown. Approximate locations of genes whose products have been shown to play a role in PrV virulence are indicated. Dashed arrows point to genes encoding enzymes that might be involved in virulence, although no data are available so far.
Abbreviations: RR = ribonucleotide reductase; TK = thymidine kinase; AN = alkaline exonuclease; PK = protein kinase; Ura = Uracil–DNA–Glycosylase

Virulence

PrV live vaccine strains attenuated by classical means have been used for several decades to cope with AD. They provided the starting point for studies to elucidate viral functions involved in determination of the virulent phenotype at the molecular level. So far, these proteins can be divided into three different classes: (*i*) viral membrane glycoproteins, (*ii*) virus-encoded enzymes, (*iii*) nonessential capsid (maturation) proteins (see Fig. 2).

Studies on the PrV vaccine strain Bartha, isolated in 1961 after passaging a field isolate in chick cells (Bartha, 1961), by DNA restriction analysis (Lomniczi et al., 1984*a*) and marker-rescue experiments (Lomniczi et al., 1984*b*; Mettenleiter et al., 1988) indicated presence of at least three defects contributing to its avirulence. An approx. 3.6 kb deletion in the U_S region eliminates expression of genes encoding nonessential glycoproteins gI and gp63 (Mettenleiter et al., 1985; Petrovskis et al., 1986*c*). It has subsequently been shown that deletion of gI in certain PrV strains leads to a significant decrease of virulence whereas other strains are less affected (Berns et al., 1985; Kimman et al., 1992; Mettenleiter et al., 1987*b*). A point mutation in the signal sequence for the glycoprotein gIII gene

interferes with efficient incorporation of this glycoprotein into the Bartha virion envelope (Robbins et al., 1989). Whereas mutation of gIII alone does not dramatically impair virulence of wildtype PrV strains, concomitant deletion of gI and gIII leads to an avirulent strain (Mettenleiter et al., 1988). A dominant virulence-determining function has been localized in the genomic Bam HI fragment 4 of the Bartha strain (Lomniczi et al., 1987). It has now become clear that the gene encoding a nonessential capsid protein homologous to UL21 in HSV which plays an accessory role in capsid maturation (de Wind et al., 1992*b*) is mutated in this fragment (Klupp and Mettenleiter, unpublished).

Recently, a PrV recombinant had been constructed where the gII(PrV)-gene had been replaced by the homologous gene from BHV-1 (Kopp and Mettenleiter, 1992). This recombinant exhibited a significantly altered virulence as compared to its parental wildtype PrV strain indicating that essential glycoproteins may play an important role in modulating PrV virulence (Lomniczi et al., 1993).

Among the first virulence functions recognized in PrV was the the virus-encoded enzyme thymidine kinase (Kit 1985*a, b*; McGregor et al., 1985). It is still used the most for construction of live vaccines. Whereas in the past TK-deficient virus mutants had been selected by their resistance against growth inhibition by nucleotide analogs such as thymidine-arabinoside, or iododesoxyuridine (Tatarov, 1968), most of the newer vaccines were genetically engineered to contain specific mutations within the TK-gene. Although the exact function of TK during virus infection is still not clear, it appears to be very important for virus replication in the CNS. In cell culture, TK is nonessential and TK-negative viruses grow as well as wildtype strains.

PrV encodes several other enzymes, whose inactivation has also been observed to interfere with viral virulence. They include the large and small subunits of the ribonucleotide reductase (UL39, UL40; de Wind et al., 1993), a protein kinase (US3; Zhang et al., 1990b; van Zijl et al., 1990), and an alkaline exonuclease (UL12; de Wind, 1992*c*). Deletion mutants in these genes can therefore also be envisaged to be used as attenuated vaccines alternative to TK-deficient viruses. A protein kinase homologous to the UL13 protein in HSV is also found (de Wind et al., 1992*a*), although no data are available on the influence of its inactivation on PrV virulence. Another enzyme encoded by PrV which is involved in nucleic acid metabolism is represented by the Uracil–DNA–Glycosylase (UL2; Klupp et al., 1993; Dean and Cheung, 1993), an enzyme that shows high sequence conservation between organisms evolutionary as far apart as *E. coli* and PrV. Deletion of UL2 in BHV-1 has recently been shown to lead to attenuation (Liang et al., 1993) which might also be true for PrV. In HSV several other enzyme-encoding genes have been described (McGeoch et al., 1988). It is likely that these are also present in PrV, adding to the number of potential virulence-associated proteins.

An important specific neurovirulence-determining function has recently been described in HSV. It has been named γ34.5 and the protein was recently shown to be important in inhibiting virus-induced apoptosis in infected neurons (Chou and Roizman, 1992). Whether PrV encodes a protein with similar functions is currently under investigation in a number of laboratories.

Latency

Establishment of latency is a characteristic feature of herpesviruses. During latency infectious virus cannot be detected, whereas viral genomes persist (Rziha et al., 1986; Belák et al., 1989). Exogenous stimuli may lead to reactivation of latent virus followed by virus excretion. Latently infected animals therefore are of major concern for controlling and eventually eradicating the disease. Latent viral DNA can be found in pigs mainly in the CNS (reviewed in Pensaert and Kluge, 1989; Wittmann and Rziha, 1991), although cells of the hematopoietic system might also harbor the viral genome (Rziha et al., personal communication). As has also been shown for HSV, during PrV latency a portion of the viral genome encompassing parts of Bam HI fragments 6 and 5, and all of fragments 14, 8' and 8 is actively transcribed in anti-sense orientation to the EP0 and IE180 genes (Cheung, 1989*b*; Lokensgard et al., 1990; Priola et al., 1990; 1991). Recently, a DNA fragment exhibiting promotor activity has been located immediately 5' of the LAT transcription unit (Schlegel et al., 1993). In HSV, LAT expression has been associated in certain instances with efficient reactivation (Leib et al., 1989) but is not requried for establishment or maintenance of latency (Sedarati et al., 1989). However, the importance of the PrV LAT transcripts for the establishment of and reactivation from latency in pigs is still unclear.

Although it is generally agreed that no existent PrV vaccine is able to prevent latency of a superinfecting field strain (Van Oirschot, 1984), recent evidence suggests that the amount of precolonization of the trigeminal ganglia by attenuated live PrV after vaccination might influence the extent of latency established by superinfecting field virus (Schang and Osorio, 1993). However, whether any PrV vaccine will be efficient in preventing subsequent field virus latency appears doubtful at present.

Vaccines

Increasing insight into the molecular biology of PrV continues to have a direct impact on the evaluation and construction of PrV vaccines. The classical means of isolating live vaccine strains, i.e. selecting for drug resistance or tem-

perature-sensitive phenotypes, or passaging in "non-natural" host cells (mostly chicken cells/embryonated eggs) have been superseded by genetic engineering methods capable of introducing specific mutations into the viral genome (Quint et al., 1987). A milestone in the development of this new generation of viral vaccines was the licensing by the USDA of the first genetically engineered live vaccine for widespread use represented by a TK-deficient PrV strain (reviewed in Kit, 1990). Currently, all genetically engineered PrV live vaccine strains carry mutations in the TK gene resulting in the attenuated phenotype. In addition to mutations leading to avirulence, all of the novel PrV live vaccines carry mutations that inactivate one (or more) of the nonessential glycoproteins gI, gX, or gIII (Quint et al., 1987; Marchioli et al., 1987*a*; Kit et al., 1987). Since in general PrV field strains have been shown to express these proteins, and since infected animals mount an antibody response against these antigens (Fuchs et al., 1991), absence of those antibodies in animals vaccinated with the deletion mutants can be used for discriminatory serological testing differentiating vaccine-induced antibody responses from wildtype virus-induced antibodies (Van Oirschot et al., 1986). Based on this serological discrimination using so-called "marker" vaccines several ambitious eradication programs have been started. Within the European Community only gI-deleted vaccines are being used, whereas in the USA vaccines containing either of the three glycoprotein deletions are still on the market.

Recently, several reports have shown that recombination between different vaccine strains may lead to novel strains with altered biological behaviour (Dangler et al., 1993; Henderson et al., 1990, 1991). In addition, there is evidence that recombination between wildtype and vaccine strains may lead to a transfer of the "marker", i.e. the glycoprotein deletion, onto a wildtype virus background (Christensen et al., 1992; 1993). It should be emphasized that deletion of any of these nonessential glycoproteins may (gI, gIII; Mettenleiter et al., 1987*c*; Mettenleiter et al., 1988*b*; Kimman et al., 1992) or may not (gX; Thomsen et al., 1987*a*) decrease virulence of the resulting virus mutant. It does, however, not abolish virulence in a way TK-mutations do. In addition, variations in the expression of gI may occur (Mettenleiter et al., 1987*a*; Katz and Pederson, 1992). Recombination between different vaccine strains leading to partial restoration of virulence, marker transfer from vaccine strains to wildtype strains, and variability in glycoprotein expression, therefore, threaten to interfere with current AD eradication protocols.

Up to now, attenuated PrV strains were still capable of being shed from vaccinated animals leading to spread of infection and persistence of the vaccine virus in the field population. In this scenario, recombination is more likely to occur than when the vaccine is confined. Recent analyses using viruses with mutations in the gp50 gene demonstrate a potential solution to this problem. As men-

tioned earlier, gp50 has been shown to be absolutely required for infectivity of free virions (Rauh and Mettenleiter, 1991; Peeters et al., 1992). After primary infection, however, gp50-negative virions can spread directly from infected to neighbouring noninfected cells. These cells release noninfectious, gp50-negative virions only. Therefore, any transmission of infectivity by cell-free virus appears impossible. Vaccination studies using gp50-negative PrV mutants that had been phenotypically complemented by propagation on gp50-expressing cell lines showed that (*i*) animals vaccinated with these strains did not shed virus nor was free infectious virus demonstrable in the body, (*ii*) despite the absence of gp50, which is regarded as a major PrV immunogen, a good protective immunity ensued as shown by challenge experiments with a highly virulent PrV strain (Heffner et al., 1993). In summary, vaccination with phenotypically complemented gp50-negative PrV leads to a natural confinement ("biological containment") of the infection to the vaccinated animal. Dissemination of the attenuated virus from vaccinated animals can not ensue.

This finding may also be of major importance for the development of a new generation of safe vector vaccines based on PrV. Large DNA viruses such as poxviruses, baculoviruses, or herpesviruses, have for some time been used as vehicles for expression of foreign genes. Indeed, expression of genes in vaccinia- and baculovirus now represent standard techniques in molecular biology. The identification of a number of nonessential sites within the PrV genome suitable for insertion of foreign DNA led to the development of PrV vectors that express e.g. the major envelope glycoprotein of classical swine fever virus (CSFV) (van Zijl et al., 1991). Vaccination with this recombinant virus protected animals from CSF, as well as AD. Other proteins expressed from recombinant PrV include reporter proteins used to monitor virus infection more easily (β-galactosidase, luciferase; Mettenleiter et al., 1990*b*; Kovács and Mettenleiter, 1991), glycoproteins from other herpesviruses (BHV-1, HSV; Kopp and Mettenleiter, 1992; Whealy et al., 1989), proteins from unrelated viruses (HIV; Whealy et al., 1988*b*), proteins from other microbial pathogens (plasmodium CS protein; Sedegah et al., 1992) or cellular proteins (tPA; Thomsen et al., 1987). Combined with the natural confinement of the gp50-negative virus and based on the large experience with PrV, these viral vectors may represent powerful tools to combat not only AD but also other viral diseases in the future.

The coming years will certainly witness an ever increasing impact of molecular biology on the study and eventual control of viral diseases. PrV can serve as a model in several respects and it represents an excellent example for effective "technology transfer" from the basic research laboratory to the animal health authorities and the industry.

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HIGH-FREQUENCY INTERGENOMIC RECOMBINATION PLAYS A SIGNIFICANT ROLE IN THE EVOLUTION OF SUID HERPESVIRUS 1 (AUJESZKY'S DISEASE VIRUS): A REVIEW

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The phenomenon of viral intergenomic recombination was experimentally demonstrated for the first time by Luria and Dulbecco (1949) and since then by many others. From time to time reports have also appeared suggesting that intergenomic recombination had taken place in field virus, and it has been reported that recombination events were responsible for the establishment of virulent vaccine-derived polio virus (Kew and Nottay, 1984; Lipskaya et al., 1991) as well as for the genesis of new viral species such as Western equine encephalitis virus (Hahn et al., 1988). In the present report some recent findings are reviewed, and the viewpoint is presented that a field strain of suid herpesvirus 1 (SHV-1), tentatively defined as the transmissible entity, is a composite population in which variants coexist, complement each other and exchange genetic material through high-frequency intergenomic recombination. This occasionally may result in a chaotic evolution of the virus.

Comprehensive molecular epizootiological studies by means of restriction fragment pattern (RFP) analyses have been performed on SHV-1 strains from different parts of the world, and an evolutionarily consistent systematization has gradually been elaborated based on differences in stable genomic characteristics (cf. Christensen, 1993). However, other differences, considered non-type discriminating, have been reported to emerge during experimental serial passage in animals and were also found among epizootiologically related cases of Aujeszky's disease (AD) in the field. The introduction of SHV-1 into herds in border areas in Denmark, known to be free of AD, and the molecular epizootiological studies of these phenomena, in particular the approach to analyze 5–10 isolates from each outbreak, revealed that the variant pool of a field strain is usually a highly stable yet eventually fluctuating characteristic (Christensen and Sørensen, 1991). The genomic diversity may be either wide or narrow and is also a highly specific feature of a strain. Thus, examples of strains are known in which certain genomic regions appear in a hypervariable fashion, while the same regions in other strains may appear invariable. So, different strains, although they may belong to the same genome type, may appear as easily distinguishable pools of related virus particles.

The strains of SHV-1 are of the so-called quasispecies nature (Eigen, 1971; Eigen and Schuster, 1979).

Indications of intergenomic recombination are occasionally seen in field isolates. However, the features combined most often appear in genomic regions known to be variable, and these indications therefore may not be considered convincing.

Further evidence was achieved through the identification and characterization of field strains derived from live attenuated vaccines. A total of four derivatives of Bartha K-61 were identified in Hungary (Christensen et al., 1992; Medveczky and Christensen, unpublished data), three of which were collected from the semen of unaffected boars. The fourth isolate appeared as a plaque clone from a nasal swab of an affected piglet, which also harboured wild type variants. Furthermore, a total of five out of 12 isolates collected from fatal cases of AD in Poland were identified as derivatives of the vaccine strain BUK-TK-900, widely distributed in Poland (Christensen et al., 1992). These data were confirmed by Kochan et al. (1992) who reported that nearly half of the isolates collected in Poland were affected in fragment *Bam*HI-7, which may be considered indicative of a vaccine origin. All isolates had been collected from unvaccinated animals or animals that had not been vaccinated during the past several months, and some were collected on farms without any record of vaccination, indicating that the vaccine derivatives are circulating in the field. By RFP analyses some of the isolates were found to be indistinguishable from the parental vaccine variants. Others exhibited only faint, yet significant, similarities with the vaccine strain (Christensen et al., 1992; Christensen and Lomniczi, 1993; Medveczky and Christensen, unpublished data). Cloning of variants from vaccine-derived field isolates diverging from the parental strain BUK-TK-900 revealed that these isolates were extremely heterogeneous and harboured virus with a wide range of deletions in fragment *Bam*HI-7 (Christensen and Lomniczi, 1993). As it is known that BUK-TK-900 consists of at least two variants both of which are affected in *Bam*HI-7 (Christensen et al., 1992; Christensen and Lomniczi, 1993), intergenomic recombination between coexisting variants in the vaccine strain was suggested as a possible mechanism that could account for the generation of this heterogeneity, in consistence with the experimental findings of Henderson et al. (1991). Alternatively, it cannot be excluded that the variants existed in the parental vaccine and a change in composition might be a means of adaptation of the strain in the field.

To further confirm the hypothesis of intergenomic recombination, mixed infection experiments were performed (Christensen and Lomniczi, 1993). Two strains with easily identifiable, stable genomic markers were mixed, installed in the nose of a pig, and this pig after two days was brought in contact with four pigs

from the same litter. Virus was isolated from nasal swabs of the pigs, and cloned by terminal dilution of the nasal swabs. The RFP analyses revealed that nearly all isolates were extremely heterogeneous populations, and the "clones" derived from these isolates represented a wide range of recombinants. A few "clones" apparently belonged to one of the genome types of the parental strains, while the majority exhibited new combinations of intertypic characteristics. None of the "clones" was identical to any of the parental strains. Another striking finding was that while the lefthand terminus of the genome, known to be hypervariable in some strains and invariable in others, was invariable in the parental strains, it was hypervariable in the progeny "clones". Thus, it must be concluded that hypervariability resulted from the coming together of variants diverging in these regions, and it was suggested that the hypervariable region, presumably consisting of various numbers of polyrepetitive sequences as seen in other herpesviruses (Hammerschmidt et al., 1986; Chowdhury et al., 1990), represented "hot spots" of high-frequency intergenomic recombination.

One other implication of these findings is that the destruction of a cell is the result of multiple invasions of the cell. This is a prerequisite of the recombination events and it would account for the survival of deletion mutants by complementation with other variants. A concept of multiple invasions is in agreement with the difficulties in achieving clonal populations from isolates exhibiting a hypervariable genomic region.

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IS RECOMBINATION OF PRV VACCINE STRAINS A REAL PROBLEM?

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Simultaneous inoculation of 50 doses of two modified live pseudorabies vaccines with either functional (strain Bartha) or inactivated (strain Begonia) thymidine kinase into piglets of 1 week of age did not induce any clinical signs of Aujeszky's disease. In only 3% of the cases could virus be re-isolated from organ specimens, namely from trigeminal ganglia or tonsils. In all samples only Bartha strain was recovered. By using a nested set of gII-specific primers viral DNA was found in 92% of the 135 samples. A deletion of 72 bp in the inverted repeats was used to differentiate Begonia from the Bartha strain. The genomes identified represented 12 times the Bartha, and 11 times the Begonia type, the remaining genome types could not (yet) be identified. We found no indications for the presence of the genomes of both viruses at the same time, nor for recombinants. The results suggest no selective pressure for recombinants by using vaccines with the same marker, i.e. gI-negative vaccines. Thus, when using gI-minus vaccines only, there are no indications that any real problems of recombination should arise.

Key words: Pseudorabies, virus, modified live vaccines, recombination

To date most pseudorabies modified live vaccines are characterized by the presence of one or more deletions either acquired "naturally" by *in vitro* passages of the virus (Bartha, 1961; Zuffa, 1963) or by rDNA deletion techniques (Van Oirschot et al., 1990; Thomsen et al., 1987). Passage of pseudorabies virus in cell cultures or embryonated eggs predominantly revealed deletions in gI and/or gp63 (Petrovkis et al., 1986). The attenuation by *in vitro* passages often results in other changes as well, e.g. for strain Bartha two mutations other than the gI deletion have been described (Mettenleiter, 1991). By using rDNA techniques other glycoproteins like gX (Thomsen et al., 1987) and gIII (Kit et al., 1985) have been deleted to obtain marker vaccines.

The absence of gI not only serves as a marker to allow differentiation between vaccination and wild-type virus infections, but deletion of gI also contributes significantly to the attenuation of the virus (Quint et al., 1987; Lomniczi et al., 1984; Card et al., 1992). In contrast, deletion of gX has no effect on virulence (Thomsen et al., 1987).

In most genetically engineered vaccines the inactivation of the TK gene contributes significantly to attenuation at an acceptable level (Kit et al., 1985; Moormann et al., 1990; Visser and Lütticken, 1989).

Recently, Henderson et al. (1990, 1991) and Katz et al. (1990) have described the possibility that in the USA in a worst-case scenario two vaccine strains like Bartha (gI^-, TK^+) and Iowa-derived recombinant vaccine (gX^-, TK^-) applied simultaneously might recombine to result for instance in a gI^+, TK^+ virulent virus. They reported that *in vivo* recombinants have been obtained, which upon re-inoculation into piglets, however, induced only very mild clinical signs. In contrast, the simultaneous administration of the two vaccines revealed quite severe clinical signs of Aujeszky's disease. This may be explained by complementation since the thymidine kinase activity provided by Bartha complements full virulence to the TK^- mutant strain in which the gX deletion is not supposed to contribute to the attenuation at all (Thomsen et al., 1987).

In Europe there is a different situation in which effectively only gI-minus vaccine strains are used and gX^- vaccines are not allowed.

In this report we describe experiments designed to evaluate the safety risks of complementation or recombination as a result of using TK positive and negative strains, both in a gI^- background.

Materials and methods

Inoculation. One-week-old Dutch Landrace hybrid SPF piglets were given intramuscularly (im.) or intranasally (in.) 1 dose (10^6 $TCID_{50}$) or 50 doses (1.7×10^7 $TCID_{50}$) of vaccine strains Bartha (gI^- , TK^+) and/or Begonia (gI^- , TK^-). Bartha (Suvaxyn, Solvay) was originally obtained by *in vitro* passages (Bartha, 1961); besides the deletion in the gI-gp63 area it has two other mutations (Mettenleiter, 1991). Begonia (Nobi-porvac, Intervet) has been derived from the wild-type strain NIA-3 and contains deletions of gI and in the TK gene (Quint et al., 1987; Visser and Lütticken, 1989).

Postmortem examination. During one week after inoculation the piglets were observed for clinical signs, before they were euthanized. Blood and tissue samples including local lymph nodes, tonsils, lungs, bulbus olfactorius, trigeminal ganglia and medulla oblongata were collected for virus reisolation. Samples from peripheral white blood cells and trigeminal ganglia were used for viral DNA analysis.

Virus re-isolation. White blood cells were prepared via Ficoll gradient purification. Organ pieces were dispersed using collagenase IV and trypsin. Cells obtained from organ specimens were cocultivated with MDBK cells. The culture

medium contained the hypomethylating agent hexamethylene bisacetamide (5 mM HMBA, Sigma) which increases herpesvirus recovery rates. If after 6 weeks of subcultures no CPE was observed, the cultures were scored negative.

Analysis of recovered virus. Southern blot analysis was performed on DNA prepared from re-isolated virus. DNA was cleaved with *Bam*HI and *Sal*I and blot hybridised with ^{32}P-labelled *Bam*HI fragment 7 of wild-type (wt) strain Ka. This blot hybridisation discriminates both parental vaccines with regard to different deletions in the gp63, gI, 11K, 28K coding region (see Fig. 1), the deletion being 3.4 kb for Bartha and 2 kb for Begonia. Single plaques of each of the isolates were tested for ^{14}C-thymidine incorporation in a thymidine kinase activity assay (Tenser et al., 1983).

PCR. Two regions of the genome were taken for amplification. Firstly, a gII-PCR of a 391 bp fragment of the gII-gene (primer pair gII-01/02 in Fig. 1). The amplification product was tested by ethidium bromide stained gels and specific blot hybridisation. To enhance the sensitivity and to improve specificity, the amplified products were used for a nested PCR with primers gII-Ne1/Ne2 (see Fig. 1) which results in a 365 bp fragment. Secondly, BEG-PCR of a 260 bp fragment located in the internal repeats of the PRV genome covers a 72 bp *Hind*III fragment, which is present in strain Bartha but absent in strain Begonia, thus resulting in a smaller PCR product 188 bp in size (Fig. 1).

Results

Clinical observations. Observations for 1 week after the combined inoculation of 1 or 50 doses of Begonia and Bartha showed no adverse effects on the health of the one-week-old piglets. Furthermore, postmortem examination revealed no macroscopic abnormalities.

Virus re-isolation. A total of 135 samples from various organs including local lymph nodes, tonsils, lungs, bulbus olfactorius, trigeminal ganglia and medulla oblongata were examined by cocultivation for the presence of virus. Surprisingly only four positive samples were found, recovered from the tonsils of two animals of the 1-dose group and one tonsil of the 50-dose group, all of which received both vaccines in. In the 50-dose single Bartha group virus was isolated from the trigeminal ganglia.

Southern blot analysis of DNA isolated from each of the four re-isolates revealed a pattern indicative of the Bartha strain. As examples the Southern blots of isolates TO-9 and TO-11 are shown in Fig. 2.

A ^{14}C-thymidine incorporation assay for demonstrating TK-activity in single virus plaques was positive for all four isolates, confirming the Bartha pheno-

type. In addition, this assay did not indicate the presence of TK-negative virus in each re-isolate.

gII-PCR. Using PCR on a 391 bp fragment of the gII gene the presence of viral genomes was established in the majority of samples of the trigeminal ganglia and peripheral white blood cells. In Fig. 3 a comparison of data from the direct (gII-01/02) and nested (gII-Ne) gII-PCR are given, indicating the much higher sensitivity of the nested PCR. The very weak or failing hybridisation signals after the first PCR (Fig. 3A) demonstrate the presence of minimum amounts of viral genome ranging between 1 genome copy in approximately 10^3 to 10^5 cells, which can be detected only after nested PCR (Fig. 3B). Summaries of the gII-PCR results are given in Tables 1–4. Overall 92% of the trigeminal ganglion samples and 72% of the white blood cells harboured viral DNA.

BEG-PCR. The controls of single-vaccine inoculated piglets showed that if a PCR signal was obtained then the correct parental strain was found (Table 1). In the 1-dose/in. group which received both vaccines, only Bartha was identified in trigeminal ganglion samples (Fig. 4 and Table 2), whereas in the 50-dose/im. group only Begonia representatives were found (Table 3). However, in the group receiving 50 doses/in. Bartha (5 times) and Begonia (1 time) were present (Figs 4 and 5, Table 4). It was not possible unequivocally to establish the parent genome type in all cases, since the sensitivity of the BEG-PCR is ca. 100-fold lower than that of the nested gII-PCR.

Table 1

PCR results of negative and positive control animals. Presence of viral DNA in trigeminal ganglia and white blood cells and identification of the parental strain(s)

#		1	2	3	4	29	30	31
vaccine		Be, 50 in				Ba, 50 in		
TG	gII	++	++	+/–	0	0	+	0
TG	gII-Ne	++	++	++	0	++	++	++
TG	BEG	Be	Be	Be	0	?	Ba	?
PBL	gII					++	++	+/–
PBL	gII-Ne					++	++	++
PBL	BEG					Ba	Ba	Ba

+/–: faint but doubtless positive (i.e. more than 3 days film exposure); +: good signal (1–3 days film exposure); ++: positive in a couple of hours film exposure; ?: signal too weak to identify unambiguously

Table 2

PCR results of animals intranasally inoculated with 1 dose of Begonia and Bartha (see legend of Table 1 for further explanation)

#		5	6	7	8	9	10	11	12
vaccine		Begonia + Bartha, 1 in							
TG	gII	nt	0	0	0	0	+	nt	nt
TG	gII-Ne	nt	++	0	0	++	++	nt	nt
TG	BEG	nt	?	0	0	Ba	Ba	nt	nt
PBL	gII	+	+/–	0	0	0	+	0	0
PBL	gII-Ne	++	++	0	0	++	++	++	++
PBL	BEG								

Table 3

PCR results of animals intramuscularly administrated with 50 dose of each strain (for further explanation see legend to Table 1)

#		13	14	15	16	17	18	19	20
vaccine		Begonia + Bartha, 50 im							
TG	gII	+/–	+	0	+/–	+	+/–	0	0
TG	gII-Ne	++	++	0	++	++	++	++	++
TG	BEG	Be	Be	0	Be	Be	Be	0	0
PBL	gII	0	0	0	0	0	+/–	+	0
PBL	gII-Ne		++	0			++	++	
PBL	BEG	Be	Be		0	0	?	?	

Table 4

PCR results of piglets given intranasally 50 doses of Begonia and Bartha (for further explanation see legend to Table 1)

#		21	22	23	24	25	26	27	28
vaccine		Begonia + Bartha, 50 in							
TG	gII	0	0	0	+	0	+	nt	nt
TG	gII-Ne	++	++	++	++	++	++	nt	nt
TG	BEG	Ba	Ba	0	?	0	Be	nt	nt
PBL	gII	nt	nt	+/–	+/–	+/–	0	+	+/–
PBL	gII-Ne	nt	nt	++	++	++	++	++	++
PBL	BEG	nt	nt	Ba	Ba	?	0	Ba	?

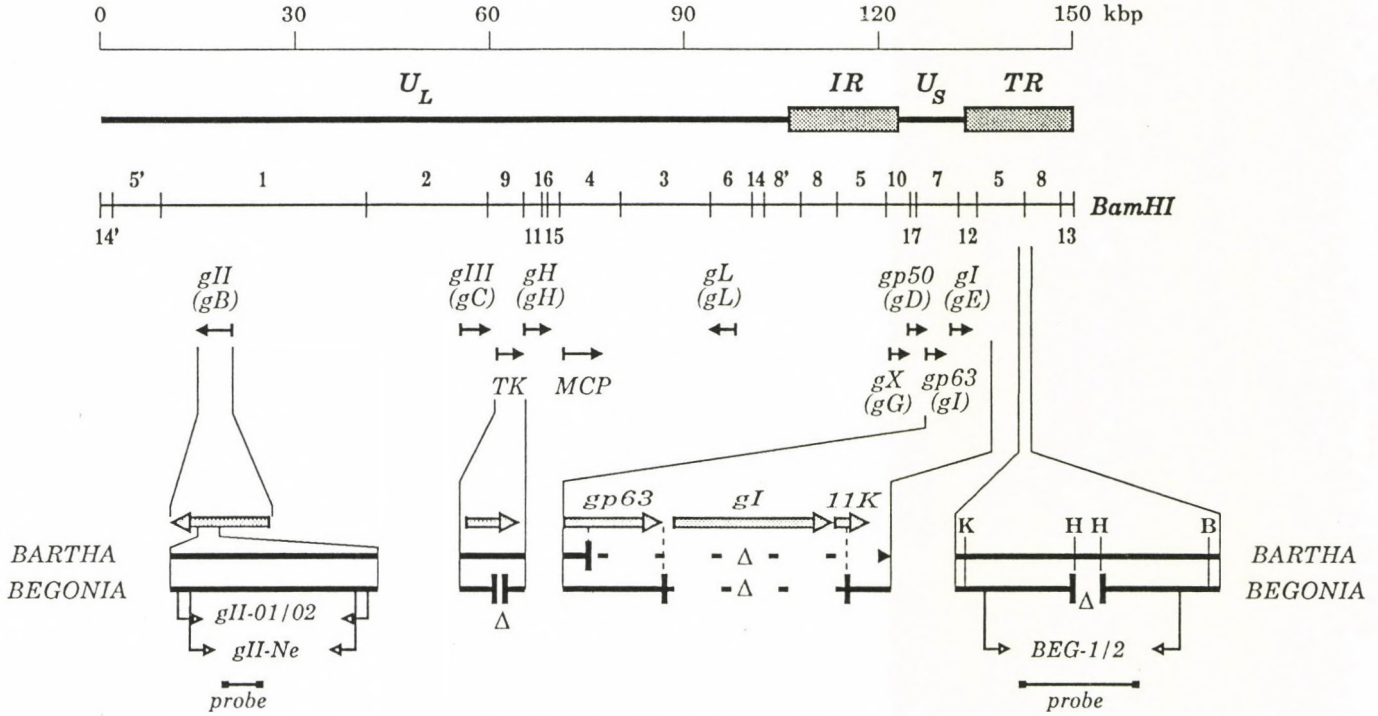

Fig. 1. Schematic outline of the PRV genome indicating the location of the primer sets and the blot fragments used. gII-01/02 and gII-ne are the first set and nested set of primers to detect the gII gene as general indicator of the presence of PRV DNA. BEG-1/2 indicated the primer set to identify the presence or absence of a deletion in the repeats resulting in a 260 bp (Bartha) or 188 bp (Begonia) PCR product

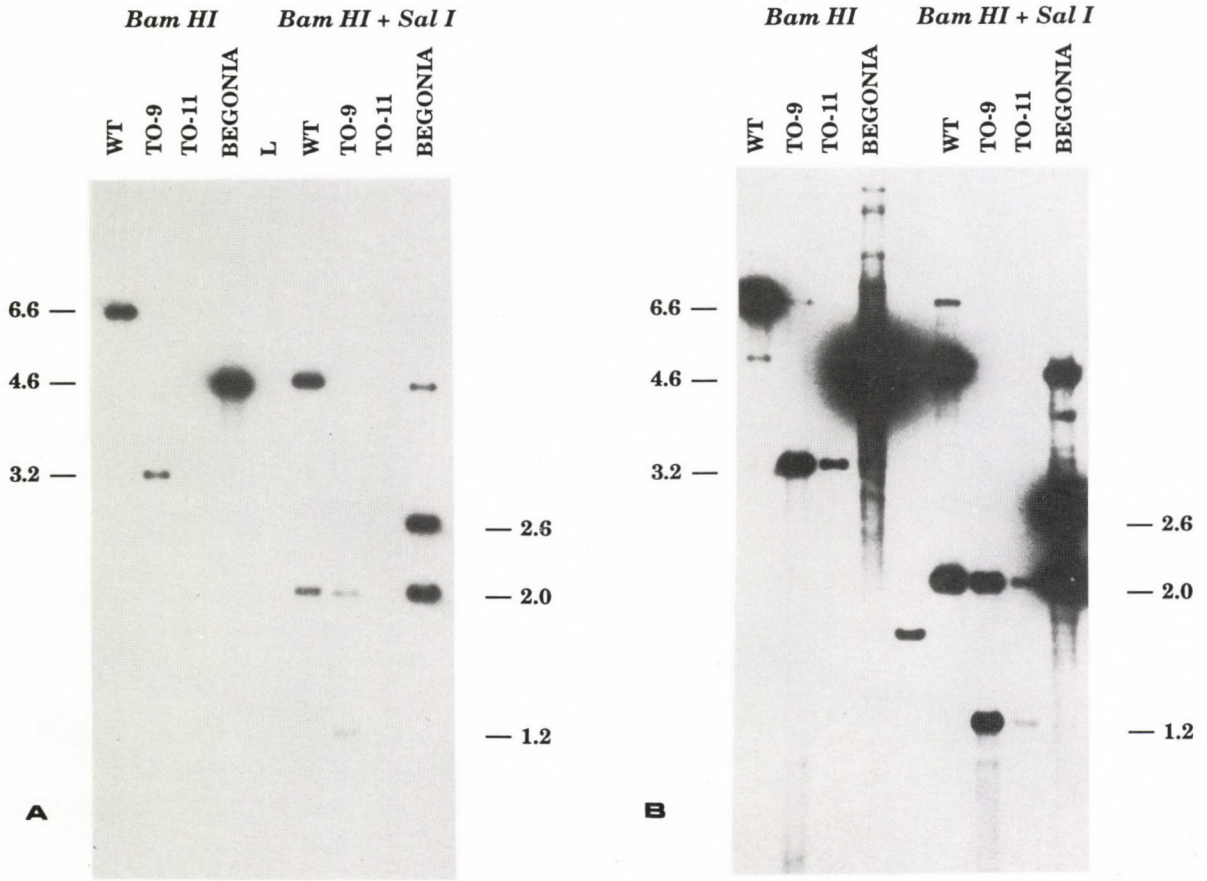

Fig. 2A. Southern blot analysis of DNA of virus re-isolates after cleavage with restriction enzyme *Bam*HI (1A and B, lanes 1–4), or double digested with *Bam*HI and *Sal*I (1A and B, lanes 5–8) and blot hybridized with ^{32}P-labelled *Bam*HI fragment 7 of wt strain Ka. *Fig. 2B.* An overexposure of the same blot as *Fig. 2A* intended to demonstrate more clearly the pattern of isolate TO-11

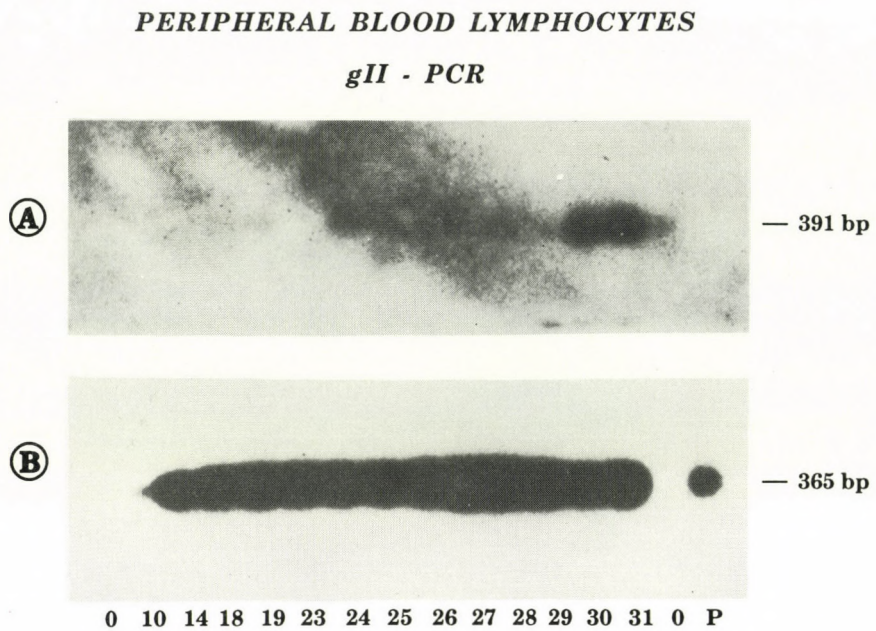

Fig. 3. Comparison of gII-PCR results of peripheral blood lymphocytes. *A*: Southern blot of ^{32}P-labelled internal primer (see Fig. 1) with the PCR products of the primer pair gII-01/02. *B*: Southern blot with the same probe after further amplification using the nested set of primers gII-Ne1/Ne2. Exposure time of film was in (A) 3 days and in (B) 1 hour

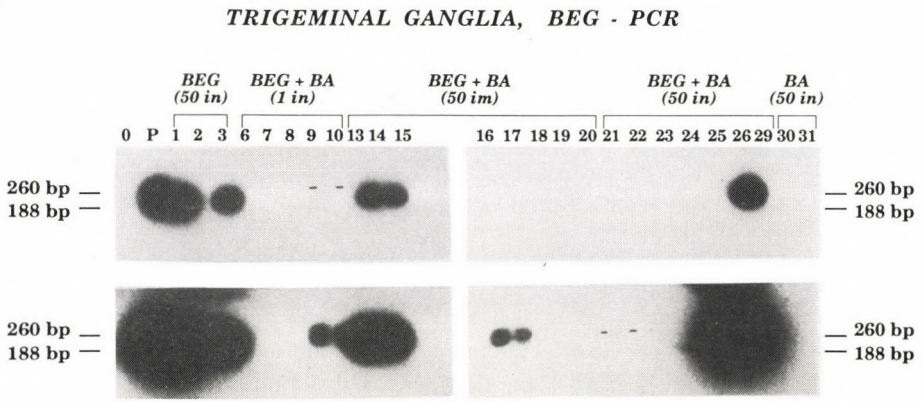

Fig. 4. A: Southern blot of PCR products using primer pair BEG-1/2 on trigeminal ganglia samples. *B*: The same blot after overnight exposure to reveal weaker bands

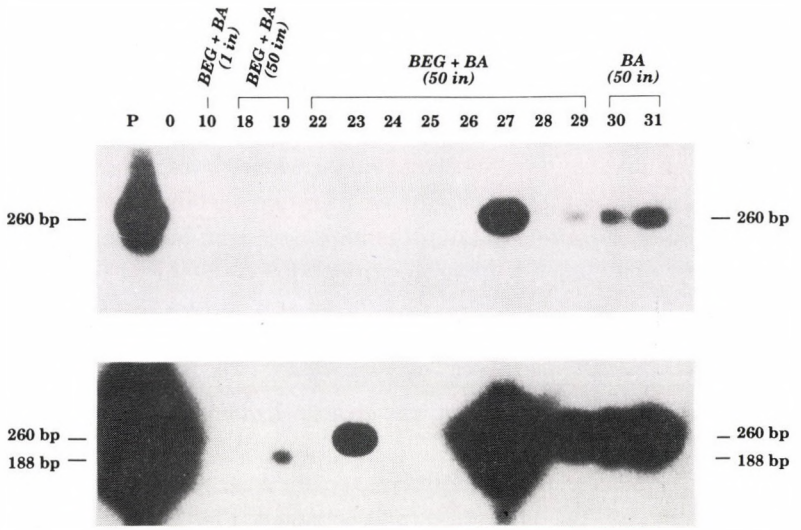

Fig. 5. A: Southern blot of PCR products using primer pair BEG-1/2 on peripheral blood lymphocytes. *B*: The same blot after 2 days exposure to show weaker bands more clearly

Discussion

Recently, Henderson et al. (1990, 1991) showed that under certain conditions recombination between vaccine viruses could occur. However, none of the recombinants found induced clinical signs comparable to wild type virus infection. In contrast, at the time of inoculation of 1 dose of Bartha and 1 dose of the Iowa-derived (gX^- , TK^-) vaccine they did note severe clinical disease. These results might be interpreted that complementation is responsible for the observed effect rather than recombination. Co-infection of a cell with both strains would give a wild-type situation in that Bartha provides the TK enzyme activity giving the other virus a fully virulent background, supposing that the gX deletion does not play a role in virulence (Thomsen et al., 1987).

Besides this aspect of complementation the use of different marker vaccines would rather complicate an eradication program, since it would no longer be possible to differentiate vaccination from infection by either marker system.

The above situation would hardly be possible in Europe where most countries only allow the use of gI^- vaccines, and then the question is: Under these circumstances would recombination be a real problem? To this end we started the

experiments described here not only with 1 dose but also including 50 doses of each vaccine to mimic a worst-case situation. The high initial dose is necessary to increase the possibility of recombination to occur, since Ben-Porat et al. (1982) showed that recombination precedes replication. It is difficult to imagine other possibilities of high vaccine doses at the same time being present in the animal, as vaccine viruses are rather rapidly eliminated from the vaccinated animals (Visser and Lütticken, 1989). We observed that the high doses did not result in any complementation or recombination leading to clinical disease within a week after vaccination.

The number of virus re-isolations was low, only 3% (4 out of 135 samples) and all 4 virus re-isolates exhibited the Bartha genome type. The diminished replication potential of both vaccine strains was clearly demonstrated by the amount of viral DNA detectable. Using PCR specific DNA was found only in the range of 1 copy per 10^3 to 10^5 cells. This is in agreement with earlier studies showing that 2 months after vaccination Begonia could only be detected by PCR, whereas Bartha was found also by the less sensitive technique of *in situ* hybridisation (Rziha et al., 1989) indicating low (latent) survival rates of vaccine virus in the host.

In none of the assays did we find any indication of the presence of both virus types, which however also might be explained by a very low prevalence of the other parental virus type.

In conclusion, our experiments show that even under worst-case conditions, applying 50 doses of two different vaccines, the viruses are rapidly cleared without inducing any clinical disease, and no evidence for recombination was found. From the results presented, the importance of using only one type of marker vaccine, such as the gI marker phenotype which also contributes to virus attenuation, can be inferred.

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A BOVINE CELL LINE STABLY EXPRESSING PORCINE TUMOR NECROSIS FACTOR ALPHA (TNF-α): GROWTH PROPERTIES AND PERMISSIVITY FOR PSEUDORABIES VIRUS REPLICATION

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To explore the feasibility of modifying the pathogenicity of pseudorabies virus (PrV), we have undertaken a program to develop recombinant PrV carrying the porcine tumor necrosis factor alpha (TNF-α) gene. We have used a cloned genomic DNA fragment containing the entire porcine TNF-α gene (Chardon et al., 1991), and have deleted the 3' non-translated region which is supposed to be a tissue-specific regulatory sequence. We have also removed the 5' non-translated region containing a TATA-box and binding sites for other transcription factors. The resulting TNF-α cassette has been inserted into a neomycin-selectable shuttle vector. This construct has been used to select MDBK cell lines harbouring the TNF-α-carrying plasmids in order to verify the production of biologically active TNF-α. One cell line thus obtained secreted 20–30 pg/10^6 cells of active TNF-α after five days in culture and exhibited normal growth kinetics. PrV titers on this cell line were the same as titers on cells not expressing TNF-α, indicating that TNF-α expression in MDBK cells does not abrogate their permissivity for virus replication. Our results show that construction of recombinant PrV expressing TNF-α should be possible using the MDBK cell line as host.

Key words: Aujeszky's disease virus, pseudorabies virus, tumor necrosis factor alpha, cytokine, shuttle vector, transfection

Pseudorabies virus (PrV), also referred to as Aujeszky's Disease Virus, belongs to the family of animal viruses designated *Herpesviridae* (Roizman et al., 1992). PrV is a member of the subfamily *Alphaherpesvirinae*, the neurotropic virus group, and causes Aujeszky's disease in domestic and wild animals (reviewed in Ben-Porat and Kaplan, 1985). In swine, the natural host of the virus, young animals die of severe neurological disease (meningoencephalitis) whereas adults usually survive the infection with fever, pneumonia or asymptomatic infection and subsequently the virus enters a latent state. In many other species, PrV often causes fatal encephalitis. Aujeszky's disease is distributed world-wide and has an economically important impact on the swine industry.

To eradicate Aujeszky's disease, or at least to minimize the spread of the virus, several countries use vaccination of swine with live attenuated vaccines. Conventional vaccines are PrV strains usually deleted in various glycoprotein genes. These vaccines prevent the development of clinical signs upon infection, because vaccinated and infected pigs produce antibodies to PrV glycoproteins. However, levels of PrV-neutralizing antibodies produced in serum frequently do not correlate with protection and are often not sufficient to control virus spread and the establishment of latency.

In the context of vaccine development, studies might profitably include genetic manipulation of the virus in such a way as to enhance its exposure to the immune surveillance system of the host and reduce the efficiency with which dissemination and/or latency occur.

Tumor necrosis factor alpha (TNF-α), also known as cachectin, is a cytokine secreted predominantly by macrophages but also by other cell types. TNF-α is a multifunctional cytokine which contributes to the defence of the host against tumors and pathogenic organisms. In addition, it can provoke inflammation, fever, cachexia and septic shock (for review: Beutler and Cerami, 1989; Semenzato, 1990).

In cell culture, TNF-α is induced in response to viral infection. It exhibits antiviral properties, selectively killing infected cells (Wong and Goeddel, 1986; Mestan et al., 1986). Recently, Sambhi et al. (1991) demonstrated the antiviral effects of TNF-α *in vivo* using recombinant vaccinia virus in a mouse model. Their experiments indicated that a localized production of TNF-α during infection could lead to enhanced clearance of the virus. These results encouraged us to explore the feasibility of modifying the pathogenicity of PrV by a similar approach. We have undertaken a program to develop recombinant PrV carrying the porcine TNF-α gene under the control of selected viral promoters. Here we report the establishment of a bovine cell line carrying the porcine TNF-α gene under the control of the PrV gX promoter and expressing biologically active TNF-α. The growth behavior and permissivity to PrV infection of this cell line are also documented.

Materials and methods

Cells. Cells were cultured at 37 °C in an atmosphere of 95% air, 5% CO_2. The Madin-Darby bovine kidney cell line (MDBK; American Type Culture Collection, Rockville, ATCC CCL22) was grown in Eagle minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS). Transfected cell lines MDBK-pREP9, MDBK-TNF, and MDBK-TNFΔ were selected, propagated

and maintained in MEM-10% FCS containing 0.5 mg per ml of G418 (Geneticin; Gibco-BRL). The mouse WEHI 164 cells (Espervik and Nissen-Meyer, 1986; ATCC CRL 1751) were propagated in RPMI 1640 medium with 10% FCS. Growth curves were determined using 6-well tissue culture plates.

Virus. We used the PrV PHY4EBG recombinant virus which harbours the LacZ gene under the control of the gX promotor (Fuchs et al., 1993). This virus is derived from the PrV Phylaxia strain (PHY) and showed no obvious differences in virus growth compared to parental wild-type PHY virus (Fuchs et al., 1993). PrV PHY4EBG has been chosen to be the recipient virus for the porcine TNF-α gene. PrV PHY4EBG was propagated in MDBK cells and purified as described elsewhere (Lukács et al., 1985).

Plasmids. The plasmid pTNFPC was a gift of P. Chardon (Lab. Radiologie Appliquée, CEA-INRA, 78352 Jouy en Josas, France). pREP9 was from Invitrogene Corporation (San Diego). Standard procedures (Sambrook et al., 1989) were used in all constructions described in this report. The vector fragments generated by restriction enzyme digestion of pTNFPC and pTFH, and the TNF-α gene module excised from pTFHX, were gel purified and ligated. Ligated DNA was used to transform Top10F' bacteria (Invitrogene Corp., San Diego) as described by the manufacturer. Recombinant plasmids were produced by the clear lysate procedure (Sambrook et al., 1989). The nucleotide sequences of the flanking region of each cloned fragment were determined to verify the constructs, using the dideoxynucleotide chain termination method (Sanger et al., 1977; Sequenase 2.0, United States Biochemical Corp., Cleveland).

Transfection of MDBK cells. At 1 day prior to transfection, approximately 3×10^6 MDBK cells were seeded in 100 mm diameter culture dishes. Plasmids pREP9 and pREP9-TNF were transfected into MDBK cells by using the calcium precipitation method (Sambrook et al., 1989). Cells were cotransfected with 2 μg of neomycin-selectable shuttle vectors pREP9 or pREP9-TNF with 3 μg of salmon sperm DNA as carrier. After 6 hours incubation at 37 °C, the medium was removed and the cells were glycerol shocked (15% glycerol). Then the transfected cells were left in G418-selection medium for 72 hours, trypsinized and split 1 to 5 into 100 mm diameter culture dishes containing the same selection medium. After 14 days, G418 resistant cells were obtained and amplified.

Semiquantitation of TNF-α in cell culture fluids. Cell-free culture fluids were harvested and stored at 4 °C until used. Serum-free RPMI 1640 was added to wells of 96-well microtiter plates, and serial dilutions were made of the cell culture samples as well as a murine recombinant TNF-α standard. Washed WEHI 164 cells were resuspended in RPMI 1640 containing 10% FCS at $1–2 \times 10^5$ cells/ml and 100 μl of the cell suspension were added to each well. After overnight incubation at 37 °C in 5% CO_2, endpoints (1 TNF unit/ml) were read as the

highest dilution of sample that caused 50% cytopathic effect as determined by microscopic examination. The concentration of TNF was approximated by comparing endpoints of samples with the endpoint of the standard TNF-α of known concentration.

Virus plaque titration assay. MDBK cells were grown in 6-well tissue culture plates. Twenty-four hours after reaching confluency, cells were washed with phosphate buffered saline (PBS). The PBS was removed and 100 µl of PBS-diluted virus were added. Following incubation at 0 °C for 60 min the inoculum was removed and replaced by MEM containing 0.5% carboxymethylcellulose. After 4-day incubation at 37 °C, an equal volume of 5% neutral red solution in PBS was added, and the cells were incubated 2 hours at 37 °C. Then the medium was removed and plaques were counted.

Results

The porcine TNF-α gene codes for a protein of 232 amino acids and is composed of four exons and three introns. The lengths of exons are 187 base pairs (bp), 46 bp, 45 bp, and 422 bp, while the introns are 586 bp, 183 bp, and 324 bp long. The region upstream of the coding portion of the gene contains a TATA-box (–25 bp) and several binding sites for other transcription factors. The region downstream of the coding portion of the gene contains a putative PolyA site 800 bp from the stop codon and an AT-rich sequence 450 bp from the stop codon (Kuhnert et al., 1991). The latter sequence is found in the 3'-region of several cytokine genes and is thought to be a tissue-specific regulatory sequence, probably influencing mRNA stability (Kruys et al., 1992).

Construction of a recombinant shuttle vector carrying the porcine TNF-α gene

We have used a 4 kilobase-pair (kbp) *Bam*HI fragment (BT), derived from swine genomic DNA (Chardon et al., 1991), containing the entire TNF-α gene (Kuhnert et al., 1991) cloned into a pUC18 cloning vector (Chardon et al., 1991; pTNFPC, Fig. 1). The published porcine TNF-α sequence is that of the mini-pig (10240 nucleotides, Kuhnert et al., 1991), and our partial sequence data show that there are few differences between the two DNA sequences (not shown). These sequence data allowed us to localize the first *Bam*HI restriction site 207 bp upstream from the TATA-box of the TNF-α gene promotor (nucleotide 6825 in Kuhnert et al., 1991). The second *Bam*HI site was found approximately 800 bp downstream from the end of the published sequence.

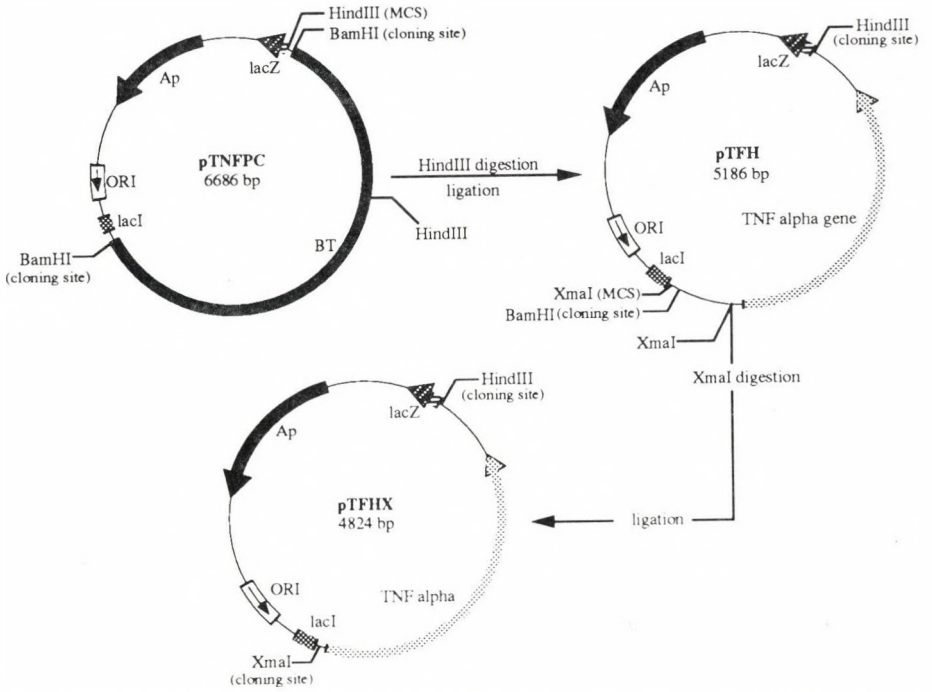

Fig. 1. Schematic representation of the construction of the recombinant plasmids pTFH and pTFHX, derived from pTNFPC

*Hin*dIII digestion of pTNFPC removed 1.5 kbp from the downstream region of BT (Fig. 1), extending from the *Hin*dIII site of the multiple cloning site (MCS) of pUC18 to that located in BT at nucleotide 9346 (Kuhnert et al., 1991). The deletion eliminated the tissue-specific regulatory sequences and the polyA site, leaving a 300 bp sequence downstream of the stop codon. Religation of the digested plasmid provided the construct pTFH (Fig. 1).

To obtain the doubly deleted plasmid pTFHX, the BT fragment was further truncated with *Xma*I to remove 0.38 kbp from the upstream region, including all transcriptional regulatory sequences. The deleted region extended from the *Xma*I site of the MCS of pUC18 to the *Xma*I site situated at nucleotide 7202 in BT, leaving a 55 bp sequence upstream of the ATG codon (Fig. 1).

Next, using the *Kpn*I site of the MCS (overlapping the *Xma*I cloning site) and the *Hin*dIII cloning site mentioned above, we removed the truncated TNF-α gene (2149 bp) from pTFHX (Fig. 2). The truncated gene was then inserted into the *Kpn*I/*Hin*dIII-digested shuttle vector pREP9 (neomycin-selectable) to obtain the pREP9-TNF recombinant construct (Fig. 2). The position of the *Kpn*I and

*Hin*dIII sites assured that the inserted gene was under the transcriptional control of the RSV LTR.

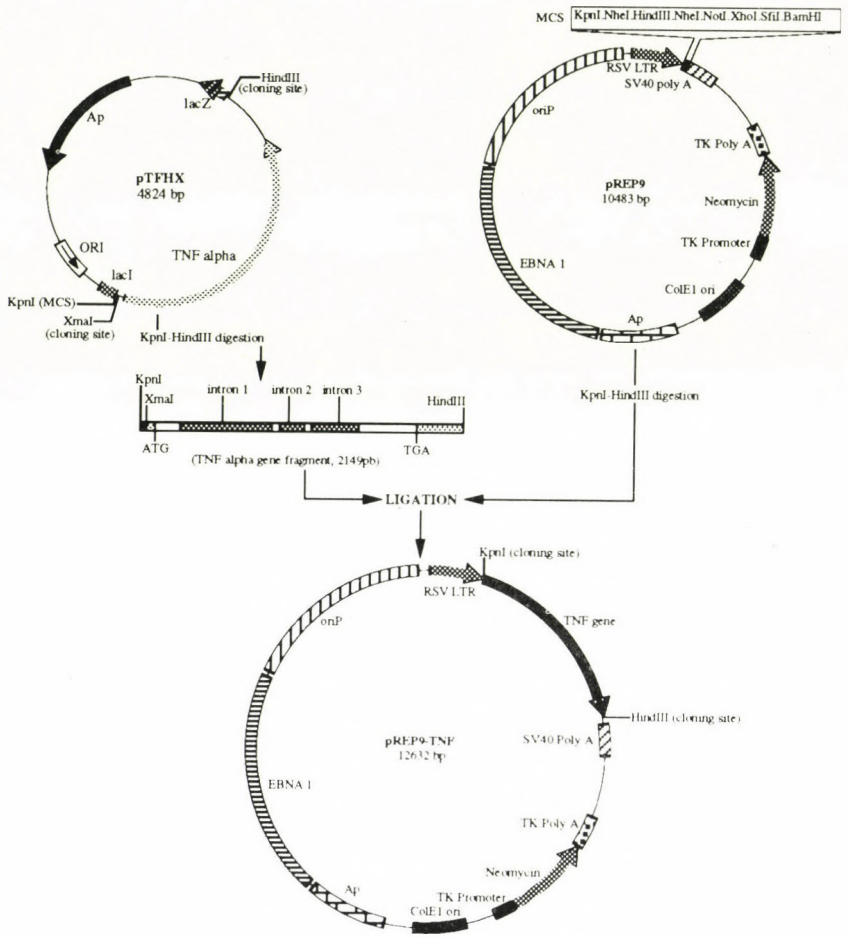

Fig. 2. Schematic representation of the construction of the recombinant shuttle vector pREF9-TNF

Isolation and characterization of bovine cells harboring the TNF shuttle vector

To verify that the cloned gene expresses biologically active TNF-α, pREP9-TNF and pREP9 were used to transfect MDBK cell line and stable transformants harboring the TNF-α-carrying (pREP9-TNF) or control (pREP9) plasmids were selected with neomycin. One cell line thus obtained (MDBK-TNF) secreted 20–30 pg of active TNF-α per milliliter of culture medium (equivalent to

10^6 cells) after five days in culture (Fig. 3C). A second cell line (MDBK-TNFΔ) was also isolated in a separate transfection experiment with pREP9-TNF. This line failed to secrete detectable amounts of active TNF-α, and has been found to harbour a deletion which is currently being characterized. In addition, a cell line obtained by transfection with the control plasmid pREP9 did not produce TNF-α.

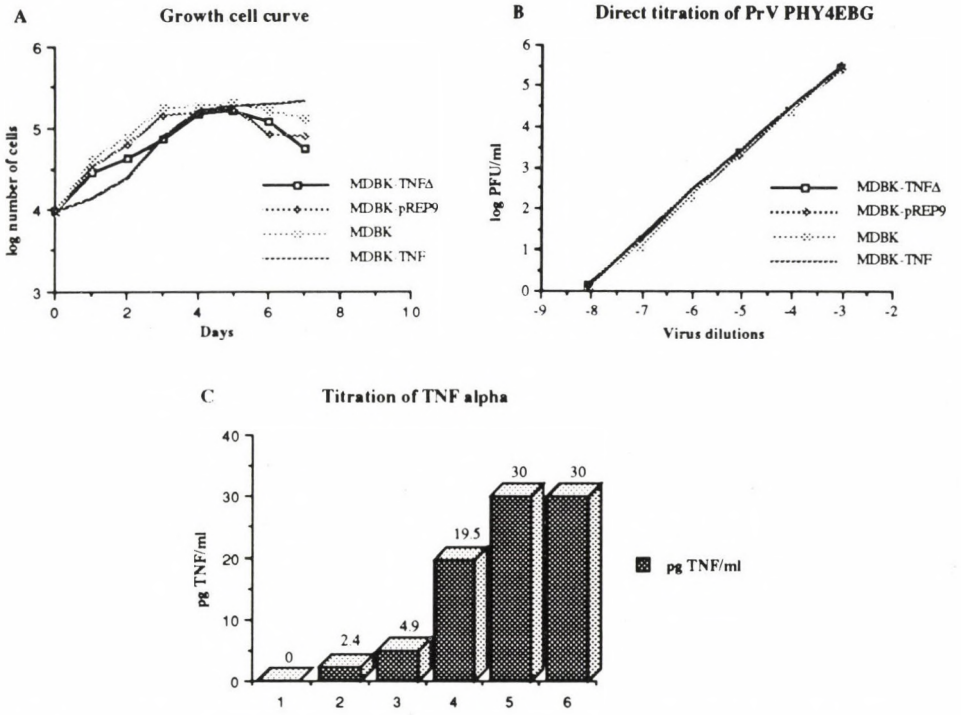

Fig. 3. A. Growth curves of the indicated MDBK cell lines; the abscissa shows the number of days in culture and the ordinate shows the logarithm of cell count per culture dish well. *B.* Plaque titration of PrV PHY4EBG virus; the abscissa shows the virus dilution ($-2 = 10^{-2}$) and the ordinate shows the logarithm of plaque-forming units per milliliter. *C.* Semiquantitation of TNF-α in MDBK-TNF cell culture fluids; the abscissa shows the number of days in culture and the ordinate shows the estimated concentration of TNF-α used as standard

Two biological parameters were determined for the cell lines: first, growth in MEM–10% fetal calf serum (500 μg/ml neomycin for the plasmid-carrying lines) medium at 37 °C (Fig. 3A), and second, permissivity for infection by PrV in a plaque assay (Fig. 3B). No significant differences were observed among the cell lines with respect to either of these parameters, except for the possible presence of

a lag period at the beginning of the growth period of cell line MDBK-TNF (Fig. 3A).

Discussion

In this report we have described the construction of a bovine cell line stably expressing biologically active porcine TNF-α. Note, however, that since we have not directly characterized the substance responsible for the biological activity, the conclusion that it is *bona fide* porcine TNF-α essentially rests on circumstantial evidence. That cells transfected with a plasmid (pREP9-TNF) containing the porcine TNF-α gene secrete the substance, while cells transfected with the same vector (pREP9) lacking the gene do not, argues for the conclusion, although the small number of independently isolated cell lines does not supply statistical support for it. Equally, the obtention, from cells transfected with (pREP9-TNF), of a TNF-negative cell line subsequently found to carry a deletion in the introduced plasmid, also comforts the conclusion. Another element of support comes from the assay of biological activity, which is known to be highly sensitive and specific for TNF (Espervik and Nissen-Meyer, 1986). The culture fluids of the MDBK-TNF cell line induced a cytolytic effect in the target WEHI 164 cells which was qualitatively indistinguishable from that induced by the murine recombinant TNF-α used as standard.

Additionally, we emphasize that the quantitative estimates of porcine TNF-α protein given here are derived from measurements performed on murine cells with mouse TNF-α as the standard of biological activity. It is not known in this system whether the specific activities of the murine and porcine proteins are equivalent, but since the murine cytokine is functioning in a homologous system it may be that the amounts of porcine TNF-α actually produced have been underestimated.

Finally, the data presented here indicate that the *in vitro* propagation of PrV is not inhibited by the presence of functional TNF-α, a result which constitutes a major prerequisite for the construction of recombinant PrV expressing TNF-α.

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MEASUREMENT OF AUJESZKY'S DISEASE VIRUS RECOMBINATION *IN VITRO* UNDER CONDITIONS OF LOW MULTIPLICITY OF INFECTION

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The natural development of Aujeszky's disease virus (ADV) vaccine-derived recombinants has been proposed as a conceivable, although unproven, outcome of the practice of using modified-live vaccines on infected herds. Herpesviral recombination has been studied *in vitro* using high multiplicity of infection (m.o.i.) to maintain synchronous co-infection of target cells and one-step multiplication cycles. However, natural *in vivo* recombination is unlikely to occur at high m.o.i. with synchronous infection of all available target cells by both parental virus strains. Using a battery of gene-specific polymerase chain reaction assays, an analysis of recombination frequency at comparatively low m.o.i. was performed, which indicates that at low m.o.i. the rate of *in vitro* genetic recombination can be expressed as a function of the number of virus–cell interactions. This finding indicates that the rate of generation of recombinant ADV genotypes in swine infected by multiple ADV strains would be modulated by factors that affect the distribution of different virus strains to common target cells, thereby limiting or enhancing the likelihood of cellular co-infection. A full description of this study is available (*J. gen. Virol.*, in press).

Key words: Aujeszky's disease virus, vaccine, recombination, *in vitro*, low multiplicity of infection

This study was undertaken because it seems unlikely that natural *in vivo* herpesviral recombination would occur under conditions of high m.o.i. with synchronous infection of all available target cells by both parental virus strains, as represented by previous *in vitro* recombination experiments (Ben-Porat et al., 1982; Ihara et al., 1982; Brown et al., 1992). This study represents an analysis of *in vitro* recombination frequency under conditions of comparatively low m.o.i., which were felt to represent *in vivo* conditions more closely than previous *in vitro* measurements of ADV recombination.

Materials and methods

Statistical prediction of cell infection and maximal viral recombination

A threshold value for the frequency of recombinant genotype recovery from a population of N_0 cells co-inoculated with two distinct parental genotypes *W* and *V*, at m.o.i.$_W = X$ and m.o.i.$_V = Y$, was calculated using a simple model equation. The model was intended to estimate the effects of infectious virus concentrations when the number of infectious viral particles is less than the number of cells in the target population. In a simplified form, the hypothetical maximal frequency of recombinant progeny as a proportion of the total number of progeny virus recovered after a single generation of viral replication would be equal to $2[P(WV)] / [P(W) + P(V)]$, when $P(W) = (1-e^{-X})$, $P(V) = (1-e^{-Y})$, and $P(WV) = P(W)P(V)$. A number of assumptions were used to simplify the analysis. Accordingly, if strains W and V were inoculated onto a cell monolayer, each at a m.o.i. of 0.1, the maximal frequency of recombinant progeny virions after a single generation of viral replication would be 9.5%.

Viruses

Two strains of ADV with unique genotypes were used in the *in vitro* co-inoculation experiments. Parental strain W had intact sequences in the thymidine kinase (TK) and glycoprotein X (gX) gene loci. Parental strain V was a deletion mutant vaccine strain with deletions in the TK and gX gene loci. A *lacZ* insert was present in the gX locus of strain V. The genotypes of these two strains will be referred to as TK^+gX^+ and TK^-gX^-, respectively. Recombination between these two parental strains will be based on the identification of TK^+gX^- and TK^-gX^+ isolates.

Co-infection of cell cultures

Four independent experiments were conducted to measure the frequency of recombinant recovery from cell cultures after inoculation with a m.o.i. of 0.1 for each parental strain. A fifth experiment was executed to test the model calculation when disparate concentrations of virus were used to co-inoculate the cell monolayers.

In each experiment, after the appropriate incubation period (four to six hours post-adsorption) the infected cell cultures were scraped to form a suspension in the culture medium. The cell suspension was sonicated, clarified by low speed centrifugation, and stored in aliquots at –80 °C. Diluted aliquots of the infected medium were inoculated onto Vero cell monolayers and individual plaques were picked and propagated by inoculation onto fresh monolayers in 24-well

plates. The plaque clone-infected cells were harvested after the cytopathic effect attained >80%.

Analysis of progeny genotypes

The progeny genotypes were analyzed using a previously described approach for sample preparation and polymerase chain reaction amplification of gene sequences from the TK and gX gene loci (Dangler et al., 1991; Dangler et al., 1993). The technique was used previously to identify recombinant ADV genotypes recovered from experimentally co-infected swine. The ADV TK PCR yielded a 953 bp amplified product from the wildtype TK sequence and a 668 bp product from the deleted TK sequence contained in strain V. The gX PCR yielded a 534 bp product and the *lacZ* PCR yields a 433 bp product.

Several hundred plaque isolates were isolated in each experiment. After sorting the distribution of the recovered genotypes from each experiment, the frequency rates for recovering recombinant genotypes, 90% confidence intervals for the frequency values, and the ratios of parental genotype recovery were calculated.

Results and discussion

In each of the first four experiments the recovery of the wildtype parental genotype (TK^+gX^+) exceeded the recovery of the other three genotypes, including the vaccine parental genotype, TK^-gX^-, even though the cells in these experiments were exposed to each parental strain at equal m.o.i. The difference in the frequency of recovered parental genotypes was thereby considered to be an indication of disparity in the extent of cellular infection and replication by the two parental strains. The ratio of parental genotype recovery was calculated as an index of this disparity. Because the two parental strains had similar single-step growth curves, with the exception of an approximately two-hour lag in the eclipse period of strain V, the unequal production and recovery of parental genotypes was attributed to asynchrony in the early viral infection processes.

Despite the asynchrony in the parental strain replication, the recovery of recombinant progeny genotypes in Experiments 1–4 ranged from 2.7% to 10% of all progeny genotypes characterized. As described earlier, a projected maximal frequency of recombinant genotype recovery of 9.5% was calculated from the simplified model equation. While the observed values seem to be good approximations of the projected value, the model also predicts equal frequencies for recovering the parental genotypes; a feature not emulated by the observed data. Furthermore, an inverse relationship exists in Experiments 1–4, between the observed frequency of

recombinant genotype recovery and the ratio of parental genotype recovery. When the model equation was manipulated to present a scenario in which disparate frequencies of parental genotype recovery would be projected, an asymptotic curve defined the relationship between the frequency of recombinant recovery and the ratio of recovered parental genotypes. In Experiment 5, reduction of the strain V m.o.i. to 0.05 was associated with a predicted reduction in recombinant genotype recovery. However, an additional reduction of strain V m.o.i. to 0.025 did not result in a further decrease in recombinant genotype recovery. This final observation was suggestive of the projected asymptotic relationship.

The simplified projection illustrates a close relationship between target cell co-infection rate and ADV recombination, and indicates that the rate of *in vitro* genetic recombination is a function of the number of virus–cell interactions. This finding suggests that the rate of generation of recombinant ADV genotypes in swine infected by multiple ADV strains would be modulated by factors that affect the distribution of different virus strains to common target cells, thereby limiting or enhancing the likelihood of cellular co-infection.

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RECOVERY OF AUJESZKY'S DISEASE VIRUS RECOMBINANTS FROM EXPERIMENTALLY CO-INFECTED SWINE

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Tissue homogenates were obtained from swine experimentally co-infected with two vaccine strains of Aujeszky's disease virus (ADV). Viral isolates were derived by serial plaque purification directly from tissue homogenates, without an intervening step of isolation and amplification on cell cultures. Use of limiting dilutions and recovery of virus isolates as individual plaques minimized the likelihood of *in vitro* recombination serving as a confounding source of recombinant ADV. The tyhmidine kinase and glycoprotein X gene sequences were classified as wildtype or deleted, using a battery of polymerase chain reaction assays. On the basis of pairwise combinations of the allelic forms of the thymidine kinase and glycoprotein X genes, the isolates were characterized as recombinant and parental genotypes. The results substantiate the observation that ADV vaccine strains can form genetic recombinants *in vivo* after experimentally induced co-infection. A full description of this study is available (*Am. J. Vet. Res.* **54** (4) 540–545, 1993).

Key words: Aujeszky's disease virus, recombination, recovery of recombinants, experimental infection, swine

The objective of this study was to review the observations of a previous study, which described the recovery of recombinant vaccine-derived Aujeszky's disease virus (ADV) isolates from experimentally infected pigs (Henderson et al., 1991). This study was performed to overcome the potentially confounding influence of *in vitro* genetic recombination on the recovery of ADV recombinants, which interfered with interpretation of the original results.

Materials and methods

Viruses

Two ADV vaccine strains were used in the original co-infection experiment. Strain A had intact thymidine kinase (TK) and glycoprotein X (gX) gene loci. Strain B was a deletion mutant vaccine strain with deletions in the TK and gX loci. A *lacZ* insert was present in the gX locus of strain B.

Tissues

Frozen tissue homogenates were obtained from the original study (Henderson et al., 1991), focusing on three experimental groups. The first group (Group ON/ON, n = 7) was inoculated simultaneously by the oronasal route with the two vaccine strains of ADV. In the second experimental group (Group IM/IM, n = 8) the pigs were inoculated simultaneously by the intramuscular route using the two ADV strains. In the third experimental group (Group ON/IM, n = 7), the pigs were inoculated simultaneously with strain A oronasally and strain B intramuscularly. Tissues harvested for virus isolation included brain, trigeminal nerve, tonsil, spleen, liver, and lung.

The tissue homogenates were sonicated and clarified by centrifugation before inoculation onto cell culture monolayers. Using limiting dilutions of the tissue homogenate inocula, individual plaques, herein referred to as primary plaques, were isolated and picked. Each picked plaque was rinsed into media and used as an inoculum for re-isolation of individual plaques, herein referred to as secondary plaques.

The secondary plaques were used to evaluate the purity of the primary plaque isolates. Five secondary plaques were picked and evaluated to establish the genotype of a primary isolate. The primary plaque data was tabulated only if all five secondary plaques had equivalent genotypes when analyzed by the polymerase chain reaction assays (Dangler et al., 1992; Dangler et al., 1993).

The amplified products generated by the PCR were assessed by electrophoresis through 3% NuSieve/1% SeaKem^{c} agarose in Tris-borate-EDTA buffer and visualized by ethidium bromide. The sizes of the amplified product bands were compared with a molecular size standard, *Hae*III restriction endonuclease digested ϕX174 DNA. The ADV TK PCR yields a 953 bp amplified product from the wildtype TK sequence and a 668 bp product from the deleted TK sequence contained in strain B. The gX PCR yields a 534 bp product and the *lac*Z PCR yields a 433 bp product.

Results

The stringency of the isolation procedure reduced the recovery of ADV from the tissue homogenates. In this study, virus was recovered from less than 100% of the pigs used in each group, although the original authors reported 100% recovery from the animals. Virus was not recovered from a majority of the tissues analyzed using the procedure described for this study.

In Group ON/ON a net total of 37 primary ADV plaques were recovered from five of the seven animals in this group. Of the five ADV positive animals, ADV isolates with recombinant genotypes were recovered from four animals. Of the 37 primary plaques in the data set, six isolates (16%) had recombinant genotypes.

In Group IM/IM a net total of 67 primary ADV plaques were recovered from seven of the eight animals in this group. ADV isolates with recombinant genotypes were recovered from all of the seven ADV positive animals. Of the 67 primary plaques in the data set, 30 isolates (45%) had recombinant genotypes.

In Group ON/IM a net total of 73 primary ADV plaques were recovered from five of the seven animals in this group. Of the five ADV positive animals, ADV isolates with recombinant genotypes were recovered from two animals. Of the 73 primary plaques in the data set, 19 isolates (26%) had recombinant genotypes.

Discussion

This study confirms that recombinant genotypes are readily generated under conditions of simultaneous exposure and site. Nonconcurrence of exposure site resulted in only a slight reduction of recombinant genotype recovery from tissue specimens. The recovery of recombinant genotypes was successful, even though the stringency of the isolation procedure reduced overall recovery of virus from the tissue homogenates.

This study and the previous report (Henderson et al., 1991) strongly support that natural generation of recombinant ADV genotypes may occur under proper exposure conditions to multiple strains of ADV. This study does not, however, offer any indication of the potential clinical or regulatory significance of recombinant genotypes.

The system described here is an interesting demonstration model for risk assessment for genetic interactions between genetically engineered agents and their wildtype counterparts. The system also demonstrates the potential usefulness of deletion mutant strains for studying the evolution of novel herpesvirus genotypes by genetic recombination.

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AUJESZKY'S DISEASE VIRUS STRAINS WITH PECULIAR FEATURES

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Aujeszky's disease (AD) virus strains isolated from weak colostrum-deprived piglets were investigated for virulence in rabbits. Most of them (5 out of 9 investigated) proved avirulent. As clinical symptoms of AD were not observed during this period on the farms of origin, one can assume that the strains had a particular affinity for the pig fetus. It is proposed that the strains may be a result of recombination between the attenuated strains MK-35 (gI–) now in use as a live vaccine and MK-35 (gI+), used as a live vaccine until 3 years ago, as well as between the vaccine strain and a wild-type strain of AD virus, followed by the acquisition of tropism for the reproductive system. Virulent as well as avirulent strains were isolated in the same herd. The evaluation of a highly immunogenic killed AD vaccine in this complicated situation is recommended.

Key words: Aujeszky's disease virus, virulence, affinity, pig fetus, transplacental infection, recombination

Aujeszky's disease (AD) was demonstrated for the first time in Bulgaria by Nachev and Petrichev in cattle (1947) and by Semerdjiev in pigs (1947). Toneva (1958) produced data demonstrating the wide distribution of AD virus and Genev and Stojanov showed its involvement in respiratory enzootics in pigs. The disease gained a notably wide distribution at the time of creation of the large swine husbandry enterprises and now almost all such farms are infected. Due to the wide use of vaccination the obvious clinical symptoms (nervous and influenza-like) are observed only in newly infected foci and are very rare events in chronically AD-infected farms.

During the last three years in many of the industrial swine farms mass reproductive disorders were observed: abortions, repeated insemination and, above all, birth of weak piglets that die soon after birth. These disturbances are observed in herds with routine vaccinations and where the sows show no clinical symptoms. We isolated AD virus from the organs of weak colostrum-deprived piglets and found antibodies in their blood sera, which provides evidence for transplacental infection.

In the present investigation we aimed to check the virulence of some of the strains of AD virus isolated from cases of transplacental infection.

Materials and methods

Nine groups of 2 or 4 rabbits each were formed and every rabbit was injected subcutaneously in one of the rear limbs with 1 cm^3 (about 10^5 $TCID_{50}$) virus. On the 15th day after injection individual blood serum was taken from the rabbits which survived injection. A 10% suspension of brain, liver and lungs from every dead rabbit was inoculated into primary cell culture of chicken embryo fibroblasts. The viruses isolated were identified by seroneutralization test and by immunofluorescent microscopy. The blood sera were investigated for AD virus antibodies by micro-seroneutralisation test.

Results

Out of 26 rabbits included in the experiment only 8 (30.77%) died between 72 and 120 hours postinfection. Only one strain (238-1) provoked scratching in the injected rabbits on the 3rd day, while the other 6 rabbits died without showing this symptom. Four out of the 9 strains investigated provoked death in the injected rabbits while the other five were avirulent. Strain 250-1 provoked death in half the rabbits, but the other three virulent strains killed all the injected animals. Ten (55.55%) of the rabbits which survived the injection had antibodies in their blood serum and the other 8 were seronegative. All the seropositive rabbits had antibodies of 1:2 titre, excluding those injected with strain 257-6 which had an antibody titre of 1:4.

Table 1

Results of testing the strains for virulence in rabbits

Strain	No. of injected rabbits	No. of rabbits with			No. of rabbits that died at			No. of rabbits with antibody titres of	
		scratching	nervous signs	indisposition only	72 h	96 h	120 h	1:2	1:4
238-1	2	2	–	–	–	–	2	nd	nd
238-2	4	–	–	–	–	–	–	–	–
250-1	4	–	1	–	–	2	–	1	–
250-2	2	–	–	2	–	–	–	2	–
250-3	4	–	–	–	–	–	–	1	–
256-2	4	–	–	–	–	–	–	4	–
257-1	2	–	–	–	2	–	–	nd	nd
257-2	2	–	–	–	1	1	–	nd	nd
257-6	2	–	–	–	–	–	–	–	2

nd = not done

Strain 238-2 did not provoke seroconversion in the injected rabbits, while strains 250-2, 256-2 and 257-6 provoked seroconversion in all the injected rabbits and strains 250-1 and 250-3 in a quarter of them. Strains 250-2 and 256-2 did not cause death but provoked seroconversion in all the injected rabbits; while both rabbits injected with strain 257-1, originated from the same herd, died at 72 h after injection (Table 1).

Discussion

All of the investigated strains were isolated from weak piglets, killed before sucking colostrum. As no clinical symptoms were observed in the farms of origin during this period, one can assume that they were strains of AD virus with marked affinity for swine fetuses. In the present investigation most of the strains (5 out of 9 investigated) proved avirulent for rabbits. In swine farms with reproductive disorders from which the strains were isolated, a sharp decrease or even complete disappearance of rodents was observed. This, together with the observation that white mice fed with organs of the colostrum-deprived weak piglets died (unpublished data), shows that the strains were fully virulent for rodents.

Kochan et al. (1992) reported that 8 out of 17 strains isolated from the field came from strain BUK, which is widely used as a live vaccine in Poland. Medveczky and Szabó (1981) isolated three strains from the semen of clinically healthy vaccinated boars, and Christensen et al. (1992) defined them as derivatives of the Bartha K-61 strain, which is used as the only vaccine in Hungary. Henderson et al. (1991) proved that in the case of simultaneous infection with two attenuated strains with different genomic and immunologic markers a recombination between them may result. If, as described above, a proportion of pigs in Bulgaria carry the attenuated strain MK-35 (gI+) used as a live vaccine until 3 years ago, injecting them with strain MK-35 (gI–) now used as a live vaccine, may result in recombination between the two strains. Rabbits injected with a mixture of both strains before and after their propagation in cell culture died 6 and 12 h earlier, respectively, than rabbits injected with strain MK-35 (gI+) (unpublished data). Another explanation of the situation can be found in the results of Christensen et al. (1992) who assume that the derivatives of strain Bartha K-61 are a result of recombination with a wild-type virus followed by the acquisition of ability to propagate in the reproductive system.

We isolated virulent as well as avirulent strains of AD virus from weak colostrum-deprived piglets. In addition, strains 250-2 and 256-2 did not cause death but provoked seroconversion in the injected rabbits, while strain 257-1 isolated from the same herd was highly virulent. This supports the suggestion of

Christensen et al. (1992) that different variants of AD virus may exist and complement each other within one farm.

All these assumptions need additional proofs but the present results are sufficient to engender the proposal that in such a complicated situation the use of a highly immunogenic killed vaccine would be beneficial.

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GENOME ORGANIZATION OF THE HERPESVIRUSES: MINIREVIEW

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Fundamentally, the members of the herpesvirus family can be divided into six genome structure types designated by the letters A–F. Most of the viruses relevant to animal diseases belong to the D or E type. Whereas the biological function of the different DNA structures is so far unknown, studies on HSV genome structures indicate a rolling-circle mechanism as a mode of virus replication. Furthermore, the terminal repetitive segments seem to be involved in cleavage/packaging mechanisms and probably in the integration process of virus into the host genome. In the well-studied D and E type viruses a great number of genes are colinearly arranged and are homologous regarding their sequences. Thus genes encoding alpha-proteins map at least in part within the reiterated sequences, whereas beta- and gamma-polypeptide genes are largely located within the unique sequences of the long and short components.

Key words: Herpesvirus, genome structure, type, sequence, arrangement, biological function, HSV-1

All herpesviruses have common morphological characteristics. Thus a typical herpesvirion consists of a core containing a linear double stranded DNA, an icosahedral capsid surrounded by a tegument and an envelope. The size of the DNA varies from 124–235 kb and is characteristic of each virus species. Individual herpesvirus genomes differ slightly in size, which may be due to about 10 kbp variations at the terminal and/or internal reiterated sequences. The G+C content varies from 32 to 75 moles/cent (for a review see Roizman et al., 1992).

An interesting feature of herpesviruses is their sequence arrangement. Fundamentally, the genome structures of the viruses studied so far represent 6 different types, designated as A to F.

This short review covers the sequence arrangement of the genome structure types A–F, the known biological functions of such genome structures, the functional features of the reiterated sequences, and finally the functional organization of the HSV-1 genome.

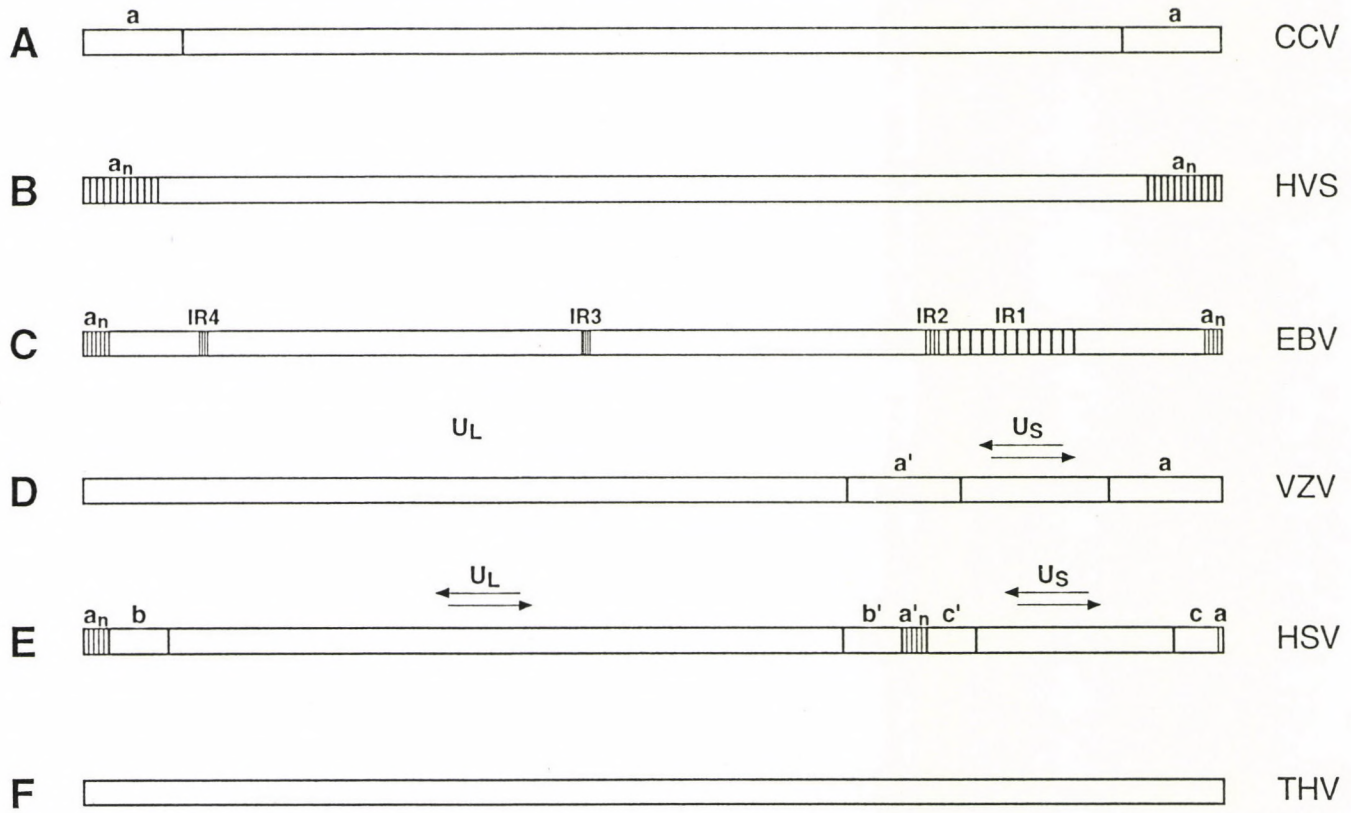

Fig. 1. Arrangement and classification of herpesvirus genomes. This schematic diagram shows the 6 different genome structure types comprising the family *Herpesviridae* according to Roizman (1990). Arrows above the US and UL, respectively, indicate their possible orientations after inversion

Sequence arrangement

Typical elements of the herpesvirus genome structure are terminal and internal repetitive segments flanking a nondisrupted long and short region (Fig. 1). The exception from this are structures A and F, which represent the simplest forms (for a review see Roizman and Sears, 1990).

Genome type A is represented by the channel catfish herpesvirus, equine herpesvirus type 2 (EHV-2) and human herpesvirus type 6 (HHV-6). In these genomes only 2 repetitive sequences are located in equal orientation, one at each terminus.

Group B is exemplified by herpesvirus saimiri (HVS) and bovine herpesvirus type 4 (BHV-4). The central unique part of the genome is flanked by stretches of tandemly arranged direct repeats which have the same orientation at both termini. The number of reiterations may differ.

In group C genomes, exemplified by Epstein-Barr virus (EBV), the number of direct terminal reiterations is smaller than that in the B-type genomes, but there are additional, internal sequences of about 2 kb length, that are directly repeated subdividing the unique sequences of the genome into several well-delineated stretches.

The structure of the D-type DNA was for the first time described by Stevely (1977) for pseudorabies virus (PRV). Subsequent studies of Ben-Porat et al. (1979) confirmed Stevely's findings and showed that the sequences at one terminus of the PRV genome are repeated in an inverted orientation internally. These repeats separate a domain consisting of the stretch of unique sequences, unique short (US), from the remaining sequences, called unique long (UL). Because of the identical ends at the repetitive segments an inversion of the US can occur relatively to the UL. This inversion results in 2 isomeric forms, which are found about 50% each in a molecule population. Additionally, there are some smaller repeats at the left end of the D type genomes (Rall et al., 1991, 1992; Chowdhury, 1987).

Group E is exemplified by the herpes simplex virus (HSV) genome, which has been studied most intensively (Sheldrick and Berthelot, 1974; Wadsworth et al., 1976), and furthermore by bovine herpes mammillitis virus (BHV-2), Marek's disease virus (MDV), herpesvirus simiae (B virus), and cytomegalovirus (CMV). Sequences from both termini are repeated in an inverted orientation and juxtaposed internally, dividing the genome into L and S segments, each of which consists of unique sequences flanked by inverted repeats. The repeats of US and UL are not identical, with the exception of a sequence. Both US and UL can independently or all together invert, therefore up to 4 isomeric forms (designated as prototype, inversion of US, inversion of UL, inversion of US and UL) can be found in

equal molar ratios (Hayward et al., 1975; Jacob et al., 1979). Thus, in contrast to the A, B and C genome structures, the D and E genome types can occur in 2 and 4 isomeric forms, respectively, due to the inverted repeats.

For the genome comprising the F group so far only one virus, the tupaiid herpesvirus 1, was found. The sequences at both termini are not identical and are not repeated directly or in an inverted orientation.

Furthermore, it should be mentioned that EHV-5, causing respiratory infections in horses and showing some homologies to EHV-2, seems to have a new genome structure. As with the tupaia herpesvirus no large internal or terminal repeats seem to exist. This is unique among the equine herpesviruses (Agius et al., 1992).

For definition of new herpesviruses genomes detailed restriction enzyme analyses are necessary. For this purpose specific enzymes cutting only in the individual and repetitive segments, respectively, were used separately or in combination. In the case of the occurrence of different isomers hypomolar fragments will result. Besides this technique electron-microscopical analysis of DNA duplex structures built after denaturation and self-annealing of complementary regions constitute another common method.

Function of the genome types

So far the function of the different virus genome types is not known and no correlation of features of the alpha-, beta- or gamma-herpesvirus subfamilies with the genome structure types seems to exist (Fig. 2). Functional studies done mainly on HSV-1 result in a more detailed understanding of the replication process and can explain the occurrence of the different isomers by inter- and intra-molecular recombination (Jacob et al., 1979; for a review see Roizman, 1979). Thus at different times of the replication circle DNA was investigated by density gradient centrifugation, electron microscopical studies and restriction enzyme analysis (Jacob and Roizman, 1977; Ben-Porat et al., 1979; Rabkin and Hanlon, 1990). From this it became clear that at the early stage of replication a circularization of the DNA molecule occurs by self-annealing. Within the circularized molecule the productive or lytic phase DNA synthesis starts at different fixpoints (origins of replication) ending in a concatemeric form (for reviews see Roizman, 1979, and Hammerschmidt and Sudgen, 1990). This DNA amplification might be the result of a rolling-circle-like mechanism, which is demonstrated in Fig. 3. For the subsequent maturation of virus particles the concatemers must be cut into virion DNA units and packaged. During this cleavage/packaging process two highly conserved blocks of sequences (called pac 1 and 2) are functionally involved, which are cor-

related to the repetitive "a" sequences, as demonstrated for HSV-1 (Vlazny et al., 1982; Mocarski et al., 1985; Deiss et al., 1986).

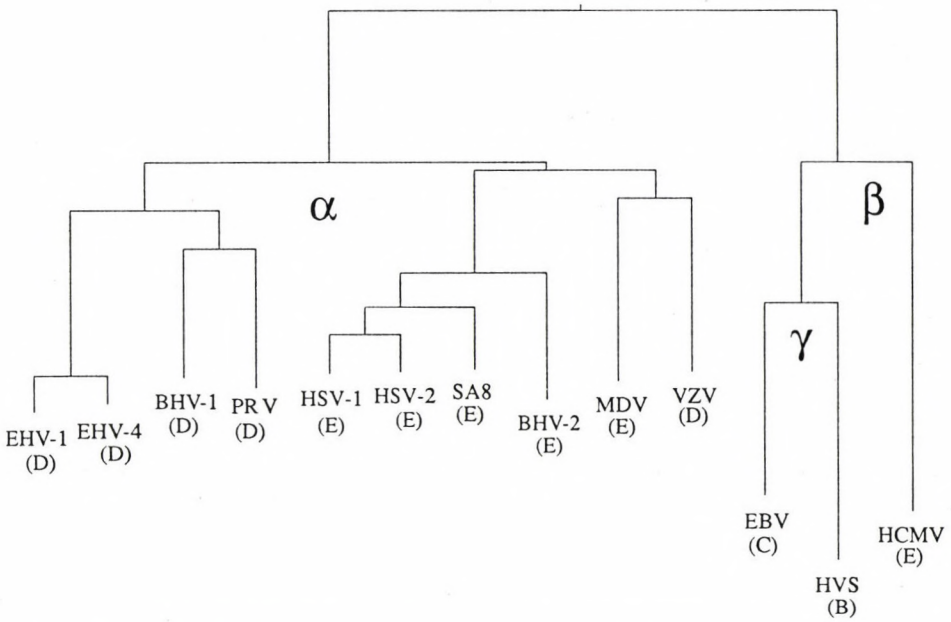

Fig. 2. Evolutionary tree of the herpesviruses. This evolutionary tree is based on homologies in their gB proteins (Borchers et al., 1991; Goltz et al., in preparation). In this tree the members of the herpesvirus family can be separated into α-, β- and γ-subfamilies. The individual genome structure types are indicated in brackets

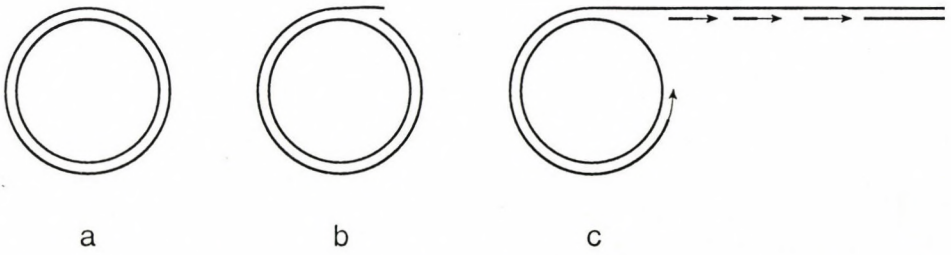

Fig. 3. Rolling-circle model of DNA replication. After circularization of the virus DNA (a) a nick in the template strand (b) leads to the rolling-circle replication (c). The replication fork is asymmetric, containing a leading and a lagging strand. The latter results in so-called Okazaki fragments. After several cycles of replication head-to-tail concatemers accumulate

For the lymphotropic EBV a second method of replication has been demonstrated. Thus in the latent state of the viral life-cycle an origin of plasmid-like replication (*ori*P) is used for cell cycle dependent replication of the episomal genome. The result is a stable copy number of virus genomes in a dividing cell population (for a review see Hammerschmidt and Mankertz, 1991).

Functional and structural relevance of the reiterated sequences

In our group the termini of BHV-1 and the junction UL/US were sequenced. Cloning experiments show that 10% of the BHV-1 ends contain additional nucleotide sequences at the terminal repeat of the right genome terminus (Hammerschmidt et al., 1990). A similar finsing has been reported for PRV by Ben-Porat et al. (1979). Such tail sequences might be of host origin, as indicated by cloning and sequencing of this region in BHV-1. The presence of such host-specific DNA sequences suggests the integration and subsequent inexact excision from the cellular genome. Integration and excision are related recombination events, which are also involved in the circulation/maturation process. It remains to be determined whether the integration is crucial for inducing latency of BHV-1.

To estimate the relevance of these results, EHV-1 genome structures were included in these experiments. Interestingly enough, the nucleotide sequence analysis of the right and left genome termini of EHV-1 done by Chowdhury (1987) showed several copies of tandemly repeated sequences, which might be important for the variability of the virus. Nucleotide sequence comparison done in EHV-1, BHV-1, PRV, VZV and HSV-1 showed conserved terminal domains, which represent the pac 1 and 2 signals, as mentioned above (Deiss et al., 1986; Hammerschmidt et al., 1988). These consensus sequences are characterized by GC- and T-rich areas.

As experienced from studies of defective particles the pac signals are necessary for the cleavage/packaging process (Mocarski et al., 1985; Yalamanchili et al., 1990). To extend the studies on repetitive regions and their role in the cleavage/packaging process the polyrepetitive (pr) DNA elements of BHV-4 were investigated in detail. The total number of elements per genome is 15 on average but the number at each end can differ. The size of a single prDNA element can vary from strain to strain within a range of 1450–2850 bp, which is a result of variable number of internal 200 bp repeats within the prDNA elements. Furthermore, the analysis of DNA preparations derived from a single strain shows this size variation in a small portion of prDNA, even after virus plaque purification. From this one can draw the conclusion that a single genome consists of a mixed population of prDNA elements with one size dominating. These facts lead

to slight but distinct differences in the restriction enzyme patterns of BHV-4 (Ehlers et al., 1985; Bublot et al., 1990; for a review see Goltz and Ludwig, 1991).

Interestingly, in HVS each of the repetitive elements contains the cleavage/packaging signal (Bankier et al., 1985). Recent sequencing of a prDNA element as well as genomic termini confirm these results also for BHV-4 (Goltz et al., in preparation). However, neither in EHV-1 not in BHV-4 could host specific sequences be identified.

Functional organization of the HSV-1 genome

The herpesvirus genes are expressed sequentially in a cascade fashion (Honess and Roizman, 1974; Roizman et al., 1975), but they are not clustered in accordance with the temporal order of their expression.

In closely related herpesviruses genes seem to be colinearly arranged. This means that most of the structural and regulatory genes are located in the same genome segment and mostly in the same orientation in HSV, BHV-1, EHV-1, and PRV. Thus, genes specifying alpha-polypeptides, made very early in the infectious cycle and having regulatory functions, map at least in part within the reiterated sequences of the L and S components (for reviews see Roizman, 1979; Roizman and Sears, 1990). The genes specifying beta- and gamma-polypeptides, representing structural and enzymatic proteins, are mainly located within the unique sequences of the L and S segments. Furthermore, genes encoding type-common properties seem to be localized in the UL, whereas type-specific features are largely encoded in the US. Additionally, most of the genes localized in the US seem to be non-essential for *in vitro* replication of the virus.

In summary, the main biological relevance of the different herpesvirus DNA structures are unknown. Functional studies mainly done on HSV-1 give a more detailed understanding of the replication process and give an explanation for the occurrence of the different isomers by inter- and intra-molecular recombination. The repetitive elements, preferentially located at the termini, seem to be involved in recombination and cleavage/packaging events.

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FUNCTION OF AUJESZKY'S DISEASE VIRUS PROTEINS IN VIRUS REPLICATION AND VIRULENCE

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Aujeszky's disease virus (ADV, pseudorabies virus) is a member of the alphaherpesvirinae. The virus is an economically important pathogen of swine, causing severe neurological and respiratory symptoms. ADV infections often have a fatal course in young piglets, but older pigs usually survive.

The genome of ADV is a linear double stranded DNA molecule of approximately 140 kbp. It is divided in a unique long (UL) sequence of 95 kbp and a unique short (US) sequence of 9 kbp, which is flanked by inverted repeat (IR) sequences of 15 kbp (Fig. 1). The viral genome has an estimated coding capacity for at least 70 proteins. In recent years sequence information has been obtained of more than 40 genes (Mettenleiter, personal communication). The gene arrangement in ADV and the prototype herpesvirus Herpes simplex virus type 1 (HSV-1) is highly colinear. The sequence data reveal an overall homology in amino acid sequence of many ADV and HSV-1 proteins, suggesting that these proteins share identical or similar functions in the biology of both viruses.

HSV-1 is the most intensively studied alphaherpesvirus. Compared to HSV-1, our information on the function of many ADV proteins is still limited. During the past few years, most progress has been made with regard to the structural and functional analysis of the viral envelope glycoproteins and the gene products involved in virulence.

In this article, we review current information on the function of ADV proteins in virus replication and virulence.

Viral proteins involved in entry

Herpesvirus entry into the host cell involves a cascade of interactions between components of the virion and the surface of the cell. According to a model proposed for HSV-1 by Fuller et al. (1992), this complex process of virus entry can be divided in three main stages, i.e., attachment, membrane fusion, and nucleocapsid release. The first step in the attachment of ADV to its host cell is mediated by the major envelope glycoprotein gIII. Mettenleiter et al. (1990) have shown that gIII binds to the cellular heparin-like substance heparan sulphate, that

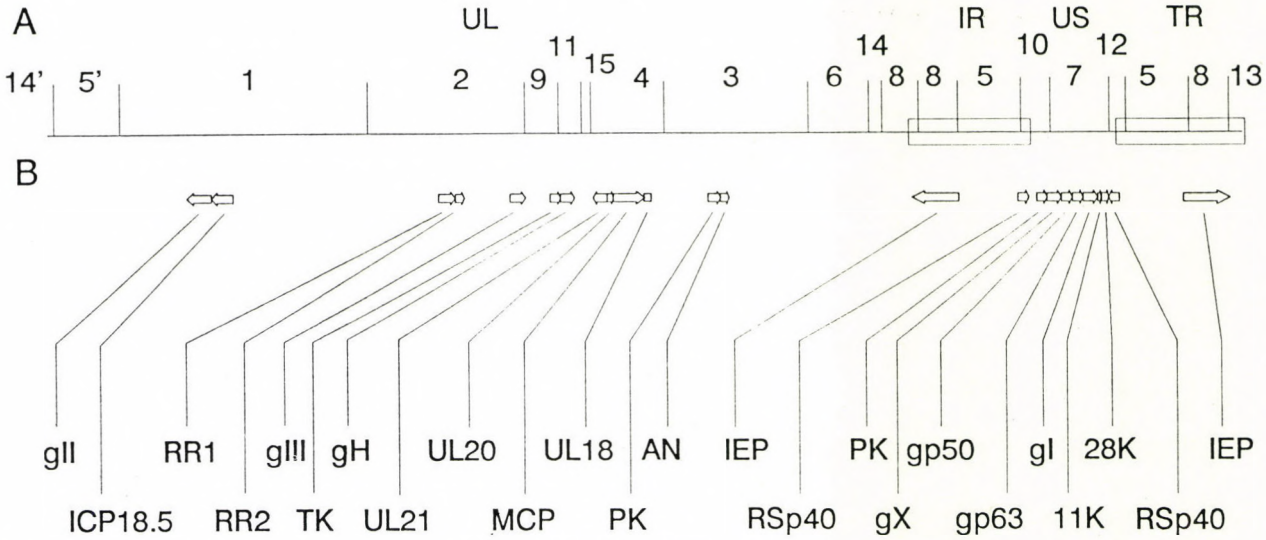

Fig. 1A: Physical map of the ADV genome. The genome consists of a unique long region (UL), which is separated from the unique short region (US) by inverted repeats (open boxes; IR: internal repeat; TR: terminal repeat). *Bam*HI restriction fragments are given in numerical order according to size

Fig. 1B: Location of some of the ADV genes that have been identified. Arrows indicate the direction of transcription. gII: glycoprotein gII (Robbins et al., 1987); ICP18.5, homolog of HSV-1 UL28 (Pederson and Enquist, 1989); RR_1 and RR_2: subunits of ribonucleotide reductase (De Wind et al., 1993); gIII: glycoprotein gIII (Robbins et al., 1986); TK: thymidine kinase (Prieto et al., 1991); gH: glycoprotein gH (Klupp and Mettenleiter, 1991; Peeters et al., 1992*b*); UL20, UL21 (De Wind et al., 1992*b*); MCP: major capsid protein UL19 (Yamada et al., 1991; Klupp et al., 1992*b*); UL18 (Klupp et al., 1992*b*); PK: protein kinase gene UL13 (De Wind et al., 1992*a*); AN: alkaline nuclease gene UL12 (De Wind et al., 1992*a*); IEP: immediate-early protein (Cheung, 1989; Vlcek et al., 1989); RSp40 (Zhang and Leader, 1990); PK: protein kinase gene US3 (Van Zijl et al., 1990; Zhang et al., 1990); gX: glycoprotein gX (US4) (Rea et al., 1985); gp50: glyciprotein gp50 (US6) (Wathen and Wathen, 1984); gp63 and gI, glycoprotein gp63 (US7) and gI (US8) (Petrovskis et al., 1986*a*); 11K: US9 (Petrovskis and Post, 1987); 28K: US2 (Van Zijl et al., 1990)

serves as a receptor for initial binding of virus. Glycoprotein gII also binds to heparin, but efficient binding of this protein is dependent on the presence of gIII. The initial attachment step brings the virus in close contact with the cell. This low affinity binding is heparin-sensitive (Karger and Mettenleiter, 1993). Stable attachment of the virus to the cells is mediated by the essential glycoprotein gp50 and results in heparin-resistant binding (Karger and Mettenleiter, 1993). Thus, both gIII and gp50 are required to allow a close and stable interaction between the virus and the target cell. It has been suggested that the heparin-resistant binding of the virus occurs by interaction with heparan sulphate or a receptor closely associated with this component in the plasma membrane (Karger and Mettenleiter, 1993).

After stable attachment, fusion of the virion envelope and the cell membrane can occur by interaction of both viral and cellular components. It is likely that several structural components of the virion play a role in mediating the fusion event. Although gp50 is required for stable virus attachment and might trigger the fusion event, this protein may not be required for the fusion process itself. This assumption is based on the observation that gp50 is required for virus penetration, but not for cell-to-cell spread of the virus (Rauh and Mettenleiter, 1991; Peeters et al., 1992*a*). Two other essential envelope glycoproteins, i.e., gH and gII, are both required for penetration as well as for cell-to-cell spread and are presumably involved in the virus-cell fusion event (Rauh and Mettenleiter, 1991; Peeters et al., 1992*a*; Peeters et al., 1992*b*; Klupp et al., 1992*a*; Kopp and Mettenleiter, 1992). It has been reported that mutations in four HSV-1 genes [UL1 (gI), UL20, UL24, and UL53 (gK)] can give rise to a syncytial phenotype. (Hutchinson et al., 1992*a*; Hutchinson et al., 1992*b*; Jacobsen et al., 1992; Baines et al., 1991). The protein products of these genes may play an accessory role in virus-induced membrane fusion events by interacting with viral proteins directly involved in the membrane fusion process. Such an interaction has been observed for glycoprotein gH and gI (Hutchinson et al., 1992*a*). In infected cells gH is tightly associated with gI. The formation of the gH/gI complex appears to be required for proper folding, transport, and surface expression of gH and gI. The heterodimer of gH is incorporated into the virion envelope. Together, these results suggest that gI plays an important (accessory) role in virus entry and cell fusion. The ADV counterparts of HSV-1 gI and gK have recently been identified by Mettenleiter et al. (Klupp et al., 1993*a*).

Of all glycoproteins involved in entry, gIII is probably the best characterized. This major component of the virus envelope is a multifunctional protein. Glycoprotein gIII plays a key role in attachment and appears to be a major immunogen. The protein induces neutralizing antibodies in the absence of complement and is a target for T-cell responses in mice and pigs (Ben-Porat et al., 1986*a*; Zuckermann et al., 1990). Glycoprotein gIII is dispensable for virus growth in tis-

sue culture cells, although the virus has a reduced infectivity and growth to lower titres than wild-type virus (Whealy and Enquist, 1988). The multifunctional properties of gIII can further be deduced from its role in virus release and in virulence. However, these phenotypes of gIII can only be observed when inactivation of gIII is accompanied by the inactivation of either glycoprotein gI or gp63 (Schreurs et al., 1988; Mettenleiter et al., 1988*b*; see below). Similar to gC of HSV-1, gIII binds to the third component of complement and interferes in this way in the complement cascade (Huemer et al., 1992). Binding of gIII was only observed with C3 of swine but not with human C3. The interaction of gIII with this pivotal component of the complement pathway may protect infected cells against complement dependent lysis and may protect the virus against complement dependent neutralization.

Viral enzymes involved in nucleic acid metabolism

To support viral DNA replication in dividing and non-dividing cells, HSV-1 encodes several enzymes involved in the supply of building blocks for DNA synthesis. These enzymes include ribonucleotide reductase (UL39 and UL40), thymidine kinase (UL23), dUTPase (UL50) and Uracil-DNA glycosylase (UL12) (Roizman and Pears, 1990).

Presently, only the homologs of HSV-1 genes UL23, UL39 and UL40 have been identified in ADV.

Ribonucleotide reductase (RR) consist of two non-identical subunits, a large subunit (α) encoded by UL39 and a small subunit (β) encoded by UL40. The two subunits associate in an $\alpha_2\beta_2$ heterodimer and together they form the active catalytic domain of the enzyme. Thus, both subunits are required for enzymatic activity (Roizman and Pears, 1990). RR occupies a key position in the *de novo* synthesis of dNTPs. The enzyme reduces the four common ribonucleotide diphosphates to the corresponding deoxyribonucleotides. The deoxyribonucleotides dADP, dCDP and dGDP are subsequently phosphorylated to dNTPs and can be used directly for DNA synthesis. Synthesis of dTTP requires a multi-step modification of dUTP. The pyrophosphatase dUTPase acts in the first step of this reaction sequence by hydrolysing dUTP to dUMP (Reichard, 1988).

Deoxyribonucleotides (dNTPs) can also be synthesized via the salvage pathway by using nucleotides obtained from degradation of DNA. The viral thymidine kinase (TK) is required for this pathway and functions to phosphorylate deoxythymidine to dTMP (Roizman and Pears, 1990).

The virus-encoded RR and TK activities are both dispensable for growth in tissue culture cells, indicating that the endogenous mammalian RR and TK will

compensate for the defective viral genes in dividing cells (De Wind et al., 1993). However, RR1 mutants grow lower titres in tissue culture than TK mutants and wild-type virus (De Wind et al., 1993). This observation indicates that the supply of dNTPs via the *de novo* pathway (RR dependent) is more important for viral DNA synthesis than the salvage pathway (TK dependent).

Although the viral RR and TK are not essential for growth in dividing cells, they are required for a productive infection of non-dividing cells such as neurons. RR and TK mutants indeed proved to be completely avirulent in mice and both had a strongly reduced virulence in pigs (De Wind et al., 1993; Kimman, personal communication). However, in pigs an RR mutant was more severely impaired (100- to 1,000-fold) in replication in nasal and oropharyngeal tissues than a TK mutant (De Wind et al., 1993; Kimman personal communication). This finding was surprising as the endogenous RR of the dividing mucosal epithelial cells was expected to compensate for the inability of the virus to supply dNTPs via the *de novo* pathway. Thus both the *in vitro* and the *in vivo* data indicate that the viral RR activity is more important for efficient viral DNA synthesis than the viral TK activity.

Viral enzymes involved in phosphorylation

Protein phosphorylation plays a major role in signal transduction and in the regulation of growth and differentiation of cells. This phosphorylation occurs by protein kinases which constitute a large family of enzymes (Hanks et al., 1988). Protein kinases are also encoded by herpesviruses. In HSV-1, three genes, US3, UL13, and UL39, specify protein kinases which phosphorylate serine and threonine residues (Purves et al., 1987; Cunningham et al., 1992; Paradis et al., 1991). The kinase activity associated with the large subunit of HSV-1 RR (UL39) maps at the N-terminal domain of the protein (Paradis et al., 1991). Because this domain is absent in the ADV homolog, it is likely that ADV RR_1 does not have kinase activity (De Wind et al., 1992*a*). The ADV homologs of US3 and UL13 have been identified and sequenced (Van Zijl et al., 1990; Zhang et al., 1990; De Wind et al., 1992*a*). Both encode a protein serine/threonine kinase that is nonessential for growth of the virus in cell culture (Van Zijl et al., 1990; De Wind et al., 1992*a*).

Although the US3 and UL13 encoded protein kinases are members of the same family, they show quite distinct biochemical and biological properties. In HSV-1 infected cells the US3 protein is localized in the cytoplasm, whereas the UL13 protein is mainly found in the nucleus (Cunningham et al., 1992; Zhang et al., 1990). The US30 protein kinase can only use ATP to phosphorylate basic substrates, whereas the UL13 protein uses both ATP and GTP to phosphorylate

basic and acidic substrates (Cunningham et al., 1992; Leader and Katan, 1988). The US3 and UL13 protein kinases of ADV and HSV-1 are present in purified virions and can be autophosphorylated (Cunningham et al., 1992; Zhang et al., 1990; De Wind et al., 1992*a*; Frame et al., 1987). Purves et al. (1991, 1992) have shown that HSV-1 US3 protein kinase phosphorylates a virion membrane protein of 30 kDa encoded by UL34. The function of UL34, which is presumably essential for virus replication, is still unknown. By using an *in vitro* assay, Coulter et al. (1993) showed that HSV-1 UL13 protein kinase phosphorylates the major tegument protein of 38 kDa which is the product of the UL49 gene. Whether these protein are the natural and most important targets for the kinases will need further study. Much less is known about the substrates of the ADV kinases. There is only one report showing *in vitro* phosphorylation by US3 PK of a major virion phosphoprotein of 112 kDa (Zhang et al., 1990).

The biological features of ADV UL13 and US3 mutants are quite different. UL13 mutants display the wild-type growth phenotype in cell culture and are virulent in mice (De Wind et al., 1992*a*; De Wind, personal communication). US3 mutants, however, show a slightly impaired growth in cell culture and a strongly reduced virulence in mice and pigs (Kimman et al., 1992; Kimman, personal communication). The information currently available on the US3 and UL13 protein kinases suggests that they play a different role in the viral replication cycle. Identification and functional analysis of their natural substrates in infected cells will undoubtedly contribute to our insight in the function of the viral protein kinases.

Capsid components

The capsid of herpesviruses is an icosahedral protein structure that encloses the core which contains the viral DNA (Roizman and Pears, 1990). Three different types of capsids have been isolated from infected cells. Type A, or empty capsids, which are devoid of a core and DNA; type B, or intermediate capsids, which contain a core but lack DNA; and type C, or mature capsids, with a core containing DNA.

Type B capsids differ from type C capsids in that they contain an abundant protein, VP22a. This protein is presumably involved in packaging of viral DNA and only transiently associated with B capsids. It is generally assumed that type B capsids are the precursors of type C capsids.

The HSV-1 capsid is composed of seven proteins (Roizman and Pears, 1990). Four proteins, VP5 (UL19), VP19C (UL38), VP23 (UL18), and VP26 (UL35) are components of the capsid cell. Three proteins, VP21 (UL26), VP22a (UL26.5), and VP24 (UL26) are presumably core proteins (Roizman and Pears,

1990; Schrag et al., 1989; Rixon et al., 1990; McNabb and Courtney, 1992; Desai et al., 1993; Liu and Roizman, 1993). VP5 is the major capsid protein and forms the main component of the capsomers. The capsid components VP19C, VP23 and VP26 presumably interact with VP5 to form the complete capsid shell. VP22a, which is transiently associated with the cores of intermediate capsids, has been proposed to be the assembly or scaffolding protein (Newcomb and Brown, 1991). This protein is able to self-assemble into torus-shaped structures, and might serve as a scaffold around which the capsid proteins assemble to form the capsid shell. Another product of the UL26 locus, the core protein VP24, has serine protease activity and might be required in either capsid morphogenesis or capsid disaggregation after penetration of the virus (Davison et al., 1992).

Ladin et al. (1982) identified three major protein components of 142 kDa, 35 kDa, and 32 kDa in capsids isolated from cells infected with ADV. In addition, most capsid preparations contained a minor 62 kDa component and in some preparations two minor proteins are found of 38 kDa and 41 kDa.

It has been shown that ADV temperature-sensitive (ts) mutants defective in capsid assembly belong to four complementation groups. By using marker rescue, these mutants have been mapped to the ADV *Bam*HI fragment 4 (Ladin et al., 1982). *In vitro* translation of mRNAs transcribed from this region, followed by immunoprecipitation with sera prepared against purified capsids indeed yielded three proteins of 142, 62 and 32 kDa (Lomniczi et al., 1987). The sequences of the genes encoding these protein have recently been elucidated (Yamada et al., 1991; Klupp et al., 1992*b*; De Wind et al., 1992*b*). The open reading frames encoding the major ADV capsid proteins of 142 kDa and 32 kDa represent the homologs of genes UL19 and UL18 of HSV-1.

The 62 kDa protein is the product of the ADV homolog of the UL21 gene of HSV (De Wind et al., 1992*b*). Although the available data suggest that this 62 kDa virion component is a structural protein of the virion capsid, this supposition still lacks definitive proof. The UL 21 product has a regulatory or accessory role is processing of viral DNA or in packaging, so it is more likely that the UL21 protein is closely associated with capsids during capsid morphogenesis. However, additional work will be required to clarify this issue definitively. Finally, it might be speculated that the minor capsid components of 38 kDa and 41 kDa are the ADV homologs of the protein specified by the UL26 domain of HSV-1.

Studies with HSV-1 ts (UL19, UL26, UL38) and null (UL18, UL19) capsid mutants have shown that the proteins encoded by these genes are essential for capsid assembly and thus for virus replication (Desai et al., 1993). These mutants fail to package the DNA and cleavage of the concatemeric DNA to unit length molecules will not occur. However, capsid formation appears not to be sufficient for cleavage and encapsidation of concatemeric DNA. In addition to the capsid

proteins, the products of at least five other genes (UL6, UL25, UL28, UL32, and UL33) are required for the formation of mature capsids (Tengelsen et al., 1993).

In ADV the function of a protein homologous to ICP18.5 (UL28) of HSV-1 has been studied by Mettenleiter et al. (1993). Because this 79 kDa protein is essential for virus replication, null mutants were propagated in cells that could provide the protein in trans. When the phenotypically complemented viruses were used to infect non-complementing cells, it was found that cleavage of concatemeric DNA did not occur. It was concluded that ICP18.5 plays an essential role in the formation of mature capsids.

A role in capsid formation has also been assigned to the 62 kDa and 41 kDa proteins of gene UL21. The 41 kDa product represents presumably the N-terminal part of the 62 kDa protein and is obtained by proteolytic processing. Both proteins are present in purified virions and, as mentioned before, experimental evidence suggest that the 62 kDa protein is associated with purified capsids (Ladin et al., 1982; Lomniczi et al., 1987). Although the UL21 protein is not essential for virus replication, it is required for efficient growth in tissue culture. ADV UL21 mutants are strongly impaired in processing of viral DNA, indicating that the UL21 encoded protein plays a role in capsid maturation. As expected, these mutants had a strongly reduced virulence in mice.

The reduced virulence of UL21 mutants might explain the defect in virulence of strain Bartha assigned to *Bam*HI fragment 4 (Lomniczi et al., 1987). Recently this defect was shown to be caused by point mutations in gene UL21 (Klupp et al., 1993*b*). The list of gene products involved in capsid maturation might also include the alkaline nuclease activity encoded by the HSV-1 UL12 gene (Weller et al., 1990; Thomas et al., 1992). Its homolog in ADV has been identified by De Wind et al. (1992*a*). Alkaline nuclease (AN) is a phosphorylated early protein that is not essential for virus replication, but is necessary for efficient growth. It has been shown that the HSV-1 AN associates with the major DNA-binding protein and localizes to the replication complex in the nucleus of infected cells (Thomas et al., 1992). Associated with the replication complex it might function in processing and packaging of viral genomes. An ADV insertion mutant deficient in synthesis of AN has a severely impaired growth in cell culture and a strongly reduced virulence in mice (De Wind, personal communication).

The role of gI-gp63 in neurovirulence

Glycoproteins g63 and gI are the homologs of HSV-1 glycoproteins gI and gE, respectively (Petrovskis et al., 1986*a*). The proteins are encoded by two adjacent genes, US8 and US9 located in the S component of the viral genome. Both

proteins have the features of membrane proteins and are structural components of the virion envelope (Petrovskis et al., 1986*a*; Whealy et al., 1993). Glycoproteins gI and gp63 can be co-immunoprecipitated by antisera against either gI or gp63, suggesting that they form a complex in infected cells (Whealy et al., 1993; Zuckermann et al., 1988). This complex is also present in purified virions (Whealy et al., 1993). The gI-gp63dimer is most likely stabilized by monovalent hydrophobic interactions (Zuckermann et al., 1988; Whealy et al., 1993). In infected cells, formation of the complex occurs in the ER probably soon after co-translational addition of high-mannose sugars (Whealy et al., 1993). Oligomerization was found to be required for rapid and efficient conversion of the gI and gp63 precursor polypeptides to mature glycoproteins and was also required for efficient transport of both proteins to the Golgi (Whealy et al., 1993). Inactivation of either gI or gp63 similarly affects the biological characteristics of the virus in cell culture and *in vivo*, indicating that the phenotype is determined by the gI/gp63 complex. Expression of either gI or gp63 is not essential for virus replication in cell culture (Petrovskis et al., 1986*b*; Mettenleiter and Rziha, 1985). Viruses lacking either gI or gp63 grow to wild-type titers in pig kidney (PK) and rabbit kidney (RK) cells (Ben-Porat et al., 1986*b*; Mettenleiter et al., 1987*a*). Furthermore, the absence of these genes does not effect the release of mutant virus from the latter cells. Remarkably, both gI and gp63 mutants show a growth advantage on chicken embryo fibroblasts (CEF), probably resulting from the more efficient release of the mutants from CEF (Zuckermann et al., 1988; Mettenleiter et al., 1988*a*; Zsák et al., 1989). Glycoproteins gI and gp63 apparently have a deleterious effect on viral growth in CEF. Passage of wild type virus on CEF cells results in the accumulation of virus mutants with a deletion in the gI/gp63 region of the viral genome (Mettenleiter et al., 1988*a*). Whereas inactivation of the genes specifying either gI, gp63 or gIII does not affect virus release from RK cells, inactivation of both gI and gIII or gp63 and gIII will do so, indicating that the three proteins can affect virus release in conjunction with each other.

Despite the growth advantage of both gI and gp63 mutants on CEF, the plaque size on these cells was significantly reduced (Zsák et al., 1992). This might be caused by the reduced capacity of gI mutants to spread via direct cell-to-cell transmission. Because gI mutants also form small syncytia, it was concluded that gI is one of the viral proteins that promote cell fusion and cell-to-cell transmission of the virus.

Inactivation of either the gI or gp63 gene will similarly affect the virulence of the virus for pigs or other ADV susceptible animals (Kimman et al., 1992; Mettenleiter et al., 1987*b*). In mice, rats and chickens these mutants had a slightly reduced virulence resulting in longer survival of the infected animals. However, a marked decrease in virulence was observed when the inactivation of either the gI

or gp63 gene was accompanied by the inactivation of gIII (Mettenleiter et al., 1987; Zsák et al., 1992; Card et al., 1992). This reduced level of virulence is probably caused by the impaired spreading efficiency of the mutant virus (Zsák et al., 1992). Wild-type virus can spread either by direct transmission from infected cells to uninfected cells or, after release from infected cells, by adsorption to and penetration of uninfected cells. However, a gI/gIII double negative mutant will be impaired both in direct cell-to-cell transmission (gI) and in adsorption (gIII) to uninfected cells (Zsák et al., 1992). ADV mutants lacking either gI or gp63 have a strongly reduced virulence for 3–10 week old piglets (Kimman et al., 1992; Jacobs et al., 1993). The decreased virulence was manifested by the absence of typical clinical signs of Aujeszky's disease (mortality, neurological and respiratory signs, weight loss) and a reduced period of fever. Mutant virus could be recovered from most peripheral tissues and the trigeminal ganglion, but the mean virus content in the trigeminal ganglion was significantly lower than in pigs infected with virulent virus. Remarkably, no virus was isolated from other parts of the CNS.

These findings indicated that the gI mutant virus (*i*) infects the same peripheral tissues as the virulent virus does, even though its multiplication is somewhat impaired, (*ii*) can enter neural cells in the nasal and olfactory mucosa and replicates locally in the trigeminal ganglion, and (*iii*) after entry of the sensory neurons does not easily spread into the CNS.

Immunohistological examination of the mucosa of the nasal cavity early after infection of pigs infected with high doses of a virulent virus or a gI negative virus confirmed that the latter virus is impaired in spreading through the mucosa (Pol et al., 1989). Virulent virus caused widespread necrotic lesions in the nasal mucosa, invaded rapidly the submucosal cells and infected olfactory neurons within 24 h post inoculation. In contrast, the gI mutant caused less necrotic lesions, showed a reduced rate of spread into the submucosa and a temporarily retarded (72 h) infection of the axons of the olfactory neurons.

A role of gI/gp63 in neurovirulence has also been demonstrated using the rat visual system as a model to study ADV transneuronal transport (Mettenleiter and Rziha, 1985; Card et al., 1991; Card et al., 1992). Striking differences were observed in the pattern of viral transport in the CNS after ocular infection of virulent virus and gI/gp63 mutants. Following inoculation, both virulent and mutant virus replicated in the ganglionic cells of the retina. The virulent virus was subsequently transported to all known retinorecipient areas in the brain, but gI/gp63 mutants only infected a subset of these neurons. It was concluded that gI and gp63 are required to infect specific classes of neurons in the rat retina, e.g. through interactions with a gI/gp63 receptor. Taken together, these observations suggest that the gI/gp63 complex might be required for the efficient completion of distinct steps in the transfer of virus from the peripheral tissues to the CNS. The function

of the complex to promote virus release and cell-to-cell transmission is presumably required for efficient spread of virus through the mucosa and submucosa. The complex may also be required for entry or axonal transport into specific classes of neurons (Mettenleiter and Rziha, 1985), and for trans-synaptic transport during neuronal spread (Kimman et al., 1992; Jacobs et al., 1993).

In conclusion, the gI/gp63 complex of ADV has an important function in neurovirulence of this virus in oronasally infected pigs.

Table 1

Non-essential ADV genes involved in virulence

		Growth in tissue culture	Virulence	
			Mice	Pigs
UL23	Thymidine kinase	wt	↓↓↓↓	↓↓↓
UL39	Ribon. reductase (RR)	↓	↓↓↓↓	↓↓↓
US3	Protein kinase (S/T)	↓	ND	↓↓
UL12	Alkaline nuclease	↓↓↓	↓↓↓	ND
UL21	Cleavage/encaps.	↓	↓↓	ND
UL20	Egress	↓	ND	ND
US7	gp63	wt	↓	↓↓
US8	gI	wt	↓	↓↓
US4	gX	wt	wt	wt
US2	28K	wt	wt	wt
UL13	Protein kinase	wt	wt	wt
UL44	gIII	↓	wt	wt

wt: wild-type growth properties in tissue culture, wild-type virulence in mice or piglets; ↓: indicates reduced growth in tissue culture or reduced virulence compared to wild-type virus in mice or piglets

Nonessential ADV genes involved in virulence

The development of efficient methods to generate ADV mutants with single inactivated genes and the rapidly increasing information on the position and sequence of ADV genes have greatly stimulated and facilitated our exploration of gene products that contribute to virulence in mice and pigs. Table 1 summarizes

the nonessential genes that have been inactivated to study their role in virulence. It appears that of the eleven genes studied so far, seven specify proteins required for full virulence. This observation underlines the conception that full expression of virulence of herpesviruses is a multigenic mechanism. The data also suggest that impaired growth in tissue culture cells, as observed for RR, PK, AN, and UL21 negative mutants, is correlated with reduced virulence in mice and pigs. However, the wild-type growth in tissue culture of TK and gI/gp63 negative mutants which exhibit reduced virulence, indicates that a correlation of both *in vitro* and *in vivo* phenotypes is not a hard rule. Finally, even though this list is still far from complete, the data suggest that many viral proteins which function during different stages of the virus life cycle contribute to full virulence.

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SENSE ANTISENSE DNA STRAND ?

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Recent evidence indicates that alphaherpesviruses express latency associated transcripts (LATs) from the antisense strand of immediate-early (IE) genes of the viral genome. It has been suggested that LATs containing extended open reading frames (ORFs), might be translated into (a) protein product(s). We found that a salient feature of some herpesvirus DNAs is a high GC preference at the third codon positions. The consequence of this feature is that the probability of a stop-codon appearing at two of the six reading frames of the DNA strand is very low. Therefore, the presence of an extended ORF does not necessarily mean that it is relevant to real translation.

Key words: Alphaherpesvirus, DNA, latency associated transcript, antisense strand, immediate-early gene, translation

Alphaherpesviruses are large DNA-containing animal viruses which have genomes of 100-150 kilobase pairs coding for 50 to 100 proteins. They can either undergo a productive lytic infection or they can enter a state of latency. In the latent state they produce antisense RNAs called latency associated transcripts, which are transcribed from the antisense strand of IE genes: ICP0 gene in the case of herpes simplex virus type 1 (HSV-1) (Stevens et al., 1987) and of the single IE gene (IE180) of Aujeszky's disease virus (ADV) (Cheung et al., 1989). LATs could play an important role in latency since they are the only detectable viral RNAs transcribed in neurons of latently infected ganglia. However, the molecular mechanisms involved in the establishment, maintenance, and reactivation of latent virus are not understood (Sawtell et al., 1992). The expression of LATs is supposed to be controlled by two different promoters. One of them has been sequenced (Vlcek et al., 1990), while in the other case only partial sequence data have been published to date (Priola et al., 1991). We have determined the nucleotide sequence of this promoter, and we present the putative consensus regulatory elements at Fig. 1. Their functional role has to be proven in further experiments. The nature of LAT function has two main components: firstly, they could act as antisense inhibitors of IE genes, and secondly, one or more LAT species may be a functional protein-expressing mRNA.

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Recently, in studies on HSV-1 (Wagner et al., 1988*b*; Sedarati et al., 1989), ADV (Vlcek et al., 1990; Cheung, 1990) and bovine herpesvirus type 1 (BHV-1) (Kutish et al., 1990), several authors hypothesized that LATs might function as protein encoding mRNAs. Vlcek et al. (1990) found an anti-parallel oriented open reading frame (ORF, 5211 bp) to the IE gene of PRV which overlapped almost entirely the long IE gene. The authors suggested that the antisense RNA containing an appreciably sized ORF can be translated into a protein product. According to Cheung (1991), the "antisense protein" may be translated from the spliced "long latency transcript" (LLT) that overlaps the entire region of IE180 and EP0 genes of ADV, as well as their HSV-1 homologues ICP4 and ICP0 genes, respectively. Experiments were carried out to detect the putative HSV-1 LAT-encoded polypeptide applying immunological methods (Wagner et al., 1988*b*), and to determine its possible function utilizing frame-shift mutations (Farrel et al., 1993), without deriving any positive results.

Materials and methods

Computer programs. DNA sequences were analyzed with the sequence analysis software package version 7.1 of the University of Wisconsin Genetics Computer Group (Devereux et al., 1984). The quasi-random sequences were generated with programs written in Pascal language.

DNA sequencing. Subclones of 7.5 kbp *Bam*HI-6 fragment of PRV strain KA, encompassing the 5' region of LAT, were made according to standard techniques. Digoxigenin labeling and detection system was used (Boehringer Mannheim) for mapping DNA fragments. DNA sequencing was performed with the dideoxynucleotide chain termination method (Sanger et al., 1977). In all reactions dGTP was substituted by 7-deaza-2' dGTP. Taq polymerase was used for extending the sequencing reaction. DNA reaction products were separated on 6% polyacrylamide gels containing 8M urea at 55 °C.

Results

In this work we present a novel explanation for the presence of gene-length antisense ORFs of alphaherpesviruses. Using the computer program "frame" of GCG package, we analyzed the available gene sequences of pseudorabies virus and found a number of extended DNA sequences without a stop-codon. They have a polarity opposite to the sense strand and are in-frame with the corresponding "sense" protein encoding frame. We also found long regions without a stop-codon

on the "sense strand" displaced with two shifts downstream of the "coding frame".

```
   1 CTCGAGGCCC AGCAGCGGGT GGTCTTCATC GTCCCATTCC AGCTCCTCGC
  51 GCCGGGGAGC GCGGGTGGGG GGCGCGGGCG TGACCGGCGG CTCGAAGATG
 101 GCATCGAGCC CATGGGGGTT TCCCGCGACG CCGGTGCCAG GAACCCCCAG
 151 CGCCGCCGAA AAAAACACCA GCACCGCCAC GAGCGGCGAC ATCGCGGCTG
 201 GTGTATCCTT TTTCGGCGCG GGGGTCCCGG CTTTTATCTC CGGAAAGAGG
 251 AAATTGAGAC ATAAACTAGA CGTCCGCATA ATCCCTCTTG ATTTACGGGG
 301 GGAAAATACG TGCCACGTGC CGGGGGGCGG GGCAACTGGA GCGTGGCGAA
 351 GCGGAACGGG GCGCGATGGG GCGCATCGAG GCGCGGACGC GTCCCGGGGG
 401 GAGTCTGGGG GGAGTCTGGG GGGAGTCTGG GGGGAGTCTG GGGGGAGTCT
 451 GGGGGGAGTC TGGGGGGAGT CTGGGGGGAG TCTGGGGGGA GTCTGGGGGG
 501 ATTCCAGCTC GGCGCGATCG CCGCATCGCG CAAAGTCCGG CTGCAGTAAA
 551 TTTACTGCGG ATGCAGTTCC CGGGACGGCC GCACCGGCCA AATGGCGCTG
 601 CAGTATAGAT ACTGCGGCTG CAGTTTACTA CAGTTGCAGT ACCGCGCGCC
 651 GCCGCCAAAT ACTACAGTAG ATTTCCTGCG GCCGCGCGGC CGCGTACTGC
 701 AGTTTACCGC GGCTGCAGTA AACTGCAGTA TCGCGCGGTA AATTGTAGTC
 751 TGGCAGCCGC GCGTTACTGC AATTAGCGGT TGGCTCCCGA CACTCTGGCC
 801 AATTGGTGCT AATGGGCCGT GATGGTCCAT GTGGGGGTGA TGTAACCGCC
 851 GGGCCCCGGT TGGGCACTCA GATGGTGGCC GGGCGGCCCC TGTCTGAGTG
 901 CCACTTTATG ACTTTGTTTT TCTCAAACAA CATCAATTAT GGATGCACAT
 951 CGTGTATATA ATCCCCGGTC CGCGCTCCGC CCACCCATCA CAGCAGCCGC
1001 GGACGCTGCG GCCGGAGCGG TCCATCTCGC CAGCCAGCCA ACCAGCCGAG
1051 CCGCCCAGCC GACCCGAGAG CCCCGAGAGC CAGACTCCCT CAGCCATAGA
1101 AGACACCGGG CGGGAGAGAC GGACTGAAAA AATATATCTT TTTTTATTTT
1151 GTCTGGGCCT GGAGACCCGC AGCAGGAGCG GAGGTGAGTG CGGGGCCGGG
```

Fig. 1. Nucleotide sequence of 5' limit of ADV LATs. The putative regulatory consensus elelments are as follows: AAGATGGC (bold, binds NF-μE1), TGGGMNNNNGCCA (bold, double underline, binds NF1), GGGRNTYYC (underline, binds NF-kB), CAAT-box (double underline), TATAA-box (bold, underline), cap site (double underline, CATCTC). There are many putative GC-boxes (GGGCGG), but their presence may be explained by the high G+C content. There are short repetitive sequences ranging from 396 to 494 base pairs

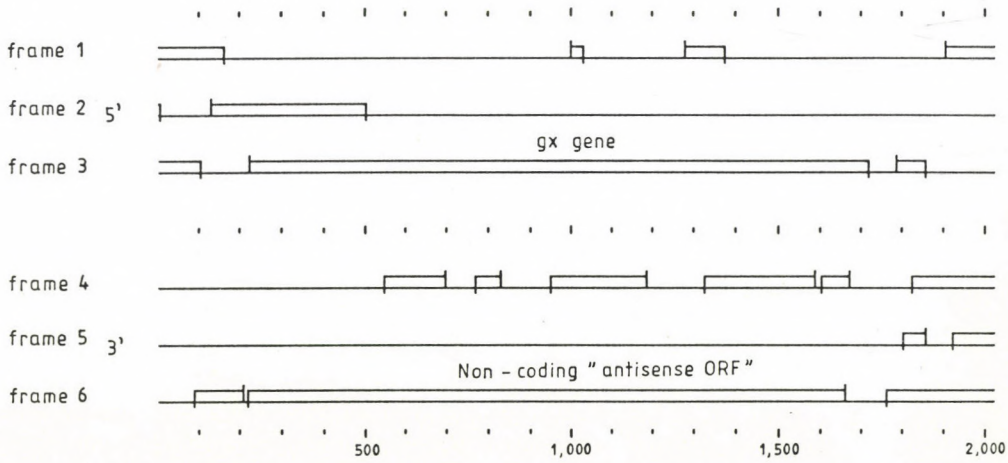

Fig. 2. The six reading frames of ADV gx gene. The "antisense ORF" (frame 6) matches almost exactly the ORF of "protein encoding frame" (frame 3)

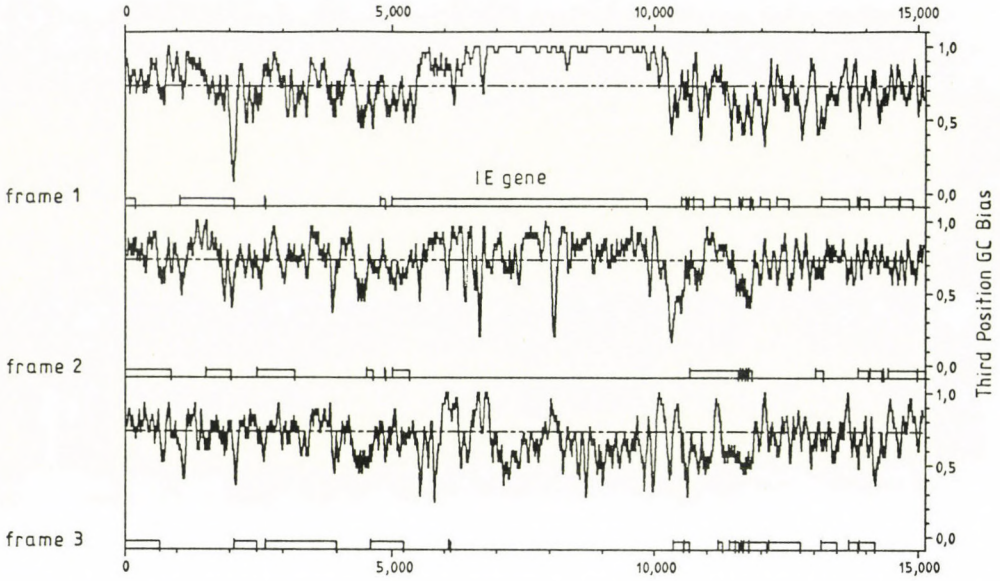

Fig. 3. The third codon positions of ADV IE gene (frame 1) possess high GC content

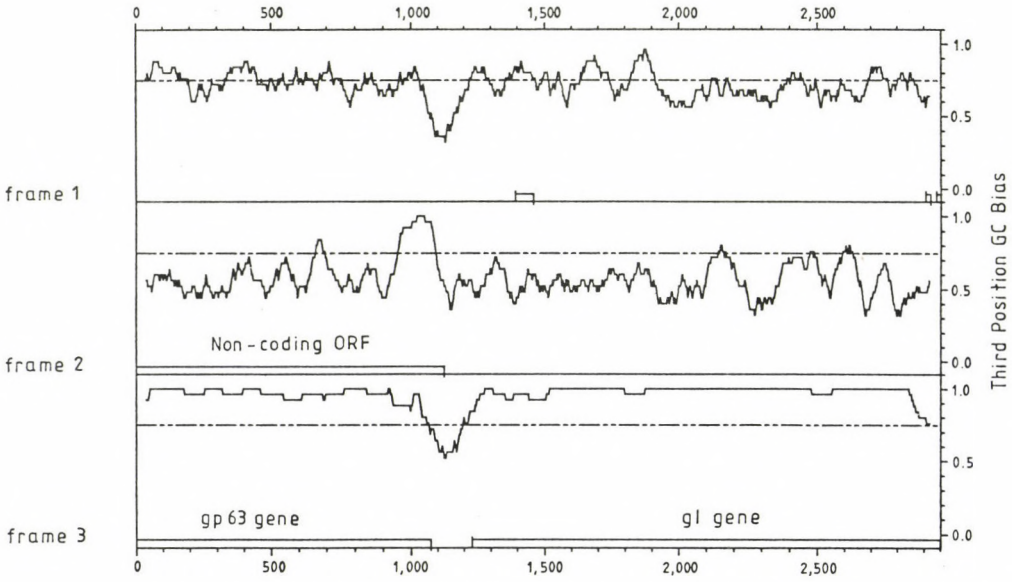

Fig. 4. gp63 and gI gene of ADV (frame 3) possess nearly 100% GC content at the third codon letter. There is no GC preference at any one of the frames at the intergenic region

Analyzing the antisense strands of available sequences, we found long (>700 bp) regions in 15 out of 16 cases and one short segment (434 bp) without a stop-codon. In length, 10 of 15 sequences exceed 1 kb. Initiation ATG codon has been localized in nine cases (see an example in Fig. 2), 4 sequences do not have start-codons, and we have no data in the case of 2 sequences. In order to determine whether the "antisense ORFs" could be expressed, we searched for transcriptional signals (TATA-box, CAAT-box, octamer and polyadenylation signal). Except for the antisense strand of the IE gene, we could find neither upstream, nor downstream consensus regulatory sequences. We can therefore conclude that these sequences are unlikely to be transcribed. Although the IE 180 and ICP0 genes produce antisense RNAs, the presence of their ORFs may be explained by the same argumentation, presented below.

The alphaherpesvirus DNAs are characterized by high variation in composition of guanine and cytosine: varicella-zoster virus (VZV): 46%; HSV-1: 67%; HSV-2: 69%; BHV-1: 72%; Equine HV-1: 57%; ADV: 74% G+C moles percent. We found a high GC preference at the silent (mainly third-) codon positions in the case of some herpesviruses. For example, ADV genes are characterized by nearly 100% G+C at the third codon positions (Fig. 3). The genomes of BHV-1 and HSV-1 are similarly ordered, particularly in the inverted repeat regions where IE genes are located, but their GC distribution is less biased than in ADV.

In addition to herpesvirus genes, we examined the available sequences of other GC-rich organisms such as *Micrococcus luteus* (74% G+C), *Streptococcus griseus* (73%), *Thermus thermophilus* (69%) and the human alpha-like globin gene (70%). We found that codons ending in G or C were used almost exclusively.

Discussion

The consequence of this base distribution type is that the first position of one of the reading frames at the antisense strand very rarely contains T (or A) bases and thus, the probability of a stop-codon (TAA, TGA, TAG) arising is very low. The G+C ratio is similarly high at the intergenic regions, but the G+C content is quite evenly distributed (Fig. 4), and therefore stop- and initiation-codons may appear. That explains why some "antisense ORFs" coincide almost exactly with the coding region of functionally active genes (Figs 2 and 4). Analysis of homologous herpesvirus genes has shown that the higher the third positional G+C content, the longer the regions which lack stop-codons at one of the frames on the antisense strand. This, of course, holds true of the IE genes as well, that is herpesviruses possessing low G+C content (VZV, EHV-1) do not have considerably

sized ORFs on the antiparallel strand of IE genes, while the sizes of "antisense ORFs" increase with G+C content in the following succession: HSV-1, HSV-2, BHV-1, and ADV. We could find long regions without a stop-codon on the genes of GC-rich bacteria and on human alpha-like globin gene.

In a model experiment we generated quasi-random sequences with constant base ratio at the different codon positions. We also generated random distribution from existing sequences keeping constant the base ratio of codon positions. In both cases the probability of stop-codon arising in frames 3 and 4 was very low (data not shown).

The functional significance of high G+C content has not yet been determined. Recently, there has been speculation on whether the evolution of G+C accumulation was directed by neutral or selective forces. Ohama et al. (1990) suggested a theory of "GC-biased mutation pressure" to explain the general increase in genomic G+C content in the case of *Micrococcus luteus*.

There can be two levels of selection in the evolution of G+C accumulation. An increase in genomic G+C content seems to confer some kind of evolutionary advantage at the DNA level. However, selective constraints arising from expression (protein) level prevent significant changes in amino acid content, which explains why the silent (mainly third-) positions are the targets of G+C accumulation.

Although LAT-encoded polypeptides have not been detected until now, we cannot exclude this possibility. We can conclude, however, that the presence of an extended ORF on the antiparallel strand of the IE gene is predictable from the high G+C content at the third codon position.

Thus, the presence of a gene-length ORF on the latency-associated transcripts does not necessarily mean that it is relevant for translation.

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STRUCTURAL FEATURES OF THE RIBONUCLEOTIDE REDUCTASE OF AUJESZKY'S DISEASE VIRUS

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A gene construct of the Aujeszky's disease virus (ADV) genome was prepared and the DNA fragment encoding the ribonucleotide reductase was structurally characterized. We determined the entire DNA sequence of two adjacent open reading frames of the ribonucleotide reductase genes with the intergenic sequence of nine base pairs. From the sequence analysis we predict that Aujeszky's disease virus encodes a ribonucleotide reductase which comprises two polypeptides — large and small subunits, with sizes of 835 and 303 amino acids, respectively. Nucleotide and amino acid sequences of the large and small subunits of the Aujeszky's disease virus ribonucleotide reductase have been compared with that of other herpesviruses, and structural features of both proteins have been characterized.

Key words: Aujeszky's disease virus, ribonucleotide reductase, structure, sequence analysis

For several human herpesviruses such as Epstein-Barr virus, HSV, VZV and CMV complete genomic sequences are available, but only approximately half of the ADV genome has been sequenced so far. Mostly the repeat regions and Us region have been characterized in detail and, in addition, a number of genes scattered in various places of Ul (gII, gIII, TK, ICP 18.5, gH, MCP and several genes with unknown function) have also been sequenced (Mettenleiter, 1991).

There is a category of genes that encode proteins which are dispensable for virus replication in tissue culture and probably play important roles in enhancing the efficient propagation of the virus in infected animals as well as in virulence. These proteins provide functions associated with the viral nucleic acid metabolism, DNA synthesis and replication. Known examples of viral genes include thymidine kinase, ribonucleotide reductase, thymidylate kinase, DNA ligase, uracil-DNA glycosylase.

We focused on the structural analysis of the ADV ribonucleotide reductase (RR), a key enzyme involved in the replication of DNA catalyzing the reduction of ribonucleoside diphosphates to corresponding deoxyribonucleoside diphosphates which are the precursors for DNA synthesis (Reichard, 1993). For several her-

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pesviruses, namely HSV-1, HSV-2, EBV, EHV-1, EHV-3 and ADV it was shown that they induce a RR activity with properties different from those of the endogenous cell enzyme (Nikas et al., 1986).

In this work, we present the primary structure of RR1 and RR2 from ADV.

Materials and methods

The ADV (strain Ka) kindly provided by Dr. B. Lomniczi was used in this study. The virus was propagated in pig kidney cells. ADV DNA was isolated from purified virions as described (Ben-Porat et al., 1974). Restriction enzyme analysis and cloning techniques were performed using standard procedures (Sambrook et al., 1989). A *Kpn*I restriction fragment library was constructed using bacterial positive-selection plasmid-vector containing palindrome (Elhai and Wolk, 1988). DNAs were sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977) using T7 and Taq DNA polymerases. In order to resolve band compressions due to high G+C content of the ADV genome (74%) deaza C-7 dGTP or dITP were also used as a substrate. For computer analysis a "GCG" program package (Devereux et al., 1985) was used.

Results

Analysis of the genetic map of sequenced herpesviral genomes (McGeoch et al.,1993) allowed us to predict the approximate location of the ADV RR1 and RR2 genes within the *Kpn*I A fragment of its physical map. To isolate these genes a *Kpn*I restriction fragment library was constructed using a bacterial positive-selection plasmid-vector (see Methods) and a plasmid containing the *Kpn*I-A fragment (29kb) was characterized using restriction enzymes. Subclones of the fragment were sequenced in order to identify the location of genes in the region. Restriction maps, preliminary sequencing of subclones as well as published data allowed us to reconstitute the genetic map of the significant part of the *Kpn*I-A DNA fragment of ADV. To sequence the RR-encoding region, corresponding subclones of the *Sal*I fragment were obtained and subsequently analyzed. All experimental data on cloning, mapping and DNA sequencing will be published elsewhere (Kaliman et al., in preparation).

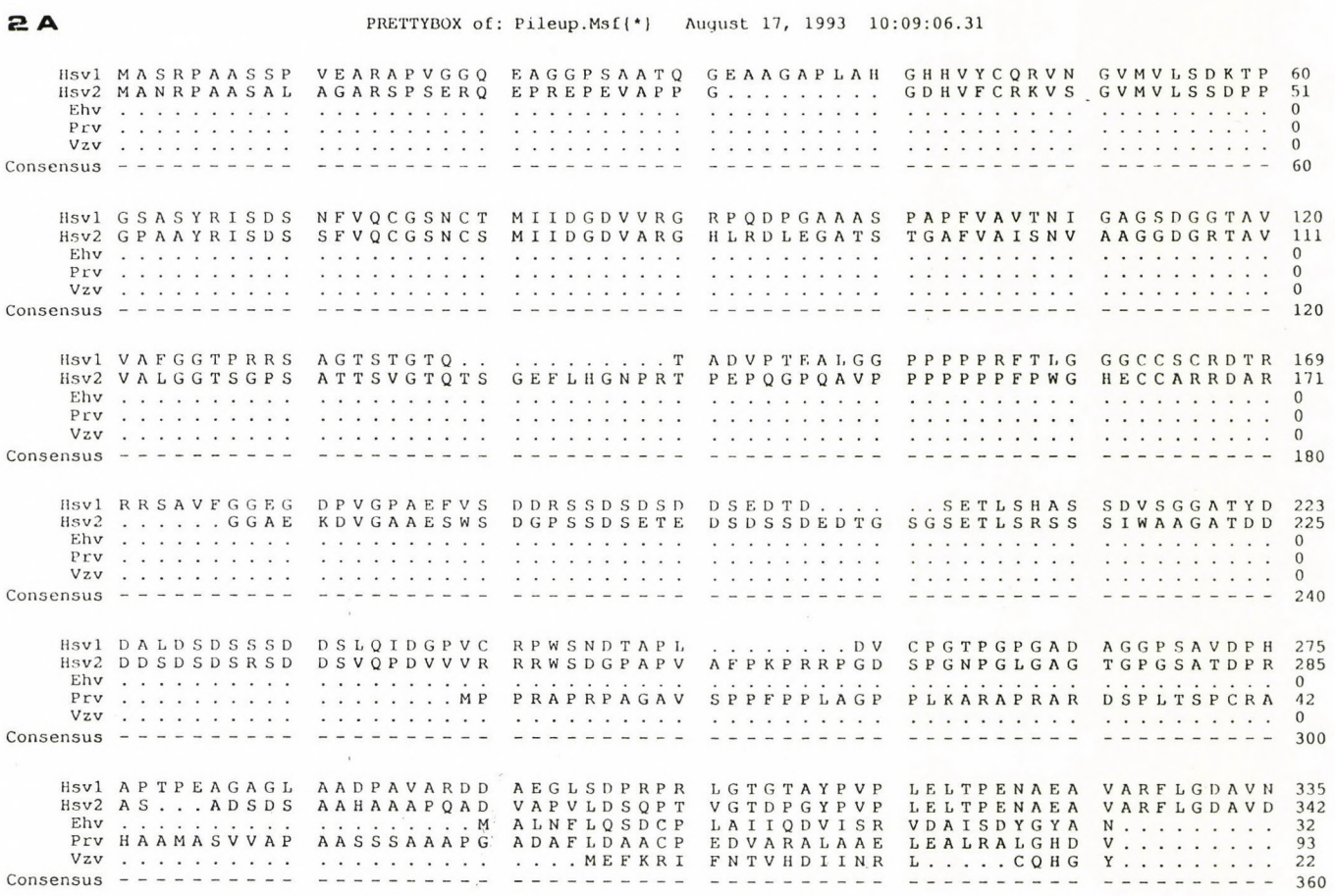
Using computer analysis it was found that two ORFs encode polypeptides of the putative large (RR1, 835 a-a) and small (RR2, 303 a-a) subunits of ribonucleotide reductase (Fig. 1).

```
RR1:
  1  MPPRAPRPAG AVSPPFPPLA GPPLKARAPR ARDSPLTSPC RAHAAMASVV
 51  APAASSSAAA PGADAFLDAA CPEDVARALA AELEALRALG HDVGAPAPGA
101  SRREAALFIT RAVDGLKAFS RVDERVYVAC GKLVHLRVRS READLDAWLA
151  SPELALIPAV AAAVRRHRAR VEAALRWFWR EAYPALYARG QSALKYEEM
201  YLARLEHGRC EAMDQFFVRL AAAAATATRR PMALVLCGSD AWPEVFDAYF
251  RALATQAIVP ATPLMLFAGR ARGSLASCYL LNPLPRTTEE AVRAITDEVA
301  PILLRRGGVG LSLQSFNRTP SGDCTRGIMA VLKALDSMTA AINSDSERPT
351  GVCVYVEPWH ADVRAVLNMR GMLAADESLR CDNIFSCLWT PDLFFQRYQR
401  HLDGERAVKW TLFDDRASHL ASLHGPDFAR EYERLERLGL GVESLPIQDM
451  AFLIVRSAVM TGSPFLMMKD ACNRHFHTDT RGAALATSNL CTEIVQRATP
501  GENGVCNLAS VNLPACLAGG AFDFAALRRA ARVAAVFVNA MMRIGNYPTG
551  ASVEGVRRSR SLGIGLQGLH TTVLALDMDM ADPAARRLNA AIAEELLYGV
601  MDASVELCER GLRPFDGFEH SRYARGVMPF DAYERVSLRE PMRWDALRVR
651  IAEHGVYNAQ FVALMPTVSS SQVTESSEGF SPTFTNMFSK VTISGELLRP
701  NLPLMETLRR LFPRECARRD AVARLERAQW SVAAAFGELP AGHPLAKFKT
751  AFEYDQELLI DMCADRAPFV DHSQSMSLFL TEPADGKLHA SRVMGLLMRA
801  YNLGLKTGMY YCKIRKATNN GVFTGGDLVC TSCHL

RR2:
  1  MEYFYTSQCP DMDHLRSLSV ANRWLETDLP LGDDAKDVAA LSEPELEFYR
 51  FLFAFLSAAD DLVNVNLGSL SELFTQKDIL HYYIEQECIE VVHSRVYSAI
101  QLMLFRGDAA ARERYVRAAL RDEAIRRKVE WLDSRVAECA SVAEKYLLMI
151  LIEGIFFASS FASISYLRTH NLFVVTCQSN DFISRDEAIH TSASCCIYNN
201  YLGDAPRPDE ARIHQLFAEA VEIECEFLRA RAPRDSLLLD LPAIISYVRY
251  SADRLLQAIG ASPLFGAPAP AADFPMALMV AEKHTNFFER RSTNYTGTVV
301  NDL
```

Fig. 1. Amino acid sequences for the Aujeszky's disease virus putative large (RR1) and small (RR2) subunits of the ribonucleotide reductase. The potential translational initiation methionines (M) are double underlined. The putative nucleotide binding site of RR1, the putative iron binding site and tyrosine free radical of RR2 are underlined

2 A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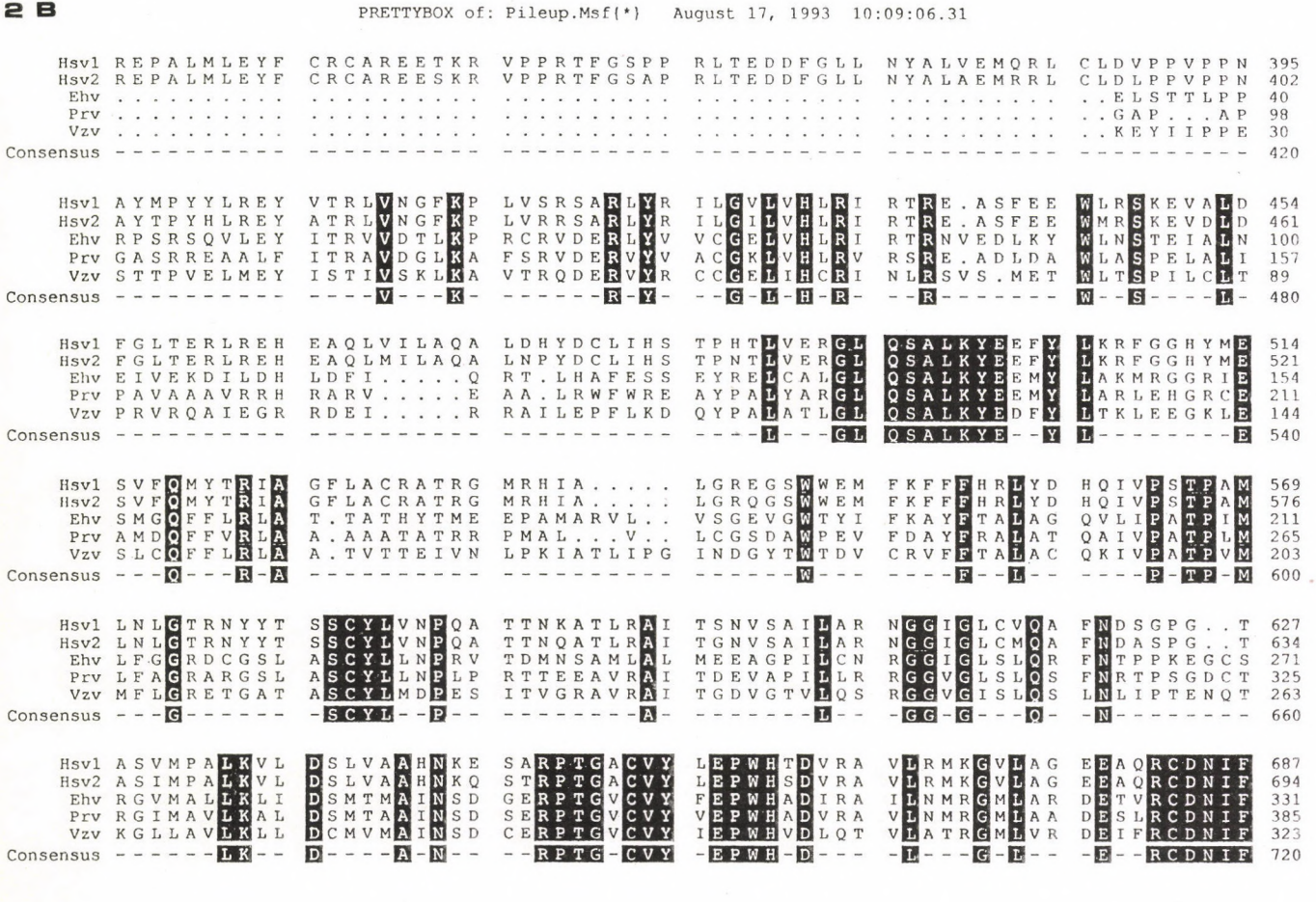
Hsv1 MASRPAASSP VEARAPVGGQ EAGGPSAATQ GEAAGAPLAH GHHVYCQRVN GVMVLSDKTP 60
Hsv2 MANRPAASAL AGARSPSERQ EPREPEVAPP G.......... GDHVFCRKVS GVMVLSSDPP 51
Ehv 0
Prv 0
Vzv 0
Consensus ----------- 60
Hsv1 GSASYRISDS NFVQCGSNCT MIIDGDVVRG RPQDPGAAAS PAPFVAVTNI GAGSDGGTAV 120
Hsv2 GPAAYRISDS SFVQCGSNCS MIIDGDVARG HLRDLEGATS TGAFVAISNV AAGGDGRTAV 111
Ehv 0
Prv 0
Vzv 0
Consensus ----------- 120
Hsv1 VAFGGTPRRS AGTSTGTQ.. ....T ADVPTEALGG PPPPPRFTLG GGCCSCRDTR 169
Hsv2 VALGGTSGPS ATTSVGTQTS GEFLHGNPRT PEPQGPQAVP PPPPPPFPWG HECCARRDAR 171
Ehv 0
Prv 0
Vzv 0
Consensus ----------- 180
Hsv1 RRSAVFGGEG DPVGPAEFVS DDRSSDSDSD DSEDTD.... ..SETLSHAS SDVSGGATYD 223
Hsv2 ......GGAE KDVGAAESWS DGPSSDSETE DSDSSDEDTG SGSETLSRSS SIWAAGATDD 225
Ehv 0
Prv 0
Vzv 0
Consensus ----------- 240
Hsv1 DALDSDSSSD DSLQIDGPVC RPWSNDTAPL ....DV CPGTPGPGAD AGGPSAVDPH 275
Hsv2 DDSDSDSRSD DSVQPDVVVR RRWSDGPAPV AFPKPRRPGD SPGNPGLGAG TGPGSATDPR 285
Ehv 0
Prv ....MP PRAPRPAGAV SPPFPPLAGP PLKARAPRAR DSPLTSPCRA 42
Vzv 0
Consensus ----------- 300
Hsv1 APTPEAGAGL AADPAVARDD AEGLSDPRPR LGTGTAYPVP LELTPENAEA VARFLGDAVN 335
Hsv2 AS...ADSDS AAHAAAPQAD VAPVLDSQPT VGTDPGYPVP LELTPENAEA VARFLGDAVD 342
Ehv ....M ALNFLQSDCP LAIIQDVISR VDAISDYGYA N..... 32
Prv HAAMASVVAP AASSSAAAPG ADAFLDAACP EDVARALAAE LEALRALGHD V..... 93
Vzv ....MEFKRI FNTVHDIINR L.....CQHG Y..... 22
Consensus ----------- 360


2 B
PRETTYBOX of: Pileup.Msf(*) August 17, 1993 10:09:06.31
Hsv1 REPALMLEYF CRCAREETKR VPPRTFGSPP RLTEDDFGLL NYALVEMQRL CLDVPPVPPN 395
Hsv2 REPALMLEYF CRCAREESKR VPPRTFGSAP RLTEDDFGLL NYALAEMRRL CLDLPPVPPN 402
Ehv . . . .ELSTTLPP 40
Prv . . . .GAP...AP 98
Vzv . . . .KEYIIPPE 30
Consensus 420
Hsv1 AYMPYYLREY VTRLVNGFKP LVSRSARLYR ILGVLVHLRI RTRE.ASFEE WLRSKEVALD 454
Hsv2 AYTPYHLREY ATRLVNGFKP LVRRSARLYR ILGILVHLRI RTRE.ASFEE WMRSKEVDLD 461
Ehv RPSRSQVLEY ITRVVDTLKP RCRVDERLYV VCGELVHLRI RTRNVEDLKY WLNSTEIALN 100
Prv GASRREAALF ITRAVDGLKA FSRVDERVYV ACGKLVHLRV RSRE.ADLDA WLASPELALI 157
Vzv STTPVELMEY ISTIVSKLKA VTRQDERVYR CCGELIHCRI NLRSVS.MET WLTSPILCLT 89
Consensus 480
Hsv1 FGLTERLREH EAQLVILAQA LDHYDCLIHS TPHTLVERGL QSALKYEEFY LKRFGGHYME 514
Hsv2 FGLTERLREH EAQLMILAQA LNPYDCLIHS TPNTLVERGL QSALKYEEFY LKRFGGHYME 521
Ehv EIVEKDILDH LDFI.....Q RT.LHAFESS EYRELCALGL QSALKYEEMY LAKMRGGRIE 154
Prv PAVAAAVRRH RARV.....E AA.LRWFWRE AYPALYARGL QSALKYEEMY LARLEHGRCE 211
Vzv PRVRQAIEGR RDEI.....R RAILEPFLKD QYPALATLGL QSALKYEDFY LTKLEEGKLE 144
Consensus 540
Hsv1 SVFQMYTRIA GFLACRATRG MRHIA..... LGREGSWWEM FKFFFHRLYD HQIVPSTPAM 569
Hsv2 SVFQMYTRIA GFLACRATRG MRHIA..... LGRQGSWWEM FKFFFHRLYD HQIVPSTPAM 576
Ehv SMGQFFLRLA T.TATHYTME EPAMARVL.. VSGEVGWTYI FKAYFTALAG QVLIPATPIM 211
Prv AMDQFFVRLA A.AAATATRR PMAL...V.. LCGSDAWPEV FDAYFRALAT QAIVPATPLM 265
Vzv SLCQFFLRLA A.TVTTEIVN LPKIATLIPG INDGYTWTDV CRVFFTALAC QKIVPATPVM 203
Consensus 600
Hsv1 LNLGTRNYYT SSCYLVNPQA TTNKATLRAI TSNVSAILAR NGGIGLCVQA FNDSGPG..T 627
Hsv2 LNLGTRNYYT SSCYLVNPQA TTNQATLRAI TGNVSAILAR NGGIGLCMQA FNDASPG..T 634
Ehv LFGGRDCGSL ASCYLLNPRV TDMNSAMLAL MEEAGPILCN RGGIGLSLQR FNTPPKEGCS 271
Prv LFAGRARGSL ASCYLLNPLP RTTEEAVRAI TDEVAPILLR RGGVGLSLQS FNRTPSGDCT 325
Vzv MFLGRETGAT ASCYLMDPES ITVGRAVRAI TGDVGTVLQS RGGVGISLQS LNLIPTENQT 263
Consensus 660
Hsv1 ASVMPALKVL DSLVAAHNKE SARPTGACVY LEPWHTDVRA VLRMKGVLAG EEAQRCDNIF 687
Hsv2 ASIMPALKVL DSLVAAHNKQ STRPTGACVY LEPWHSDVRA VLRMKGVLAG EEAQRCDNIF 694
Ehv RGVMALLKLI DSMTMAINSD GERPTGVCVY FEPWHADIRA ILNMRGMLAR DETVRCDNIF 331
Prv RGIMAVLKAL DSMTAAINSD SERPTGVCVY VEPWHADVRA VLNMRGMLAA DESLRCDNIF 385
Vzv KGLLAVLKLL DCMVMAINSD CERPTGVCVY IEPWHVDLQT VLATRGMLVR DEIFRCDNIF 323
Consensus 720

2 C

PRETTYBOX of: Pileup.Msf(*) August 17, 1993 10:09:06.31

```
     Hsv1 SALWMPDLFF KRLIRHLDGE KNVTWTLFDR DTSMSLADFH GEEFEKLYQH LEVMGFG.EQ  746
     Hsv2 SALWMPDLFF KRLIRHLDGE KNVTWSLFDR DTSMSLADFH GEEFEKLYEH LEAMGFG.ET  753
      Ehv ACMWTPDLFF DRYQRYLDGE SGVMWTLFD. DTASHLCHMY GKEFEEEYER LEQCGFGVDS  390
      Prv SCLWTPDLFF QRYQRHLDGE RAVKWTLFD. DRASHLASLH GPDFAREYER LERLGLGVES  444
      Vzv CCLWTPDLFF ERYLSYLKGA SNVQWTLFD. NRADILRTLH GEAFTSTYLR LERFGLGVSS  382
Consensus ---W-PDLFF -R----L-G- --V-W-LFD- -----L---- G--F---Y-- LE--G-G---  780

     Hsv1 IPIQELAYGI VRSAATTGSP FVMFKDAVNR HYIYD.TQGA AIAGSNLCTE IVHPASKRSS  805
     Hsv2 IPIQDLAYAI VRSAATTGSP FIMFKDAVNR HYIYD.TQGA AIAGSNLCTE IVHPSSKRSS  812
      Ehv IPIQDMAFII VRSAVMTGSP FLMFKDACNK HYHFDLRRKG AIMGSNLCTE IIQHADETQN  450
      Prv LPIQDMAFLI VRSAVMTGSP FLMMKDACNR HFHTD.TRGA ALATSNLCTE IVQRATPGEN  503
      Vzv VPIQDIAFTI IRSAAVTGSP FLMFKDACNR NYHMN.TQGN AITGSNLCTE IVQKADAHQH  441
Consensus -PIQ--A--I -RSA--TGSP F-M-KDA-N- ---------- A---SNLCTE I---------  840

     Hsv1 GVCNLGSVNL ARCVSRQT.. ......FDFG RLRDAVQACV LMVNIMIDST LQPTPQCTRG  857
     Hsv2 GVCNLGSVNL ARCVSRRT.. ......FDFG MLRDAVQACV LMVNIMIDST LQPTPQCARG  864
      Ehv GVCNLASINL PKCLAIPPPH TAGVPYFDFA ALGRAAATAT IFVNSMMRAG TYPTVKSQRG  510
      Prv GVCNLASVNL PACLAGGA.. ......FDFA ALRRAARVAA VFVNAMMRIG NYPTGASVEG  555
      Vzv GVCNLASINL TTCLSKGPVS ......FNLN DLQLTARTTV IFLNGVLAAG NFPCKKSCKG  495
Consensus GVCNL-S-NL --C------- ------F--- -L-------- ---N------ --P------G  900

     Hsv1 NDNLRSMGIG MQGLHTACLK LGLDLESAEF QDLNKHIAEV MLLSAMKTSN AILC.VRGAR P  916
     Hsv2 HDNLRSMGIG MQGLHTACLK MGLDLESAEF RDLNTHIAEV MLLAAMKTSN ALCC.VRGAR P  923
      Ehv VDENRSLGLG IQGLHTAFLM LDLDMASPEA RQLNKQIAER LLLNSMKASA TLCCRL.GMK P  569
      Prv VRRSRSLGIG LQGLHTTVLA LDMDMADPAA RRLNAAIAEE LLYGVMDASV ELCCER.GLR P  614
      Vzv VKNNRSLGIG IQGLHTTCLR LGFDLTSQPA RRLNVQIAEL MLYETMKTSM EMCCKIGGLA P  555
Consensus ----RS-G-G -QGLHT--L- ---D------ --LN--IAE- -L---M--S- --C---G--P  960

     Hsv1 FNHFKRSMYR AGRFHWERFP DARPRYEGEW EMLRQSMMKH GLRNSQFVAL MPTAASAQIS  976
     Hsv2 FSHFKRSMYR AGRFHWERFS NASPRYEGEW EMLRQSMMKH GLRNSQFIAL MPTAASAQIS  983
      Ehv FKGFEDSKYS LGELPFDSYP GVTLANRNAW RRLRTEIKQH GLYNSQFVAY MPTVSSSQVT  629
      Prv FDGFEHSRYA RGVMPFDAYE RVSLREPMRW DALRVRIAEH GVYNAQFVAL MPTVSSSQVT  674
      Vzv FKGFTESKYA KGWLHQDGFS TISYLD.LPW CTLRDDICAY GLYNSQFLAL MPTVSSAQVT  614
Consensus F--F--S-Y- -G--------- ----------W --LR------ G--N-QF-A- MPT--S-Q--  1020

     Hsv1 DVSEGFAPLF TNLFSKVTRD GETLRPNTLL LKELERTFS. GKRLLEVMDS LDAKQWSVPQ  1035
     Hsv2 DVSEGFAPLF TNLFSKVTRD GETLRPNTLL LKELERTFG. GKRLLDAMDG LEAKQWSVAQ  1042
      Ehv ESSEGFSPVY TNLFSKVTAT GEVLRPNLLL MRTIRSIFPR ECARLQALST LEMAQWSVVG  689
      Prv ESSEGFSPTF TNMFSKVTIS GELLRPNLPL METLRRLFPR ECARRDAVAR LERAQWSVAA  734
      Vzv ECSEGFSPIY NNMFSKVTTS GELLRPNLDL MDELRDMYSC EEKRLEVINI LEKNQWSVIR  674
Consensus --SEGF-P-- -N-FSKVT-- GE-LRPN--L ---------- ---------- L---QWSV--  1080
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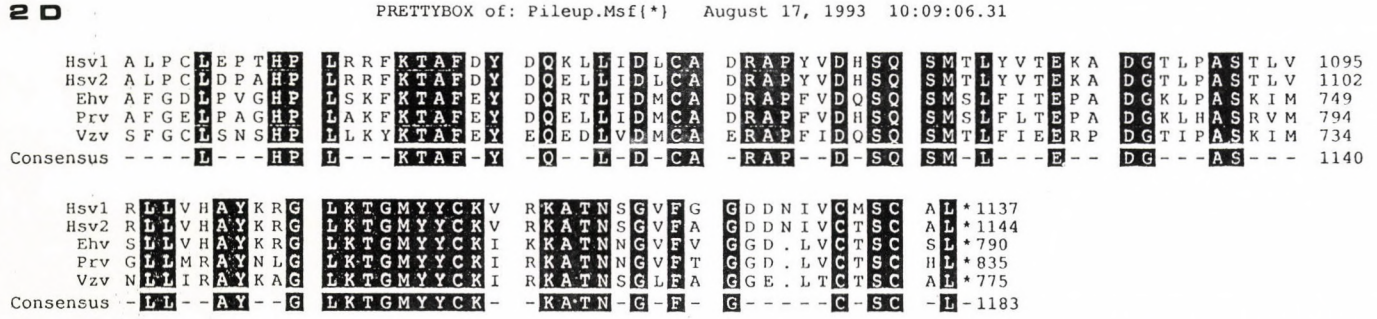

Fig. 2. Alignment of RR1 amino acid sequences from herpes simplex virus types 1 (Hsv1, 1st row from the top) and 2 (Hsv2, 2nd row), equine herpesvirus 1 (Ehv, 3d row), Aujeszky's disease virus (Prv, 4th row) and varicella zoster virus (Vzv, 5th row). Sequences were aligned employing the computer program described in Methods. Identical amino acids in all 5 viruses shown as a consensus (6th row) have been shaded

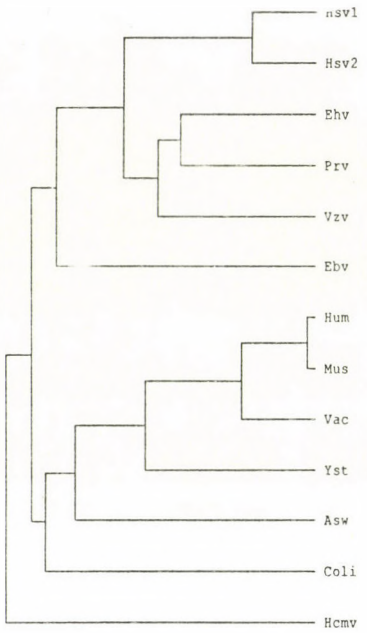

Fig. 3. Phylogenetic tree of herpesviruses based on the structure of RR1: herpes simplex virus types 1 (Hsv1)and 2 (Hsv2), equine herpesvirus 1 (Ehv), Aujeszky's disease virus (Prv), varicella zoster virus (Vzv), Epstein-Barr virus (Ebv), human cytomegalovirus (Hcmv), and of several other viruses and organisms: human (Hum), mouse (Mus), vaccinia virus (Vac), yeast (Yst), african swine fever virus (Asw), *E. coli* (Coli)

RR1 contains two potential translational initiation codons resulting in Met located at positions 1 and 46. At positions 563-568 RR1 contains three conserved glycine residues with the sequence G-x-G-x-x-G, corresponding to nucleotide binding site (Nikas et al.,1986). From the amino acid sequence comparison of ADV, HSV-1, HSV-2, VZV and EHV the RR1 from ADV can be divided into the heterogeneous N-terminal portion and several conserved regions (Fig. 2). In the N-terminal part of RR1 the complete homology begins at ADV a-a 113 and blocks of complete homology are divided by extended stretches of lower homology of the regions 157-185, 222-241, 626-643,705-725.

In the structure of RR2, positions His93 and Tyr97 represent the location of the putative iron binding site and tyrosine free radical (Nikas et al.,1986). These, and several other highly conserved amino acid residues involved in the active site of the small subunit polypeptide of ADV (Asp60, Glu90, Glu153, Glu187, His190) can be identified from the structure-functional analysis of the *E. coli* RR2 (Nordlund et al. ,1990).

A phylogenetic tree of herpesviruses and representatives of several other

organisms based on the structure of RR1 is shown in Fig. 3.

Discussion

We have cloned and sequenced ADV genes encoding the large and small subunits of the ribonucleotide reductase and from the structure of genes the putative structure of both subunits of the enzyme have been deduced. Similarly to HSV-1 RR (McGeoch et al.,1988), genes RR1 and RR2 are arranged in tandem in the unique long region of the genome directed in the same orientation. The enzyme of ADV presumably consist of two subunits in an $\alpha_2\beta_2$ configuration (Ingemarson and Lankinen, 1987).

Comparative analysis of the ADV RR1 and RR2 with other herpesviral homologous proteins revealed highly conserved sites associated with the catalytic process. Further structural studies using NMR and X-ray crystallography should greatly facilitate the understanding of the catalytic process and ongoing drug design projects. For this purpose a sufficient supply of pure enzyme is needed. Since the yield of RR1 and RR2 in virus-infected cells is low, the use of genetic engineering techniques and efficient expression of the cloned RR genes in various prokaryotic or eukaryotic vector systems would allow us to obtain the enzyme in sufficient quantity.

The predicted molecular weight of the large subunit is 91K, which is significantly lower than identified *in vivo* (110K, Cohen et al., 1987). A similar observation was reported for HSV-1 RR1 (Preston et al., 1984). The discrepancy between the predicted and apparent MW remains to be explained.

The viral enzyme is not essential for growth in actively dividing cells maintained at 37 °C. However, it is required for efficient viral growth and DNA replication in nondividing cells or in cells maintained at 39.5 °C (Furlong et al., 1991). Compared to the wild type the efficiency of replication of RR^- ADV is lower too. It was also shown that HSV-1 RR is required for pathogenicity and virulence (Cameron et al., 1988; Brand et al., 1991; Idowu et al., 1992). Furthermore, the enzyme appeared to be critical in establishing acute and latent infections in mice (Jacobson et al., 1989) and may play a role in the recovery of latent virus from nerve ganglia in both mice and guinea pigs (Idowu et al., 1992).

Recently de Wind et al. (1993) isolated ribonucleotide reductase-deficient mutants of ADV and assayed them for virulence and immunogenicity in pigs. RR^- mutants were avirulent, were shed in very low titres in the oropharyngeal fluid by the animals and induced low titres of neutralizing antibodies. However, protection against clinical signs after infection with virulent ADV was induced. Thus, RR^- ADV strains could be considered as potential vaccine candidates. It was also

demonstrated that RR^- mutants are useful HSV-1 vectors for gene delivery into neuronal cells (Chang et al., 1991).

RR enzyme was shown to be specifically inhibited *in vitro* by synthetic peptides corresponding to the C-terminus of the small subunit (Cohen et al., 1987). Thus, the nonapeptide YTGTVVNDL can serve as a lead compound for anti-ADV drugs.

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THE NEUROBIOLOGY OF ALPHAHERPESVIRUS INFECTIONS

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In this review we have tried to outline some of the recent experimental data which has contributed to our understanding the neurobiology of herpesviruses. We have attempted to draw together the threads of several different but related disciplines, i.e. virology, immunology, neurobiology and cell biology, to demonstrate how all are inextricably linked and may be used in combination to address some of the issues surrounding the molecular basis of herpesvirus latency and reactivation. In conclusion, the interaction between virus and host and in particular the neuron, is very complex. We have probably only scratched the surface here in revealing some of the many pathways and factors which are thought to impinge on this unique relationship. Ultimately, it is likely that a full understanding of that relationship will prove to be one of the most exciting and important milestones in biology.

Key words: Alphaherpesviruses, neurobiology, herpesvirus latency, reactivation, molecular biology, virus–host relationship, neuron

The *Alphaherpesvirinae* are one of three sub-families of the *Herpesviridae* and comprise a number of human and veterinary pathogens, including the best studied member of this family, herpes simplex virus type 1 (HSV-1). Although herpesviruses have been classified on the basis of multiple criteria, the definition of the alphaherpesviruses is that they should exhibit a variable host range, relatively short replicative cycle, rapid spread in culture, efficient destruction of infected cells and the capacity to establish latent infections, non-exclusively, in sensory ganglia (Roizman et al., 1981). In addition, analysis of the complete DNA sequences for three of these viruses, namely HSV-1, equine herpesvirus 1 (EHV-1) and varicella-zoster virus (VZV) clearly distinguishes the alphaherpesviruses from the beta- and gammaherpesviruses on the basis of genome organisation (McGeoch et al., 1988; Telford et al., 1992; Davison et al., 1986).

HSV-1 infects its natural host at mucosal surfaces resulting in local replication. Subsequently, the virus is able to enter sensory nerve endings whence it is transported to neuronal cell bodies by retrograde axonal transport. Once in the sensory ganglia, a much curtailed phase of virus replication ensues, generally resulting in a latent infection of neurons at this site (Nash and Lohr, 1992). Thus, in man, the primary sites of HSV-1 latency are the trigeminal ganglia which inner-

vate maxillary, mandibular and ophthalmic dermatomes. In our murine experimental system, infection of ear or neck skin involves the dorsal root ganglia (cervical II–IV). In addition, it is clear that in certain circumstances, HSV-1 will establish a latent infection at sites within the CNS (Stroop, 1986). Indeed, invasion of the CNS by herpesviruses can cause severe disease, e.g. encephalitis (Goldsmith and Whitley, 1991; Stroop and Schaefer, 1989).

In man, as in other animals, the pathogenesis of herpesvirus encephalitis may vary between individuals. This may involve new "primary" infections caused by other strains of HSV, or reactivation of latent virus in the peripheral nervous system (PNS) leading to invasion of the CNS via olfactory and/or trigeminal nerves. The latter are thought to represent the major routes of access to the CNS for HSV (Johnson, 1984). Indeed, it has been shown in the rat, that pseudorabies virus (PRV), like HSV-1, spreads in a projection-specific manner via retrograde axonal transport from the periphery to pre-ganglionic, ganglionic and higher nuclei in the brain, and also transneuronally within the CNS (Card et al., 1990).

Undoubtedly, a major feature of HSV infections in sensory ganglia is that although some neurons are destroyed, the majority are spared (Speck and Simmons, 1992). This situation contrasts with that seen during infections of mucosal epithelium for example, in which these viruses are extremely lytic. Thus, one conclusion that can be drawn from this is that a special relationship appears to exist between neurons and this group of herpesviruses which serves to limit the destructive capacity of the virus.

A possible explanation for this observation would be that the neuron possesses various mechanisms which enable them to be protected against virus-induced necrotic or programmed cell death. In this article we examine the behaviour of alphaherpesviruses in neurons, using HSV-1 as an example and emphasize the molecular interactions between virus and host-cell factors which may influence the course of infection. We also consider how the host immune system may impinge upon and influence events in the infected neuron. Finally, we consider experimental approaches for modulating the pathogenesis of alphaherpesvirus infections via the use of herpesvirus genes.

Role of the immune system in the establishment and maintenance of latency

We know that during primary infections and recrudescences, the host immune system normally mounts a vigorous response. Further, this response is multifunctional involving both the production of neutralising antibodies against the "cocktail" of viral glycoproteins present in the viral envelope (Morrild, 1985;

Martin et al., 1988) and the cell-mediated component of the immune system. In terms of the latter, it is clear that a variety of different cell types play a role in the clearance of the infection (Nash et al., 1985). We know, for example, that $CD4^+$ T cells are involved in eliminating virus from the skin, probably mediated via gamma interferon (IFN-γ)-induced recruitment of macrophages (Nash and Cambouropoulos, 1993). It is also clear that $CD8^+$ T cells are important in viral clearance, both in the periphery and in sensory ganglia (Nash et al., 1987; Simmons and Tscharke, 1992). Pre-existing immunity, as typified by IFN-α/β production and the presence of natural killer (NK) cells, may also play a role in helping to control both primary and recurrent infections in the immunocompetent host. Indeed, both of these mechanisms, together with $CD4^-$, $CD8^-$ (γδ) T cells, are thought to be involved in the clearance of PRV-infected peripheral blood lymphocytes (Kimman et al., 1993).

During latency, the host is apparently unable to clear virus from infected neurons. Indeed, Sekizawa et al. (1980) demonstrated that the latent state appears to be maintained in the absence of neutralising antibody and, although there is some evidence to suggest that T cells may persist in sensory ganglia until the disappearance of late viral antigens, there is no further lymphocytic infiltration into the PNS. One possible reason for the lack of immune involvement during latency may be the apparent absence of professional antigen presentation in sensory ganglia. Although satellite cells and glial cells are known to express major histocompatibility complex (MHC) class I and II (Kastrukoff et al., 1993), neurons themselves appear to be deficient in both of these molecules (Joly et al., 1991; Joly and Oldstone, 1992). Recent evidence suggests that neurons can produce MHC class I mRNA, but there is no expression of "classical" MHC proteins (Pereira et al., 1993).

The events which surround the reactivation of virus from latency remain unclear and may be triggered by various stimuli which alter the physiology of infected neurons (see section *d* of this chapter). The potential role of other cell types within ganglia and the CNS, e.g., glial cells and satellite cells, is still under investigation, although it is known that glial cells can be induced to produce cytokines under certain conditions (for a review see Benveniste, 1992), and as such could influence the local cellular microenvironment. Interestingly, a key role for astrocytes, microglia and macrophages in restricting the trans-neuronal spread of PRV in the rat CNS has recently been reported (Card et al., 1993).

Many groups have shown that during the lytic phase of ganglionic infection, late virus antigens can be detected in >50% of neurons. However, this appears to result in the death of only a small proportion of neurons, the majority being spared and approximately 1% becoming latently infected (Stevens, 1989). $CD8^+$ T cells are clearly involved in the protection of neurons, since their removal from mice

results in a significant decrease in neurons in dorsal root ganglia (Simmons and Tscharke, 1992). In view of the absence of MHC class I molecules on neurons, the mechanism of protection is unclear; however, this may involve cytokines.

Thus, we are presented with two of the most tantalising questions in the study of the virus–host relationship i.e., "What are the factors which control the outcome of a herpesvirus infection in neurons?" and, "What are the mechanisms which permit ganglionic neurons to survive both lytic and latent herpesvirus infections?". In order to answer these questions it is necessary to briefly examine, both the status of the viral genome during latency and the transcription factor environment in the neuron.

The latent infection

a. Status of the viral genome

The latent viral genome persists extrachromosomally in the nucleus and in addition is in a different form from that present in virions (Rock and Fraser, 1983). During latency, the viral DNA exists in a circular concatameric form (Rock and Fraser, 1985; Efstathiou et al., 1986) which may be tightly associated with host nucleosomal protein (Deshmane et al., 1989). The latter observation to some extent probably explains why, until very recently, latent viral genome had escaped detection and quantification by *in situ* hybridisation techniques. However, it is possible that not all alphaherpesviruses share this pattern of latent virus DNA structure; there is some evidence to suggest that the latent pseudorabies virus genome exists in neurons predominantly as linear, non-integrated molecules (Rziha et al., 1986).

We know that latently infected ganglionic neurons can harbour between 20 and several hundred copies of the entire viral genome and that the viral load is highest in cells which have previously supported viral replication (Simmons et al., 1992). Some estimates, based on a quantitative polymerase chain reaction (PCR) even put this figure up to 10^6 genomes per cell (Sawtell and Thompson, 1992*a*). However, we also know that viral DNA replication is not a pre-requisite for the establishment of latency (Margolis et al., 1992).

b. Latent viral gene expression

When HSV-1 enters the neuron, a divergence occurs between those cells which will become productively infected and those which will become latently infected. The former is characterised by virus replication and expression of viral antigens of all temporal classes, leading ultimately to lysis of the cell. In the latent

infection, however, the cell survives and viral gene expression is limited to a single class of transcripts, LATs. Recent evidence suggests that latency may progress via one of three pathways i.e., (*i*) immediately, without any viral gene expression, including LATs, (*ii*) following limited viral gene expression, or (*iii*) by a third mechanism characterised by the co-expression of virus antigen(s) and LATs. Further, it has been shown that neurons in all three of these states can exist synchronously following acute infection in sensory ganglia (Speck and Simmons, 1992).

As mentioned above, the only viral transcripts which can be detected in neurons during latency are the family of latency-associated transcripts of LATs (Stevens, 1987; Fraser et al., 1992). The major LATs comprise three non-polyadenylated nuclear RNAs of 2.0, 1.5 and 1.45Kb, which are alternatively spliced products of the same open reading frame (ORF) which partially overlaps the 3' end of the viral immediate-early 1 (ICP0) gene (Spivack and Fraser, 1988; Wechsler et al., 1988). The expression of an 8.5Kb, "full-length", polyadenylated minor LAT species, produced by read-through of splice donor and acceptor sites, has also been described (Dobson et al., 1989). It is possible that this transcript represents the only biologically active LAT produced during latency. However, aside from a single, as yet unverified observation, no viral proteins have been identified in latently infected neurons. In the latter experiment, Doerig et al. (1991) described the use of an antiserum directed against a bacterially expressed fusion protein, containing part of a LAT ORF-encoded polypeptide, to apparently detect an 80kDa protein in infected cells.

Despite intense efforts by many groups, a definite function has yet to be ascribed to LATs. Many experiments have been carried out in a variety of animal models using viruses with miriad deletions in the LAT ORF. For example, we know that these viruses still establish latency although some appear to reactivate less efficiently than wild-type virus (Ho and Mocarski, 1989; Leib et al., 1989; Steiner et al., 1989; Block et al., 1990). Further, Sawtell and Thompson (1992*b*) have shown that LATs actually promote efficient establishment of latency in trigeminal ganglia but not at other sites.

Thus, whilst not essential for virus replication, it is possible that LATs could play a role in the establishment of latency and reactivation; theories abound. It was initially proposed that LATs could sequester ICP0 mRNA by complementary base pairing to the region of sequence overlap. It now seems more likely that LATs might function via direct interactions with cellular transcription factors. Alternatively, LATs could serve to maintain a transcriptionally active region of the genome during latency. This would permit cellular transcription factors to enter the tightly knit virus DNA–nucleoprotein episome and gain access to the promoter regions of the viral immediate early genes.

c. Cellular factors involved in the establishment of latency

Much of the recent experimental work aimed at understanding the molecular basis of latency has focussed on the examination of the transcription factor environment in a number of neuronally derived cell lines. A great deal of work has also been carried out in ND cell lines, produced by fusing mouse neuroblastoma cells with rat primary ganglionic neurons (Wood et al., 1990). Through these studies, a family of octamer-binding POU-domain proteins able to regulate the behaviour of HSV-1 in the neuron have been identified.

Oct-1 and Oct-2 are both members of this group which transactivate or repress gene expression via binding directly to the octamer-related TAATGARAT motifs present in the HSV-1 immediate-early (IE) promoters (Kemp et al., 1990). The ubiquitous factor, Oct-1, is known to play a role in activating ICP0 expression via the formation of a tripartite complex involving a viral tegument protein, Vmw65 and another cellular factor, CFF (O'Hare, 1993). Oct-2 on the other hand is found only in neurons and B cells and is absent from cell lines permissive for HSV-1 replication, which is able to repress viral IE genes by preferentially binding to the TAATGARAT motif (Lillycrop et al., 1991). Thus, one might consider that the ratio of these two factors in any given neuron will be important in determining whether or not HSV replication is activated or repressed.

It is known from studies of HSV encephalitis in the mouse, that the virus displays a predilection for certain regions of the brain (Anderson and Field, 1983). Esiri (1982) also demonstrated, in a *post mortem* study of human encephalitis, that HSV antigens localize to certain areas of the brain, in particular the dentate granule cells of the hippocampus. Further, in the rat it has been shown that PRV selectively infects a population of medullary cardiomotor neurons in the brain (Standish et al., 1993). Thus, it may be that different cell populations in the CNS vary in their susceptibility to HSV infection and/or HSV-mediated damage. If this is the case, it is conceivable that host cell proteins might play a role in determining the pattern of infection. Interestingly, in the mouse, two alternatively spliced products of the Oct-2 gene, Oct-2c and Oct-2d appear to be differentially expressed in neurons, both spatially and temporally (Stoykova et al., 1992).

Thus, the pathological changes observed *in vivo* might be determined by cellular transcription factors which in turn may directly affect the permissiveness of the cell for viral replication. This introduces yet a further variable into the equation i.e., that the transcription factor environment is different in distinct populations of neurons. Indeed, Margolis et al. (1992) were able to define a number of subsets within a ganglion using a set of histochemical markers. Efforts are now underway in a number of laboratories to characterise these neurons in terms of their behaviour when infected with HSV-1.

We have only recently begun to discover some of the other inherent protective mechanisms which may enable neurons to survive virus infections. One of the most striking findings has been that the cellular proto-oncogene, *bcl*-2 is able to selectively rescue nerve growth factor (NGF), brain-derived growth factor (BDNF) and neurotrophin-3 (NT-3) dependent sensory neurons from apoptosis (Allsopp et al., 1993). Further, experiments carried out in several laboratories have demonstrated that *bcl*-2 is capable of protecting neurons from death caused by Sindbis virus infection (Levine et al., 1993).

Some neurotropic paramyxoviruses, e.g. Newcastle disease virus, can induce astrocytes to produce cytokines (Lieberman et al., 1989). This may either confer some protection upon surrounding cells or, conversely, may contribute to immune-mediated damage. Currently there is little information regarding the production of cytokines, such as IFN-γ, by neurons themselves or the response of neurons to locally produced cytokines. It is likely that cytokines play a significant role in intracellular communication between the nervous and immune system and influence the outcome of viral pathogenesis.

d. Reactivation of latent virus

In man, both HSV-1 and HSV-2 reactivate spontaneously, resulting in limited viral replication in the sensory ganglia and often the appearance of recrudescent lesions at the primary site of infection in the periphery. Reactivation can be induced in a number of different animal models; by UV irradiation, prostaglandin E_2, corneal scarification and hyperthermic stress (Blyth et al., 1976; Stanberry, 1980; Valyi-Nagy et al., 1991; Sawtell and Thompson, 1992*a*). EHV-1 can be induced to reactivate from a variety of latent sites in the mouse following either X-irradiation or corticosteroid injection (Field et al., 1992). The latter has also been used successfully to reactivate BHV-1 in cattle and in a rabbit eye model of herpesvirus infection (Whetstone et al., 1992; Rock et al., 1992).

The triggers which initiate reactivation *in vivo* have yet to be defined, but these could include local trauma and/or inflammation in the periphery, NGF deprivation and co-infection with other herpesviruses (Wilcox et al., 1988; Harris et al., 1898). It is possible then, that cytokines or other soluble factors associated with these kinds of damage may directly affect neuronal physiology by binding to receptor molecules on the surface of the neuron. For example, one of the pro-inflammatory cytokines, interleukin-6 (IL-6) is known to induce the same secondary messenger pathway in neurons as nerve growth factor (NGF) following binding to its receptor, a tyrosine kinase, gp130 (Yawata et al., 1993).

In addition to IL-6, TNF-α will also induce the cellular transcription factor *c-jun*. This is a highly significant observation since *c-jun* induction, together with

c-fos and *Oct-1* are known to be upregulated in response to corneal scarification in the rabbit eye model (Valyi-Nagy et al., 1991); these same factors have been postulated to directly transactivate HSV immediate-early gene promoters (see section *e*). Indeed, from recent experiments in neuronal cell cultures, it has been possible to elucidate some of the signal transduction pathways which are activated in response to various external stimuli (Smith et al., 1992; Roberts, 1992). Thus, a picture is beginning to emerge as to the mechanisms which may underly the reactivation of latent herpesviruses (Hayward, 1993).

e. Cellular factors involved in reactivation

As we have already discussed, any external forces such as the host immune system, superinfecting viruses and/or direct physical events are known to induce a variety of so-called immediate-early response (IER) genes, e.g. *c-jun, c-fos* and various heat-shock proteins (Sheng et al., 1990). Thus, alterations in the physiological state of the neuron can directly alter the transcription factor environment of the cell. Upregulation of *c-jun* and *c-fos*, the components of the ubiquitous transcriptional activator AP-1, may directly affect latent viral gene expression since binding sites for this and several other transcription factors, namely Sp1, AP-2, LPBF and CREB have been identified in both the ICP0 and LAT promoters (Batchelor and O'Hare, 1990). The exact mechanisms by which proteins of this type transactivate viral and cellular promoters are still being elucidated, although it is known that some appear to interact with components of the basal transcription apparatus, on or near TATA box motifs (for a review see Martin, 1991). Likewise, the BHV-1 LAT promoter is known to be positively regulated by cellular factors binding to *cis*-acting sequences (Bratanich and Jones, 1992).

Novel approaches towards modulating the neuron–virus interaction

In our laboratory, a unique approach towards controlling the establishment of latency has involved the generation of transgenic mice which constitutively express the viral immediate-early gene, ICP0, under the control of a neuron-specific promoter. The presence of the viral protein, Vmw110, has been demonstrated in both DRG neurons and in the brain, using a variety of immunohistochemical techniques. Preliminary evidence from infection studies carried out in F_2 transgenic mice suggests that the presence of the viral gene product may result in a difference in the amount of latent virus within sensory ganglia. This type of strategy represents one way in which the normal course of events in HSV-1 infected neurons might be modulated. Future experiments will examine the effects of either overexpressing or deleting individual transcription factors and could pave the way

for the development of strains of domestic livestock in which the balance has been altered towards latency negative animals.

Alternative strategies have been applied to the modulation of alphaherpesvirus pathogenesis in animals. Experiments by Shang and Osorio (1993) have shown that pre-colonisation of a natural host with attenuated strains of PRV will significantly impair the establishment of latency by wild-type PRV.

Alphaherpesviruses as potential vectors for gene therapy in the nervous system

The advent of somatic gene therapy has, for the first time, opened up the possibility of treating a number of previously incurable inherited disorders (Friedman, 1989). In many cases the strategies used have involved supplementing cells containing a mutant or defective gene with one or more copies of the normal gene. The latter can be introduced into cells by gene transfer using one of several recombinant, apathogenic viruses. Inevitably, the most successful strategy will combine a discrete population of target cells with a well-characterised and suitable vector. Many workers have proposed that alphaherpesviruses represent an ideal vehicle for introducing genes into the CNS (for a review see Leib and Olivo, 1993).

In order to render a virus "safe" for use in humans or animals and to minimise the possibility of recombination with latent viruses already present in the target cell, attenuation of virulence is required (Geller and Freese, 1990; Chiocca et al., 1990). In addition, deletions of the genes encoding Vmw65 and thymidine kinase from the virus should also preclude cell to cell spread and reactivation of latent viral vectors (Ace et al., 1989; Volz et al., 1992; Ecob-Prince et al., 1993). Preliminary evidence suggests that heterologous promoter-gene cassettes inserted into non-essential regions of the HSV-1 genome do not function during latency. However, it is known that long-term expression of the bacterial beta-galactosidase gene from the human cytomegalovirus major immediate-early promoter can be achieved if re-located downstream of the LAT ORF. Finally, another key issue to be addressed prior to the use of any herpesvirus-based vector, is the route of delivery. There is now quite a detailed understanding of natural and experimental modes of HSV-1 spread. Experiments in our laboratory have demonstrated that the large motor neurons of the spinal cord can be targeted by intramuscular injection, which enables virus to enter the sciatic nerve. We have also shown that stereotaxic injections can be used to deliver very small quantities of virus to precise nuclei within the brain.

It is not inconceivable then, that herpesvirus vectors could be developed which will establish life-long latent infections, and therefore ensure continued expression of the transferred gene, in selected populations of neurons of the PNS and/or CNS without causing any adverse pathology. With such tools we will be in a much better position to devise novel strategies for addressing the motor neuron diseases, Parkinson's disease, Alzheimer's disease and many of the other neurodegenerative conditions afflicting both humans and animals.

HSV-1 is not the only alphaherpesvirus which has been manipulated to permit the insertion of novel genes albeit for other reasons. It is interesting to note that recombinant pseudorabies viruses containing reporter genes have now been constructed to facilitate the study of virus spread (Kovács and Mettenleiter, 1991).

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PSEUDORABIES VIRUS LATENCY: A QUANTITATIVE APPROACH BY POLYMERASE CHAIN REACTION

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Some data dealing with the establishment of a quantitative PCR assay are presented. The assay is based on the use of an internal standard (mimic) which differs from the target by a deletion of a few base pairs and which is co-amplified with the target DNA. The resulting PCR products are labelled with fluorescent primers and then separated and detected by an automated sequencer. A highly specific and sensitive PCR assay for the envelope glycoprotein gp50 gene has been developed. This assay is highly reproducible with a detection limit of one copy of PRV DNA. Several mimics were then constructed. As a result we can confirm that the strategy that we have chosen is adequate for the quantification of low amounts of virus DNA present in latently infected swine.

Key words: Aujeszky's disease, pseudorabies virus, quantitative PCR

Aujeszky's disease is a significant cause of economic loss in the swine industry and is the target of eradication or control campaigns in many countries. The causative agent of this disease is a virus belonging to the alphaherpesvirus family, the pseudorabies virus (PRV), which can persist latently in the host. Latent infection is one of the main problems for the eradication of this virus because virus present in latently infected pigs can reactivate under different conditions and spread throughout the environment (Wittmann, 1991). The polymerase chain reaction (PCR), a major recent advance in molecular biology, has been successfully used to detect low amounts of viral DNA in latent and productive infections (Belák et al., 1989; Jestin et al., 1990*a*; Maes et al., 1990; Galeota-Wheeler and Osorio, 1991; Lokensgard et al., 1991). Some authors have reported the use of PCR to differentiate between wild type and some vaccinal strains of pseudorabies virus (Katz and Pedersen, 1992; Scherba et al., 1992; Hasebe et al., 1993; Dangler et al., 1993).

The aim of this study was to take advantage of the powerful PCR technology to quantify minute amounts of PRV virus present latently in different tissues. We report the development of an assay based on the use of an internal standard which is co-amplified with the target DNA. The use of an automated sequencer enabled the detection and the quantification of the standard and target DNAs

which are labelled with fluorescent primers (Porcher et al., 1992; Pannetier et al., 1992).

Materials and methods

Nucleic acid preparation. Porcine DNA was obtained from leukocytes isolated from the peripheral blood. The isolated leucocytes were lysed by incubation overnight at 42 °C in a buffer containing: 2M Tris (pH 7.6), 5M NaCl, 20% SDS, 0.5 M EDTA (pH 8) and 0.02% proteinase K. The DNA was recovered by precipitation with isopropanol after phenol-chloroform extraction. Viral DNA was obtained from purified virions grown on an established porcine kidney (PK15) cell line as described previously (Jestin et al., 1990*b*).

Amplification of the gp50 gene. Primers were selected with computer assistance using the Oligo Software (National Biosciences) and synthesized using a 391 DNA Synthesizer (Applied Biosystems). Both primers had a length of 25 necleotides:

Primer A (498) 5' CCTGGTGTCCGTCGACGGCGTGAAC 3';
Primer B (669) 5' CCGGTGCGGGGGCTGCTGGTAGAAC 3',

where the numbers in brackets represent the positions at which each primer sequence begins with respect to the initiation codon. Amplification reactions were performed in a 50 µl mixture containing 0.5 unit of Taq polymerase (Boehringer), 0.1 mM of each dNTP, 0.4 µM of each primer, and 10% DMSO in Boehringer buffer. Some amplifications were performed in the presence of 1 µg of porcine DNA as a reconstruction experiment. The reaction mixtures were overlaid with a drop of mineral oil. The amplification reactions were performed using a Perkin Elmer 9600 Thermocycler with the following program: one cycle 4 min at 94 °C; forty cycles each consisting of 30 sec at 94 °C, 30 sec at 64 °C, 30 sec at 72 °C; and a final extension step of 10 min at 72 °C.

Construction of an internal standard. The internal standards (mimic DNAs) were produced by PCR, under the same conditions as described above using mutagenic primers designed to create a small deletion located immediately downstream from the sequence of primer A or primer B. Two mimic DNAs containing respectively a deletion of 6 base pairs or 10 base pairs were constructed with the following mutagenic primers:

Primer 669Δ6: 5'CCGGTGCGGGGGCTGCTGGTAGAACAGGAA3',
Primer 498Δ10: 5'CCTGGTGTCCGTCGACGGCGTGAACACTTCATGGT3'.

The mimic DNAs were purified using spun columns (Centricon 100 from Amicon) and their concentrations were determined by spectrophotometry.

Run-off reaction. In order to detect the PCR products with an automated sequencer a fluorescent labelling step was needed. This was achieved by perform-

ing a few cycles of PCR in the presence of a fluorescent primer, which was synthesized directly with the DNA synthesizer using an amidite covalently linked with a fluorescent dye (Fam-Amidite, from Applied Biosystems). Aliquots of 10 µl from the PCR reactions were mixed in a final volume of 50 µl containing 0.5 unit of Taq polymerase (Boehringer), 0.1 mM of each dNTP, 0.4 µM of both dye-labelled and unlabelled oligonucleotides, and 10% DMSO in Boehringer buffer. Five cycles of amplification were performed under the same thermal and temporal conditions as detailed above.

Detection and separation of fluorescent PCR products. Five µl of the run-off reaction products were mixed with an equal volume of a 20 mM EDTA-formamide solution and heat-denatured at 80 °C for 10 min. Three µl of each sample were loaded on a denaturing gel (6% acrylamide, 8M urea) and electrophoresed for 6 hours using an Applied Biosystems 373A DNA Sequencer. For each detected DNA peak, a software specially designed to measure its length (with a precision below 0.2 nucleotides) and its area (with a precision below 5%) was used (a generous gift from C. Pannetier, Pasteur Institute, Paris, France).

Results

1. PCR detection of the PRV gp50 gene

Viral DNA was purified as described, digested with *Bam*HI, and the concentration was estimated by comparison of band intensities with known amounts of *Hin*dIII-digested lambda DNA on a 1% agarose gel stained with ethidium bromide. The sensitivity of the PCR procedure was assayed in a reconstruction experiment where serial dilutions of PRV DNA were prepared in the range 1 to 10^6 equivalent copies of viral genome, and subjected to amplification with the gp50-specific primers in the presence of up to 1 µg of porcine DNA. The amplified products were visualised after electrophoresis of 10 µl of each reaction on a 2% agarose gel stained with ethidium bromide. As shown in Fig. 1, an equivalent of 5 to 10 copies of PRV genome was detected after amplification in these conditions, by the presence of a 196 base-pair band as expected for the amplification of the gp50 gene using our primers. When the PCRs were performed in absence of porcine DNA, a sensitivity of 1 to 5 equivalent copies was obtained (not shown).

2. Mimic DNA construction and validation

Our quantitative PCR approach necessitated the use of an internal standard which would be co-amplified with the target DNA. As an automated sequencer was used to detect the amplified products, the size difference between the two

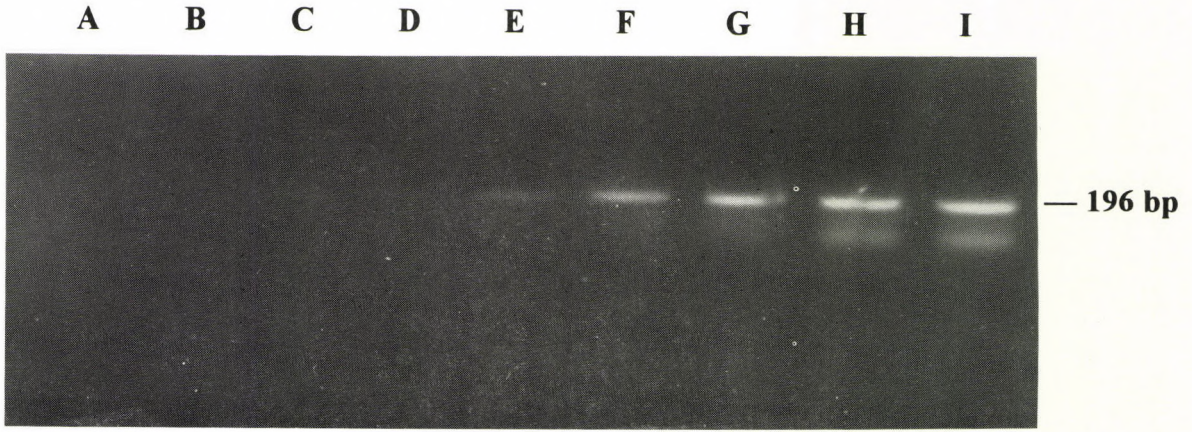

Fig. 1. Gp50 gene amplification. Serial dilutions of a stock solution of PrV DNA were PCR-amplified using Gp50 specific primers in a final volume of 50 µl (see Materials and Methods). One fifth of the reaction product was visualised by agarose gel electrophoresis. Starting quantities of PrV-DNA were 1 copy (A), 2 copies (B), 5 copies (C), 10 copies (D), 10^2 copies (E), 10^3 copies (F), 10^4 copies (G), 10^5 copies (H), 10^6 copies (I)

species could be as little as a few base pairs. Recently, two groups reported independently a quantitative PCR method using an automated sequencer (Porcher et al., 1992; Pannetier et al., 1992). Different methods were used by these authors to generate internal standards. Both methods needed several steps of cloning, enzyme digestion (Pannetier et al., 1992) or PCR mutagenesis with several primers (Porcher et al., 1992). Therefore we sought a more simple and systematic way to create mimic DNAs suitable for quantitative PCR. A PCR protocol was developed using a mutagenic primer (Fig. 2). The mutagenic primer contained a deletion, and was constructed by adding between 5 and 10 bases of the gene at the 3' end of the sequence of one of the two primers, after skipping a number of bases equal to the length of the desired deletion. The mimic DNA produced after amplification of the initial template using both the mutagenic primer and the other unmodified primer contained both primer sites which were conserved during the process, and a small deletion immediately downstream the sequence of one of the primers. The amplified products were then purified using spun columns and the concentrations were determined by optical density measurements. Mimic DNAs of 190 base pairs and 186 base pairs were constructed. They contained respectively a deletion of 6 base pairs and 10 base pairs compared to the initial gp50 amplified product. Both constructions were sequenced directly by the dideoxy chain termination method to ensure the presence of the deletions and that no other sequence modification was introduced. The amounts of product obtained after amplification of identical amounts of mimic DNA (190 base pairs) and viral DNA were compared. Both species were amplified with the same efficiency in the range 1 to 10^4 copies of template before amplification (Fig. 3). This last result indicated that the mimic DNA that wads produced according to our procedure was a valid internal control in quantitative PCR experiments.

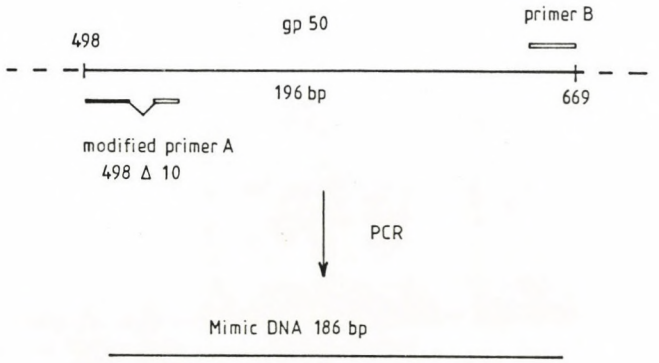

Fig. 2. Schematic representation of the PCR protocol designed for mimic construction

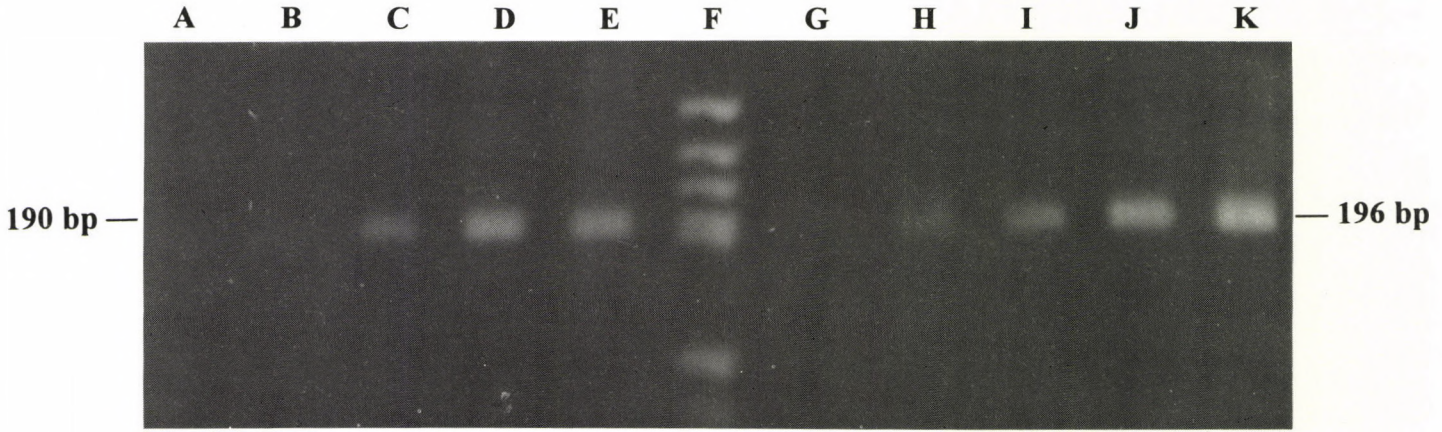

Fig. 3. Comparison of PrV and mimic DNA amplifications. Amplification of different quantities of a mimic DNA of 190 base pairs (lanes A–E) and PrV (lanes G–K) were performed separately. Quantities subjected to amplification were 1 copy (A, G), 10 copies (B, H), 10^2 copies (C, I), 10^3 copies (D, J), 10^4 copies (E, K). Lane F: size standard (DNA from pBR 322 digested with *Hae*III)

3. Fluorescence detection of PCR products

Run-off reactions were performed as described in Materials and methods using Fam-labelled primer A in order to label the PCR products obtained after amplification of PRV DNA (in the range 1 to 10^6 copies). The fluorescence of each sample was recorded using an automated sequencer (Applied Biosystems), and the amount of fluorescence was plotted as a function of the number of copies of PRV subjected to amplification. From the obtained data, it was clear that the amount of fluorescence (i.e. the amount of amplified product) was proportional to the initial amount of template before amplification (Fig. 4). There was a strong linear relationship between the amount of fluorescence and the logarithm of the initial copy number of PRV from 1 to 100 copies and 10^3 to 10^6 copies with a sudden change of the slope between 100 and 1000 copies. The cause of this slope variation is not yet understood but it emphasizes the fact that accurate quantitation can only be achieved when an internal control is used. However, this result gave a good indication of the sensitivity that is obtained using fluorescence detection. Such an experimental design should be adequate for the quantification of low amounts of virus present in latently infected tissues.

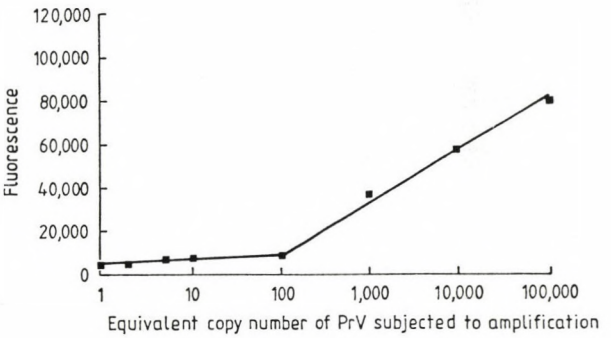

Fig. 4. Fluorescence detection of Gp50-specific PCR products. Serial dilutions of PrV DNA were amplified, labelled with a fluorescent oligonucleotide using 5 cycles of run-off and detected using an automated sequencer. Intensities of fluorescence were plotted as a function of the number of Gp50-DNA molecules subjected to amplification

4. Separation of mimic and target DNAs

In order to test the performance of the separation with the automated sequencer, amplification reactions were performed on 10^4 copies of each mimic and PRV DNA. The products obtained after PCR were labelled by run-off reactions and equal volumes were mixed. The mixtures were loaded on a sequencing gel,

electrophoresed and the peaks of fluorescence associated with each DNA fragment were recorded and analyzed as described in Materials and methods. Figure 5 depicts the results of this experiment. Peaks were well resolved allowing a good separation between wild type DNA and mimics bearing a 6 base pair or 10 base pair deletion. The size determinations were accurate.

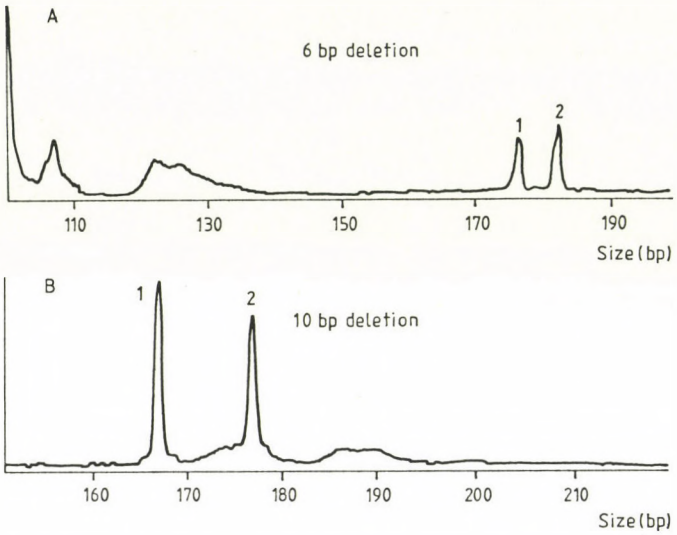

Fig. 5. Separation of PCR products with an automated sequencer. Gp50 PCR-products and mimic DNAs containing deletion of 6 base pairs or 10 base pairs were labelled with internal fluorescent oligonucleotides, and the resulting products were separated with an automated sequencer. Panel A: mimic DNA (peak 1), Gp50 PCR-product (peak 2). Panel B: mimic DNA (peak 1), Gp50 PCR-product (peak 2)

Discussion

In this paper we describe a novel PCR approach to quantify the extent of latent PRV infection in swine.

Several quantitative PCR methods have been described recently. The "External Control" method relies on the establishment of a standard curve in parallel to each experiment (Abbot et al., 1988; Katz et al., 1990; Nooman et al., 1990). The "No-Control" method uses a regression curve where the amount of amplified DNA is plotted against the number of PCR cycles, the level of DNA in the sample before emplification being determined by extrapolation to the initial conditions (Chelly et al., 1990). The "Internal Control" method (Becker-André and Hahlbrock, 1989; Wang et al., 1989; Gilliland et al., 1990) is based on the co-

amplification of an internal standard and the target DNA allowing the drawing of a titration curve in parallel to each experiment (amount of standard / amount of target versus the initial copy number of standard before amplification). Among those methods, we chose to develop an assay based on the use of an internal standard coupled with fluorescence detection and quantification using an automated sequencer (Porcher et al., 1992; Pannetier et al., 1992–1993). Several reasons guided our choice. The "External Standard" method was found to be inaccurate (Nedelman et al., 1992) and the "No Control" method requires too much handling of the material, which is a potential source of contamination in PCR experiments. On the other hand, the "Internal Control" method was shown to be accurate (Pannetier et al., 1993), particularly when the mimic DNA is as close as possible to the target DNA and when the same primers are used to amplify both species. Then, it is possible to rule out the potential cause of artifacts due to differences between the amplification efficiencies of the standard and the target DNA. Additionally, we chose to use fluorescence-based detection and quantification with an automated sequencer because it eliminates the need for radioisotopes and is particularly well suited for the detection of minute amounts of viral DNA among a large number of non-infected cells.

During the course of this study, a highly specific and sensitive PCR was developed allowing the detection of 5 to 10 copies of the gene coding for the envelope glycoprotein gp50 of PRV. A fast and efficient method to generate internal standards (mimic DNAs) was also developed. A PCR strategy was adopted for this purpose, which involves a mutagenic primer that can be used under the same conditions set to detect the gene of interest. This provided a systematic way to produce mimic DNA as a three-step procedure: (*i*) Amplification using a modified primer; (*ii*) Purification using a spun column; (*iii*) Copy number determination by optical density measurement. The efficiencies of amplification of the target DNA and of an internal standard, constructed according to our method, were compared. Both species were amplified with similar efficiencies, indicating that such mimic DNAs are suitable for use in quantitative PCR experiments.

Fluorescence labelling of the PCR products and their detection with an automated sequencer were evaluated in terms of sensitivity and performance of the separation. Five cycles of PCR were performed in our experiments to label the PCR products, allowing a very good sensitivity. Under those conditions, the fluorescence signal associated with DNA obtained after amplification of one equivalent copy of PRV was well above the background, indicating that fewer cycles of Run-off could be used to label the PCR products. Actually, Pannetier et al. (1993) used only one cycle of run-off. However, the possibility of performing more than one run-off cycle in order to improve sensitivity could be kept in mind. Mimic

DNA, containing deletions of 6 or 10 base pairs, could be easily separated from the target DNA under our experimental conditions.

In summary, we present here some data dealing with the establishment of a quantitative PCR method designed for accurate determination of low copy number of latent PRV virus in infected porcine tissues. From these preliminary experiments, we can conclude that the strategy that we chose is adequate for this purpose. Our future investigations will focus on the use of this quantitative PCR method to map precisely the sites of latent PRV infection in pigs and to study the establishment of latency by various mutants. In addition, as specificity is an intrinsic property of PCR, we believe that such a quantitative tool will be very powerful for studying vaccine efficacy.

Acknowledgements

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GLYCOPROTEIN I OF PSEUDORABIES VIRUS (AUJESZKY'S DISEASE VIRUS) DETERMINES VIRULENCE AND FACILITATES PENETRATION OF THE VIRUS INTO THE CENTRAL NERVOUS SYSTEM OF PIGS

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In the present study we investigated the virulence and neural spread of pseudorabies virus (PRV) strains with mutations in the gene encoding glycoprotein I (gI) in 3-week-old pigs which were intranasally infected. Mutant M303 (Δ 125, 126) lacks amino acids valine-125 and cysteine-126 in an immunodominant antigenic region of gI which contains 2 discontinuous antigenic domains, whereas mutant virus M304 (Δ59, 60) lacks amino acids glycine-59 and aspartic acid-60 in a continuous antigenic domain. Mutant M301 contains a frame shift mutation. Both mutants M301 (gI–) and M303 (Δ125, 126) were not virulent for pigs, whereas mutant M304 (Δ59, 60) was as virulent as wild-type PRV. All gI mutant viruses replicated in the oropharyngeal mucosa, although M304 (Δ59, 60) and wild-type PRV replicated to higher titres than M303 (Δ125, 126) and M301 (gI–). In contrast to M304 (Δ59, 60) and wild-type PRV, both mutant viruses M301 (gI–) and M303 (Δ125, 126) were not recovered from any part of the central nervous system at day 6 after infection. To study the spread of M301 (gI–) in the central nervous system in more detail, a second experiment was done in which 100-fold more virus was intranasally administered and virus was recovered from various tissues at day 4 after infection. Again, no gI-negative virus was isolated from the central nervous system. We concluded that deleting the amino acids valine-125 and cysteine-126 decreases virulence and reduces neurotropism to the same degree as deleting the gI protein. In addition, gI-negative virus does not spread in the central nervous system of pigs, probably because the transport of the virus across the synapse is hampered.

Key words: Aujeszky's disease (pseudorabies) virus, virulence, neural spread, gene mutations, glycoprotein I

Glycoprotein I (gI) is one of at least 9 glycoproteins of pseudorabies virus (PRV, family *Herpesviridae,* subfamily *Alphaherpesvirinae*) (Mettenleiter, 1991;

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Peeters et al., 1992; Mettenleiter et al., personal communication). The correct taxonomic name of PRV, the causative agent of Aujeszky's disease, is suid herpesvirus-1 (Roizman, 1992). Although gI is not required for the virus to grow *in vitro* or *in vivo* (Berns et al., 1985; Mettenleiter, 1991), all of numerous virulent field strains examined so far from pigs possess gI (Van Oirschot, 1989). Therefore gI appears essential for PRV to survive in pigs.

Glycoprotein I is important for virulence of PRV (Lomniczi et al., 1987; Mettenleiter et al., 1985, 1987). Recent findings suggest that gI affects virulence by facilitating the transport of the virus through the central nervous system. Transport of PRV in the rat brain mainly occurs through synaptically linked neurons (Rineman et al., 1993; Whealy et al., 1993; Card et al., 1993), and is facilitated by gI (Card et al., 1991, 1992). After infection in the eye of the rat, a gI-negative PRV strain selectively infected a subset of retinal ganglion cells. Consequently gI-negative virus was only found in restricted parts of the visual system. Kimman et al. (1992) suggested that gI is necessary for the transport of PRV through the porcine central nervous system.

In the present study, we investigated the virulence and the neural spread of 3 gI mutant viruses in pigs. One of the gI mutant viruses had a deletion of valine-125 and cysteine-126 (M303; Δ125, 126). This deletion is located in an immunodominant region of gI. The mutant carrying this deletion was less virulent for mice than wild-type PRV (Jacobs et al., 1990, 1993*a*). Mutant M304 (Δ59, 60) had a deletion of glycine-59 and aspartic acid-60, and was as virulent for mice as wild-type PRV. A gI-negative mutant was constructed by the introduction of a frame shift at amino acid position 124. Pigs were intranasally infected and signs of disease and virus shedding in the oropharyngeal fluid (OPF) were monitored. Tissue specimens were collected for pathological examination.

We found that deleting the amino acids valine-125 and cysteine-126 in gI decreases virulence and neurotropism to the same degree as deleting gI. In addition, M301 (gI–) and M303 (Δ125, 126) did not spread in the central nervous system, probably because transsynaptic transport was hampered.

Materials and methods

Cell cultures and plaque titration

A pig kidney cell line (SK6) was cultivated in Dulbecco's modified essential medium (DMEM) supplemented with 5% fetal calf serum, L-glutamine (0.3 mg/ml), penicillin (90 U/ml), streptomycin (100 U/ml), and nystatin (45 U/ml). For quantification, virus was plaque-titrated on SK6 cells. Virus was allowed to

adsorb for 1 h at 37 °C before the cells were overlaid with 1% methylcellulose in DMEM supplemented with 5% fetal calf serum and antibiotics. About 64 h after infection, the overlay was removed and the cells were stained with a solution containing 0.1% amidoblack, 6% acetic acid, 0.8% sodium acetate, and 8.5% glycerol, and plaques were counted.

Virus strains

The construction of the gI mutant viruses has been described earlier (Jacobs et al., 1993). Mutant M303 (Δ125, 126) has a deletion of amino acids valine-125 and cysteine-126, which results in the loss of the discontinuous antigenic domains C and E. Mutant M304 (Δ59, 60) has a deletion of amino acids glycine-59 and aspartic acid-60 which results in the loss of a continuous antigenic domain B (Jacobs et al., 1990, 1993*a*). A gI-negative virus strain (M301) was constructed by the introduction of ten nucleotides at amino acid position 124, which resulted in a frame shift. The NIA-3 strain of PRV (McFerran and Dow, 1975) was used as the parent strain for the development of the gI mutant viruses. In experiment 1, wild-type PRV was used, obtained after transfection of NIA-3 DNA into SK6 cells. In experiment 2, wild-type PRV was a rescue mutant of M301. Both wild-type PRV strains were as virulent for mnice as their parent strain NIA-3 (data not shown).

Animals

Dutch Landrace pigs (3 weeks old) were from the specific pathogen free herd of the Central Veterinary Institute. The pigs were born from unvaccinated sows. Before the start of the experiments, the pigs had no antibodies directed against PRV. Pigs of different litters were randomly assigned to experimental groups. During the experiments, each group was housed in a separate isolation room and had continuous access to food.

Experimental design

In the first experiment, ten 3-week-old pigs were infected intranasally with 10^5 PFU of a gI mutant virus or wild-type PRV. The virus suspension (0.5 ml) was slowly administered into each nostril during inspiration. Six days after infection, 4 pigs were killed and examined for the presence of virus and lesions in tissues.

In the second experiment, 3 pigs were infected intranasally with 10^7 PFU of M301 (gI–) or wild-type PRV. At day 4 after infection pigs were killed and examined for the presence of virus and lesions in tissues.

Collection of samples

Oropharyngeal fluid (OPF) was collected with swabs daily from 1 day before to 10 days after infection. Swabs were extracted with 4 ml DMEM supplemented with 5% fetal calf serum and antibiotics. To determine the virus content per gram of OPF, the weight of the collected fluid was measured after centrifugation of the swabs in a special container.

Clinical signs of disease

Clinical signs of disease were monitored as described by Kimman et al. (1992). The period of growth arrest was defined as the number of days needed for the animal to regain its weight as measured on the day of challenge. Fever was defined as a rectal temperature above 40 °C. Respiratory signs were defined as nasal discharge, sneezing, coughing and laboured breathing. Neurologic signs were defined as vomiting, itching, ataxia, paralysis, tremor, and convulsions.

Virological examinations

To determine the virus content of OPF, we diluted the OPF in DMEM supplemented with 2% fetal calf serum, L-glutamine (0.3 mg/ml), penicillin (90 U/ml), streptomycin (100 U/l), and nystatin (45 U/ml). The OPF was plaque titrated and virus titres were expressed as ^{10}log PFU/g of sample. In the first experiment, tissue specimens were collected at day 6 after infection from the trigeminal ganglion, bulbus olfactorius, brain stem, pons cerebri, cerebellum, medulla oblongata, pharyngeal mucosa, nasal mucosa, tonsil, retropharyngeal lymph node, subparotideal lymph node, mandibular lymph node, lung and spleen. In the second experiment, tissue specimens were collected at day 4 after infection from nasal mucosa, pharyngeal mucosa, olfactory epithelium, tonsil, soft palate, mandibular lymph node, subparotideal lymph node, trigeminal ganglion, bulbus olfactorius, brain stem, medulla oblongata, and cerebellum. To determine the virus content of tissues, a 10% (w/v) tissue suspension was made in DMEM supplemented with 2% fetal calf serum, the antibiotics mentioned above, and kanamycin (100 µg/ml). A complement-independent neutralization assay using a specific PRV antiserum made in rabbits was used to confirm that plaques were caused by PRV.

Pathologic examination

A part of the tissue specimens collected for virus isolation (see *Virological and serological examinations*) were used for pathologic examination. The specimens were fixed in 10% neutral buffered formalin, embedded in paraffin and cut

in 6-μm sections. Viral antigen was detected in an indirect immunoperoxidase assay (Pol et al., 1991) using a polyclonal rabbit serum directed against PRV. Sections were counterstained with haematoxylin for 10 sec.

Results

Virulence of gI mutant viruses

In experiment 1, all pigs infected with M304 (Δ59, 60) and wild-type PRV developed severe respiratory and neurologic signs characteristic of PRV infection. They had fever, lost their appetite, lost weight and died.The mean time to death for pigs infected with M304 (Δ59, 60) was 4.4 ± 1.4 (mean ± SD), and for pigs infected with wild-type PRV 5.7 ± 1.0 days. In contrast, none of the pigs infected with the gI-negative virus M301 or M303 (Δ125, 126) developed signs of PRV infection. These pigs had no fever, their appetite was normal, and they gained weight as the uninfected control group. Thus, M304 (Δ59, 60) and wild-type PRV were equally virulent for 3-week-old pigs, whereas M303 (Δ125, 126) and M301 (gI–) were not at all virulent. In experiment 2, pigs were infected with M301 (gI–) and wild-type PRV. Pigs infected with M301 (gI–) showed no signs of pseudorabies. In contrast, pigs infected with wild-type PRV showed severe signs of pseudorabies.

Virus content in OPF and tissue specimens at day 6 after infection

In experiment 1, virus was recovered from the OPF of pigs infected with mutants M301 (gI–) and M303 (Δ125, 126) until day 10 after infection, and from pigs infected with M304 (Δ59, 60) and wild-type PRV until they died from PRV infection (Fig. 1). All gI mutant viruses replicated in the oropharyngeal tissue, although M301 (gI–) and M303 (Δ125, 126) always produced lower virus titres than M304 (Δ59, 60) and wild-type PRV.

Virus was recovered from numerous tissue specimens collected from infected pigs killed on day 6 after infection or when the pigs died of PRV infection (Table 1). Virus titres in tissues of pigs infected with M304(Δ59, 60) or wild-type PRV were higher than virus titres in tissues of pigs infected with M301 (gI–) or M303 (Δ125, 126). Virus was recovered only from the trigeminal ganglion and not from other parts of the central nervous system of pigs infected with M301 (gI–) or M303 (Δ125, 126). Thus, these mutants did not spread to or replicated in the central nervous system. In contrast, virus was recovered from several parts of

the central nervous system of pigs infected with M304 (Λ59, 60) or wild-type PRV.

Table 1

Virus content in tissue specimens (6 days after infection) of 3-week-old pigs intranasally infected with gI mutants of PRV

Tissue	Virus titres (^{10}log of pfu/g [SD])[1] after infection with:			
	M301[2]	M303[3]	M304[4]	wild-type PRV
Trigeminal ganglion	1.0 (1.2)	1.8 (0.4)	5.3 (0.3)	5.6 (0.5)
Bulbus olfactorius	–[5]	–	4.1 (1.7)	5.3 (1.2)
Brain stem	–	–	2.6 (0.7)	3.7 (1.1)
Pons cerebri	–	–	3.2 (0.4)	4.4 (0.9)
Cerebellum	–	–	3.3 (1.0)	4.8 (0.8)
Medulla oblongata	–	–	2.8 (0.4)	3.8 (1.0)
Pharyngeal + nasal mucosa	1.6 (1.3)	2.4 (1.9)	5.9 (0.2)	6.0 (0.1)
Tonsil	0.9 (1.9)	3.9 (1.7)	5.7 (0.4)	5.8 (0.2)
Retropharyngeal lymph node	–	1.3 (2.0)	5.0 (0.7)	5.1 (0.8)
Subparotideal lymph node	0.9 (1.8)	0.9 (0.9)	3.0 (2.9)	3.8 (2.8)
Mandibular lymph node	2.0 (1.8)	–	4.0 (2.8)	5.7 (0.5)
Lung	2.3 (2.9)	2.5 (2.4)	4.7 (1.1)	3.7 (0.6)
Spleen	0.5 (0.9)	–	2.7 (2.0)	0.6 (1.2)

[1] Mean number of 4 pigs; [2] M301 had a frame shift in gI; [3] M303 had val-125 and cys-126 deleted; [4] M304 had gly-59 and asp-60 deleted; [5] no virus detected

Virus content in tissue specimens at day 4 after infection

To examine the influence of gI on the spread of virus in the central nervous system in more detail, a second experiment was done in which a 100-fold higher infective virus dose was used (10^7 PFU) and tissue specimens were collected at day 4 after infection. Virus was recovered from numerous tissue specimens (Table 2). Virus titres in tissues of pigs infected with wild-type PRV were higher than virus titres in tissues of pigs infected with M301 (gI–). However, from pigs infected with M301 (gI–) no virus was recovered from the bulbus olfactorius, brain stem, medulla oblongata and cerebellum. In contrast, from pigs infected with wild-type PRV, virus was recovered from all tissues that were examined. In conclusion,

even when more virus is intranasally administered no gI-negative virus is detected in the central nervous system at day 4 after infection.

Table 2

Virus content in tissue specimens (4 days after infection) of 3-week-old pigs intranasally infected with PRV mutant M301 (gI–) or wild-type PRV

Tissue	Virus titres (^{10}log of pfu/g [SD])[1] after infection with:	
	M301 (gI–)	wild-type PRV
Trigeminal ganglion	2.0 (0.5)	5.6 (0.1)
Bulbus olfactorius	–[2]	4.2 (0.3)
Brain stem	–	4.2 (0.5)
Medulla oblongata	–	2.9 (0.3)
Cerebellum	–	3.6 (0.6)
Nasal mucosa	2.9 (1.7)	6.0 (0.6)
Pharyngeal mucosa	2.1 (0.8)	5.8 (0.8)
Olfactory epithelium	0.8 (1.4)	6.2 (1.2)
Tonsil	4.3 (1.9)	6.6 (0.6)
Soft palate	4.2 (2.9)	6.5 (0.5)
Mandibular lymph node	2.0 (1.4)	5.8 (0.5)
Subparotideal lymph node	2.4 (0.7)	4.9 (0.6)

[1] Mean number of 3 pigs;[2] No virus detected

Pathogenesis of gI mutant viruses

In experiment 1, mutants M301 (gI–) and M303 (Λ125, 126) both caused slight ganglionitis in the trigeminal ganglion with some degeneration and necrosis of neurons, but no lesions were found in other parts of the central nervous system. In contrast, wild-type PRV and M304(Λ59, 60) caused ganglionitis with extensive necrosis of neurons in the trigeminal ganglion and in other parts of the central nervous system. Degeneration of neurons was accompanied by perineuronal gliosis and inflammatory infiltrates.

Viral antigen of M304(Λ59, 60) and wild-type virus was detected in the trigeminal ganglion, the bulbus olfactorius, and the cerebellum. No viral antigen

of M301 (gI–) and M303 (Λ125, 126) was detected in the trigeminal ganglion or in other parts of the brain. The results of the indirect immunoperoxidase assay were thus consistent with the virological findings, with the exception of the trigeminal ganglion.

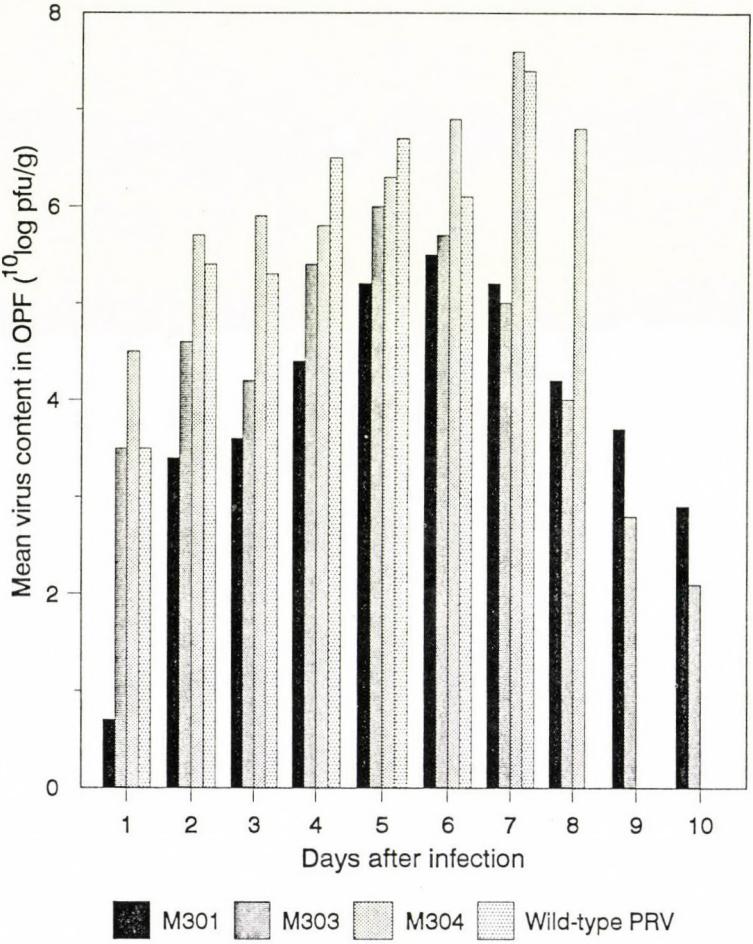

Fig. 1. Virus content of OPF of pigs intranasally infected with gI mutant viruses of PRV and wild-type PRV. The infecting PRV mutant strains are indicated at the bottom of the figure

In experiment 2, a ganglionitis with degeneration and necrosis of neurons in the trigeminal ganglion of pigs infected with M301 (gI–) was detected. This was accompanied by the detection of viral antigen in the trigeminal ganglion (Fig. 2A,

B). in contrast to wild-type PRV no viral antigen of M301 (gI–) or signs of a viral infection were found in other parts of the central nervous system. Hence, the results of the virological findings were consistent with the results of the indirect immunoperoxidase assay in this experiment.

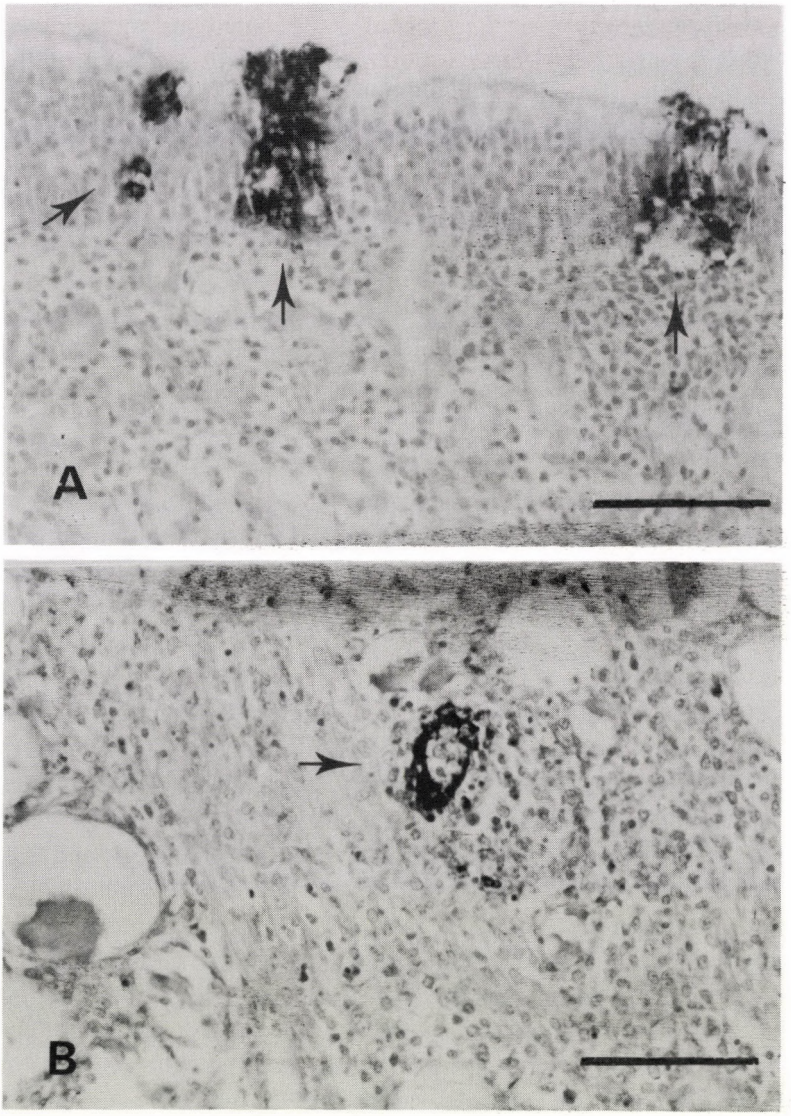

Fig. 2. Photomicrograph of nasal mucosa (A), and trigeminal ganglion (B), stained by the immunoperoxidase method, after infection with M301 (gI–). Viral antigen is indicated with an arrow. The bar represents a length of 100 μm

Discussion

The virulence and neuropathogenesis of 3 PRV mutant viruses were investigated in 3-week-old pigs. Two mutant viruses contain deletions of two amino acids in antigenic domains of gI. M303 (Δ125, 126) has a deletion of valine-125 and cysteine-126 resulting in the loss of 2 discontinuous antigenic domains; M304(Δ59, 60) has a deletion of glycine-59 and aspartic acid-60, resulting in the loss of a continuous antigenic region. A gI-negative PRV mutant was constructed by the introduction of a frame shift. The construction, characterization and immunogenicity of the mutant viruses have been described earlier (Jacobs et al., 1993*a*, 1993*b*).

Mutant viruses M301 (gI–) and M303 (Δ125, 126) were not virulent for pigs. In contrast, M304(Δ59, 60) and wild-type PRV were highly virulent: all pigs infected with the latter two died within 8 days after infection. Thus the virulence of PRV for young pigs was eliminated when the amino acids valine-125 and cysteine-126 were deleted. These findings agree with earlier results obtained in mice (Jacobs et al., 1993*a*).

M304(Δ59, 60) and wild-type PRV were isolated from several parts of the brain, in contrast to M303 (Δ125, 126) and M301 (gI–). The latter two were isolated only from the trigeminal ganglion. Glycoprotein I is clearly important for the virus to spread through the central nervous system; deleting valine-125 and cystine-126 eliminated this function of gI, and as a consequence, decreased the virulence of the virus.

Deleting valine-125 or, more likely, cysteine-126, may disturb the conformational structure of the gI or the gI–gp63 complex. Glycoprotein I is noncovalently linked to gp63 and this complex forms a functional entity (Zuckermann et al., 1988; Whealy et al., 1993). Consequently, inactivation of gI can lead to inactivation of the functional complex of gp63 with gI.

Cysteine-126 is located in an immunodominant region of gI, and is part of a well-conserved cluster of cysteines in homologous proteins of other alphaherpesviruses such as varicella-zoster virus, simian varicella virus, equine herpesvirus and bovine herpesvirus (Davison and Scott, 1986; Fletcher and Gray, 1993; Telford et al., 1992; Rijsewijk et al., personal communication). Probably this region has a (conserved) biological function.

In mice, invasion of the central nervous system by wild-type PRV only occurred by the trigeminal route (Heffner et al., 1993). In pigs, invasion of the central nervous system occurred both by the trigeminal and olfactory routes (Kimman et al., 1992; Jacobs et al., 1993*b*; Mulder et al., personal communication). However, gI-negative PRV was only recovered from the trigeminal ganglion and not from the bulbus olfactorius. This suggests one blockade of transport of gI-

negative PRV from the olfactory receptor neurons to the bulbus olfactorius, and a second blockade from the trigeminal ganglion to neurons in the brain stem.

Possibly, M301 (gI–) and M303 (Λ125, 126) spread too inefficiently in the oropharyngeal mucosa and the trigeminal ganglion to enter the central nervous system. *In vitro*, gI induces cell fusion and, as a consequence, promotes cell-to-cell spread of the virus (Zsák et al., 1992). In mice, direct cell-to-cell spread is the pathogenetically most important way of virus spread of PRV (Heffner et al., 1993). However, M303 (Λ125, 126) and M301 (gI–) replicated in the nasal and pharyngeal mucosa, albeit to somewhat lower titres than wild-type PRV. In addition, PRV is taken up and transported very efficiently along peripheral nerves to the central nervous system (Kuypers and Ugolini, 1990; Strack and Loewy, 1990). Even when 100-fold more gI-negative PRV was intranasally administered, no virus was detected in the bulbus olfactorius and in other parts of the brain.

Another possibility is that there is an impaired transport of gI mutant viruses M301 (gI–) and M303 (Λ125, 126) through neurons or across synapses. The virus has to cross one synapse from the olfactory receptor neurons in the olfactory epithelium to the periglomerular and mitral neurons in the bulbus olfactorius. In contrast, the virus has to cross no synapse to spread to the trigeminal ganglion. Transneuronal transport of PRV mainly occurs through synaptically linked neurons (Rineman et al., 1993; Whealy et al., 1993; Card et al., 1993).

In summary, deleting the amino acids valine-125 and cysteine-126 decreases virulence and neurotropism of PRV to the same degree as deleting gI, and gI-negative virus does not spread in the central nervous system. Possibly, the transport of gI-negative PRV across the synapse is hampered. Further experiments will be needed to confirm these findings.

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INTERACTIONS OF AUJESZKY'S DISEASE VIRUS AND PORCINE BLOOD MONONUCLEAR CELLS *IN VIVO* AND *IN VITRO*

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Nine pigs were inoculated oronasally with $10^{8.0}$ $TCID_{50}$ Aujeszky's disease virus (ADV). Blood was examined daily for the presence of a viremia during 7 days post inoculation. Cell-free and/or mononuclear cell (MC)-associated ADV was demonstrated in 7 out of the 9 pigs. The number of days on which MC-associated ADV was detected per pig ranged from 1 to 4 days. The number of infected cells ranged between 1 and 200 per 10^8 examined MC. Monocytes were found to be the most ADV-susceptible MC *in vivo*. Interactions of ADV with MC were also examined *in vitro*. Subpopulations of MC were inoculated with ADV at a multiplicity of infection of 20. Depending on the method of cell separation, 2 or 21% of the monocytes were infected. Lymphocytes were rather refractory to ADV-infection.

Key words: Aujeszky's disease virus, porcine blood mononuclear cells, viraemia

In the field, abortions caused by Aujeszky's disease virus (ADV) have been reported in well-vaccinated herds by de Muêlenaere and Pensaert (1989) and Dieuzy et al., (1987). These reports have indicated that ADV is able to reach the fetus in a vaccination-immune sow without being eliminated by neutralizing antibodies. Because it was possible to reproduce abortion in vaccinated sows with ADV-infected MC, it was shown that cell-associated ADV plays a pivotal role in the pathogenesis (Nauwynck and Pensaert, 1992). Based on this finding and data from Wittmann et al. (1980), the following pathogenetic mechanism of abortion is proposed in the vaccination-immune sow. ADV replicates, albeit to a lesser extent, in the respiratory tract of vaccinated animals. Hereafter, it can be transported from the primary replication sites throughout the whole body in circulating infected blood leukocytes. Finally, ADV spreads from infected blood MC through the maternal placenta in order to reach the fetuses despite the presence of a vaccination immunity.

The adhesion of infected MC to the endothelium of uterine capillaries is postulated to be the first step in the spread of ADV from the infected MC to the fetuses. The existence of an ADV-antigen-dependent adhesion of infected cells to

uninfected cells has already been demonstrated in a model using cells of the continuous swine kidney cell line SK-6 (Hanssens et al., 1993). In future studies, it will be examined whether a similar adhesion can be demonstrated between infected MC and endothelial cells. Before this is performed some interactions between ADV and mononuclear cells were examined.

In the present study, the subpopulation of MC which is most susceptible to an infection with ADV was determined both *in vivo* and *in vitro*.

Materials and methods

Determination of susceptible MC subpopulation in vivo

Nine ADV-seronegative pigs (A to I), weighing 80–90 kg, were inoculated oronasally with $10^{8.0}$ $TCID_{50}$ of the ADV strain 89V87. Blood was taken from each pig at 0, 1, 2, 3, 5 and 7 days post inoculation (PI) and mononuclear cells (MC) were isolated on Ficoll-Paque (Pharmacia, Uppsala, Sweden). The MC were washed and treated with citrate (pH 3) in order to remove extracellular virus (Mettenleiter, 1989). The number of infected cells was determined by cocultivation with cells of the continuous swine kidney cell line SK-6. The presence of cell-free ADV was determined in plasma by virus isolation on cells of the continuous swine testicle cell line ST. On 3 and 5 days PI, MC of pigs A, B, C, G, H and I were further separated. Monocytes were first isolated by adherence on plasma-coated polystyrene dishes. The adherent subpopulation consisted of more than 92% of monocytes, as determined by an indirect immunofluorescence technique using monoclonal antibody (mAb) 74.22.15 which is directed against a marker on monocyte/macrophages, as primary antibodies and FITC-labeled goat-anti-mouse as secondary antibodies (Pescovitz et al., 1984). The non-adherent cells were further depleted of monocytes by an indirect immunopanning technique. They were incubated for one hour at 4 °C with mAb 74.22.15 and subsequently washed three times. The labeled cells were layered on dishes, coated with purified goat anti-mouse IgG. After depletion, less than 3% of monocytes were detected. The number of infected cells in the subpopulations of positively selected monocytes and negatively selected lymphocytes was determined by cocultivation on SK-6-cells.

Determination of susceptible MC subpopulation in vitro

Isolation of MC from whole blood. Blood without antibodies against ADV was taken from pigs at a weight of 100 kg. MC were isolated from blood by Ficoll-Paque and washed twice. MC were separated into subpopulations using the

direct immunopanning combined with plasma-mediated adhesion or using indirect immunopanning.

Isolation of B-lymphocytes, T-lymphocytes and monocytes from MC by direct immunopanning combined with plasma-mediated adhesion. MC were layered on dishes coated with purified rabbit anti-swine IgG (Fc and Fab) antibodies for one hour at 4 °C. Afterwards, the dishes were gently swirled, the non-adherent cells were collected and the remaining cells were washed three times. The adherent cells consisting of enriched B-lymphocytes were detached after an incubation in phophate buffered saline solution with 10% porcine serum without anti-ADV antibodies for 1.5 hours at 37 °C. The non-adherent cells consisting of enriched T-lymphocytes and monocytes, were further layered on plasma-coated culture dishes. The plasma-adherent cells represented the enriched monocytes and the plasma-non-adherent cells represented the enriched lymphocytes. The composition of all subpopulations was determined by indirect immunofluorescence using mAb 5C9 directed against IgM on B-lymphocytes (Paul et al., 1985), mAb MSA_4 directed against CD_2 on T-lymphocytes (Hammerberg and Schurig, 1986) and mAb 74.22.15 directed against a marker on monocytes, as primary antibodies and FITC-labeled goat-anti-mouse as secondary antibodies. The subpopulation of enriched B-lymphocytes contained 69 to 78% B-lymphocytes, 13 to 21% T-lymphocytes and 6% monocytes. The subpopulation of enriched T-lymphocytes contained 0 to 2% B-lymphocytes, 84 to 92% T-lymphocytes and 0 to 3% monocytes. The subpopulation of enriched monocytes was 96 to 100% pure.

Isolation of lymphocytes and monocytes from MC by indirect immunopanning. MC were incubated for one hour at 4 °C with mAb 74.22.15 which is directed against a marker on monocytes and subsequently washed three times. The labeled cells were layered on dishes, coated with purified goat anti-mouse IgG. The non-adherent fraction representing MC depleted of monocytes was collected. In this fraction 13–17% were B-lymphocytes, 62–67% were T-lymphocytes and less than 3% of the cells were monocytes as determined by indirect immunofluorescence as described above. The adherent fraction representing enriched monocytes was finally washed twice with PBS. The subpopulation of monocytes was 70–75% pure.

Viability and inoculation of MC and different MC-subpopulations. The viability which was assessed by trypan blue (0.05%) staining was found to be more than 85% in all cell populations. MC and MC-subpopulations were inoculated with the virulent 89V87 ADV strain at a MOI 20.

Percentage of cells with ADV antigen expression. Eighteen hours PI, smears were made of MC and the different MC subpopulations. The smears were dried, fixed in acetone and stained with a direct immunofluorescence method, using an FITC-conjugated hyperimmune ADV-antiserum.

Results

Determination of susceptible MC subpopulation in vivo

The frequency of detection of cell-free or MC-associated ADV is given in Table 1. A viremia was demonstrated in 7 out of 9 ADV-inoculated pigs. MC-associated ADV was detected on a single day in pigs A, C, D and E; on two days in pigs B and G and on four days in pig F. The number of infected MC ranged between 1 and 5 per 10^8 examined cells in 8 samples but reached up to 33 in pig B on 2 days PI, 26 in pig A on 3 days PI and 58 and 2000 in pigs C and G respectively on 5 days PI.

After separation of MC into lymphocytes and monocytes and cocultivation of these MC subpopulations on 3 and 5 days PI, both infected monocytes and lymphocytes were found in pig A on 3 days PI and in pig C on 5 days PI whereas only infected monocytes were found in pig G on 5 days PI. The number of infected cells on a total of 10^8 cocultivated monocytes was 62 in pig A, 500 in pig C and 14 in pig G. The number of infected cells on a total of 10^8 cocultivated lymphocytes was 14 in pig A and 100 in pig C.

Table 1

Cell-free and mononuclear cell-associated viraemia after an oronasal inoculation of pigs with $10^{8.0}$ $TCID_{50}$ ADV

Pig	* Cell-free viraemia at PI day ■ Mononuclear cell associated viraemia at PI day				
	1	2	3	5	7
A			* ■	Dead	
B	* ■	* ■		*	
C	NT	NT	*	* ■	Dead
D				* ■	*
E	■		Dead		
F		■	■	* ■	* ■
G				■	■
H					
I					

NT: not tested; examined quantities: * 2 ml plasma
■ 10^8 mononuclear cells (cocultivation)

Determination of susceptible MC subpopulation in vitro

The results of the inoculation of the different MC subpopulations with ADV are summarized in Table 2. Monocytes were the most susceptible subpopulation to an infection with ADV. A difference was noticed in percentage of ADV-infected cells depending on the separation technique. When monocytes were separated on plasma-coated polystyrene, 21% were infected. Only 2% were infected when monocytes were separated with the indirect immunopanning technique. T-lymphocytes and the subpopulation consisting of both B- and T-lymphocytes contained 0.5 and 0.4% of infected cells respectively. In the enriched B-lymphocyte subpopulation 4.3% were shown to be infected.

Table 2

Percentage of infected cells in subpopulations of mononuclear cells after an *in vitro* inoculation with ADV at M. O. I. 20

Separation technique	Enriched cell type	% purity **	% ADV infected* **
none	mononuclear cells	–	9.0
direct immunopanning + plasma-adherence	B-lymphocytes	75	4.3
	T-lymphocytes	90	0.5
	monocytes	98	21.0
indirect immunopanning	T- and B-lymphocytes	83	0.4
	monocytes	72	2.0

* based on immunofluorescence; ** average of three experiments

Discussion

The results of the present study demonstrate that ADV replicates in blood mononuclear cells both *in vivo* and *in vitro* and that the monocyte is the most susceptible cell type.

As in previous studies of Wittmann et al. (1980), Wang et al. (1988) and Page et al. (1992), it was found that a low number of infected MC is frequently detected in acutely infected pigs. However, some discrepancies exist on the susceptibility of the different subsets of blood MC *in vivo*. Wittmann et al. (1980) found infected cells in the subpopulation enriched for lymphocytes but not in selected adherent monocytes. This is in contrast with the present report, where five

times more monocytes were infected than monocyte-depleted cells which mainly consist of lymphocytes. Because three times less monocytes were cocultivated than lymphocytes and the number of infected lymphocytes was on the detection limit, it is possible that the low number of monocytes tested by Wittmann et al. (1980) may not have enabled them to detect infected cells in the monocyte enriched cell population.

Viraemia may occur early after inoculation of ADV. In the present study and in the study of Wittmann et al. (1980), cell-associated and cell-free virus were demonstrated in some pigs as soon as 24 hours PI. Although it is unknown precisely how ADV gets that quickly into the blood circulation, some evidence exists that this occurs by cell-to-cell spread through the epithelium of the respiratory tract and underlying connective tissue to reach the endothelium in less than 24 hours. This infected endothelium may be then the source of cell-free ADV in the blood and the place where blood monocytes may become infected. Such a high invasion capacity of a virulent ADV strain has previously been reported by Pol et al. (1989). They found viral antigens of the virulent NIA_3 ADV-strain at 24 hours PI in groups of neighbouring cells, not only in the nasal epithelium but also in the underlying connective tissue with numerous capillaries.

As in the *in vivo* study, the main subpopulation of peripheral blood MC that was infected with ADV *in vitro* also consisted of monocytes. The percentage of infected cells in the subpopulation of enriched monocytes strongly differed according to the technique of isolation. When monocytes were isolated on plasma coated dishes, ten times more cells were found to be infected than when monocytes were isolated by an immunopanning technique. Several possible causes have to be considered for this difference in permissiveness for an infection with ADV. The purity of the enriched monocytes may have had an effect but cannot be imputed as the only cause since the percentage of monocytes differed only by 20%. The monoclonal antibody 74.22.15 used in the indirect immunopanning technique did not affect the susceptibility to infection. This was demonstrated in a supplementary experiment. Plasma-isolated monocytes which were incubated with the monoclonal antibody 74.22.15 were as susceptible as non-incubated control monocytes. It is also possible that the permissiveness may be affected by a phenotypic change induced during the isolation technique. This has earlier been described for another member of the *Alphaherpesvirinae*, herpes simplex virus (HSV) (Albers et al., 1989). Human monocytes were refractory to HSV-infection when they were separated by counterflow centrifugal elutriation and inoculated in suspension. After plastic adherence these monocytes underwent spontaneous differentiation and showed an increased susceptibility to infection with HSV. The two techniques for the separation of porcine monocytes may also have induced two stages of differentiation resulting in a difference in susceptibility to an infec-

tion with ADV. Other explanations such as the isolation of different monocyte subpopulations during the two isolation techniques can, however, not be excluded.

From the results of the present study and the reports of others (Wang et al., 1988; Page et al., 1992), it was shown that a very limited number of resting lymphocytes were susceptible to an infection with ADV *in vitro*. The identity of the 0.5% of cells which were infected in the subpopulation of T-lymphocytes and the 0.4% of cells which were infected in the subpopulation consisting of both t- and B-lymphocytes may be of two origins: contaminating monocytes or a susceptible subset of lymphocytes. Such a subset of lymphocytes may exist when one considers that after mitogen-stimulation, the number of infected T-lymphocytes considerably increases (Wang et al., 1988 and Page et al., 1992). Surprisingly, many infected cells were detected in the subpopulation of enriched B-lymphocytes. Because six per cent of the enriched B-lymphocytes consisted of monocytes, it is not unlikely that the ADV-infected cells were monocytes.

Because monocytes represent the MC which are most permissive to an infection with ADV, they will be examined as possible carrier cells for transporting ADV in blood to the uterus. In this regard, future studies will focus on replication of ADV in blood monocytes and, moreover, on possible interactions of infected monocytes with endothelial cells.

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ROLE OF gp63 AND gIII OF AUJESZKY'S DISEASE VIRUS IN THE INVASION OF THE OLFACTORY NERVOUS PATHWAY IN NEONATAL PIGS

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The purpose of this study was to examine in which way envelope glycoproteins gp63 and gIII of Aujeszky's disease virus (ADV) are involved in neuropathogenesis in pigs. The Ka strain of ADV and its single deleted mutants were examined with respect to invasion and spread in the olfactory nervous pathway after intranasal inoculation in neonatal pigs. The olfactory mucosa, olfactory bulb and lateral olfactory tract representing the 1st, 2nd and 3rd neuronal level of the olfactory pathway respectively, were examined for virus by isolation and for antigen by immunocytochemical localization. The Ka undeleted strain and its gIII deleted mutant invaded and spread to all the neuronal levels in a similar way. The gp63 deleted mutant invaded and spread in the olfactory mucosa similarly to the Ka parental strain but its replication and degree of spread were diminished in all the levels of the central nervous system (CNS). It is, therefore, concluded that gp63 is involved in the neurotropism of ADV, while gIII is not.

Key words: Aujeszky's disease virus, envelope glycoproteins, neuropathogenesis, pig

Aujeszky's disease virus (ADV) is a virus that causes nervous signs in neonatal pigs. Helpful tools for the study of the viral neuropathogenesis are, nowadays, mutants deleted in specific viral glycoproteins. These mutants were inoculated so that the exact role of the deleted part could be evaluated. In this way, it was found that a deletion of either gI, gp63 or gIII glycoproteins reduces or completely abolishes the neurological signs of ADV in 3- and 10-week-old pigs (Mettenleiter et al., 1987*b*, 1989; Kimman et al., 1992). More specifically, a gI$^-$ mutant can replicate in peripheral tissues but not in tissues of the central nervous system (CNS) and thus it was suggested that gI plays a role in the tissue tropism of the virus for the nervous system (Kimman et al., 1992).

Examination of gIII$^-$ and gp63$^-$ mutants with respect to their capacity to invade and spread in the nervous system of the pig has not been performed up to now. Such an examination could provide more information on the importance of these glycoproteins in viral neuropathogenesis. For this reason, the virulent Ka strain and its genetically engineered derivatives Ka gp63$^-$ and Ka gIII$^-$ were com-

pared with respect to their ability to induce neural invasion and spread. The study was performed in the olfactory nervous pathway because this is one of the important ways for ADV to reach the CNS of the pig in the natural infection (Sabo et al., 1969; Baskerville et al., 1973; Wittmann et al., 1980).

Schematic diagram of the olfactory pathway. A schematic diagram of this pathway is presented in Fig. 1. For more information, the reader is referred to Brodal (1969). The *1st neuronal level* of this pathway consists of the olfactory (neuroepithelial) cells which are located amongst the other non-neural cells of the olfactory epithelium. The dendrites of the olfactory cells extend up to the nasal cavity, while their axons cross the lamina propria and enter the olfactory bulb. In the olfactory bulb, the axons of the olfactory cells end up in the glomeruli, forming synapses with the dendrites of the mitral neurons. The latter neurons represent the *2nd neuronal level* of the pathway. The axons of the mitral neurons leave the olfactory bulb and end up in various structures, one of which is the lateral olfactory tract (LOT). The neurons of this structure represent the *3rd neuronal level* of the olfactory pathway.

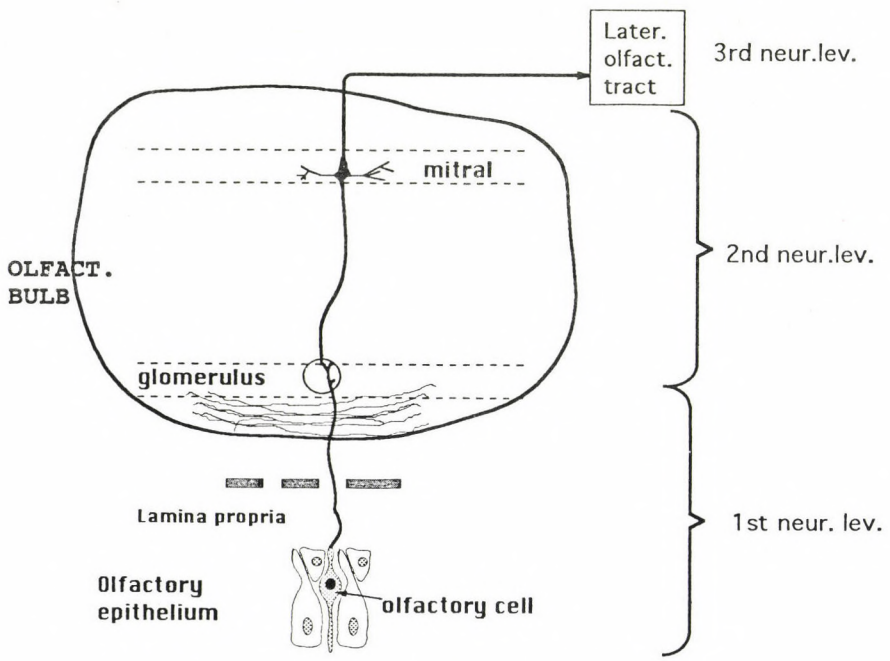

Fig. 1. Schematic diagram of the olfactory pathway

Materials and methods

The Ka parental strain and its single deleted mutants Ka gp63 and Ka gIII were kindly provided by Dr. Mettenleiter. The origin of the parental strain and the construction of the mutants described in detail elsewhere (Kaplan and Vatter, 1959; Mettenleiter et al., 1987*a*; 1988).

Six 1-week-old seronegative pigs were inoculated, 2 with each virus strain. Inoculation was performed with $10^{7.0}$ $TCID_{50}$ intranasally, half of the quantity in each cavity. For inoculation, a catheter was inserted in each nostril up to the level of the ethmoidal labyrinth. The piglets were kept in a backwards position for 5 min to ensure contact with the olfactory epithelium. They were observed for the appearance of symptoms and were killed at 5 days post inoculation (DPI). The olfactory mucosa, olfactory bulb and LOT were collected and processed for virus quantitation and immunocytochemical examination.

For virus quantitation, 20% suspensions were made from tissues in phosphate buffered saline (PBS). The suspensions were centrifuged at 3,000 rpm for 20 min at 4 °C. Subsequently, 0.1 ml, 10-fold dilutions of the supernatant were inoculated onto confluent swine testicle (ST) cells. The cell cultures were observed for 5 days for cytopathic effect. For immunocytochemical (ICC) examination, tissue samples were alcohol (50%) fixed for 5 days and embedded in paraffin. Sections were cut at different sites equally distributed so that a tissue depth of 0.5 to 1 cm was covered. After deparaffinization of the sections, the indirect peroxidase staining method was performed using a rabbit polyclonal serum against ADV in the first step and goat anti-rabbit immunoglobulins conjugated with peroxidase (Dakopatts, Denmark) in the second step. Visualization was performed with H_2O_2 and 3,3'-diaminobenzidine tetrachloride (Sigma Chemicals, St Louis, MO, USA). The sections were counterstained with haematoxylin, dehydrated, cleared in xylene, mounted in DPX and examined under the microscope.

Results

Clinical signs. The pigs inoculated with the Ka strain and the Ka gIII$^-$ mutant showed anorexia and developed vertigo and vomiting. Around 4 DPI, the pigs were found sitting on their hind legs ("dog sitting"), needed support from the wall and kept their heads down. When placed standing, they moved backwards while keeping their heads low, or when falling in a lateral position, they showed paddling movements. None of the pigs inoculated with the Ka gp63$^-$ mutant showed neurological signs.

Virus quantitation. As can be seen from the results of virus quantitation in Fig. 2, the Ka strain was isolated at high titers at all the neuronal levels of the olfactory pathway. With this strain, titers have a tendency to decrease with increasing neuronal level. Both the ka gp63$^-$ mutant and the Ka gIII$^-$ mutant invaded all the neuronal levels. Compared to the parental Ka strain, the Ka gIII$^-$ mutant had lower titers but a parallel decrease was observed with increasing neuronal level. The Ka gp63$^-$ mutant had titers similar to the Ka strain in the olfactory mucosa (0 and 1st neuronal levels) but titers at the 2nd and 3rd neuronal levels were much lower. Also, the decrease in titers with progressing neuronal level was much more pronounced than with both the Ka and Ka gIII$^-$ strains.

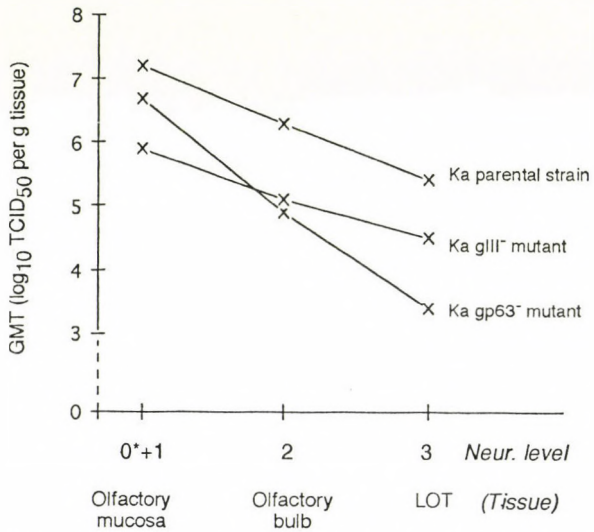

Fig. 2. Virus titers of Ka strain of ADV and of its single deleted mutants in the tissues of the olfactory nervous pathway of neonatal pigs at 5 days post inoculation. GMT = geometric mean titer of 2 pigs. *0 = non-neuronal cells of the olfactory mucosa

Antigen localization. The results of antigen localization are presented in Tables 1 and 2.

In the olfactory mucosa (Table 1), the Ka strain infected the epithelium both as single cells and as large (>0.5 mm) foci. Desquamated cells were also observed. In the lamina propria, most foci of infection were in contiguity with foci of epithelium and reached the underlining bone or the cartilage. Infected nerve fibers and nerve bundles were observed both in epithelium and lamina propria.

Table 1

Antigen localization in the olfactory mucosa with the Ka strain of ADV and its deletion mutants in neonatal pigs at 5 days post inoculation as was determined by immunocytochemistry

Virus strain	No.* of infected foci in				No. infected nerve bundles
	Olfactory epithelium		Olfactory lamina propria		
	S**	L**	S	L	
Ka parental	15	>30	17	>30	>100
Ka gp63$^-$	14	>30	13	>30	>100
Ka gIII$^-$	17	>30	16	>30	>100

* Average of 2 pigs
** Small =< 0.5 mm; Large => 0.5 mm

Table 2

Antigen localization with the Ka strain of ADV and its deletion mutants in the tissues of the olfactory pathway of neonatal pigs at 5 days post inoculation as was determined by immunocytochemistry

Virus strain	Number* of infected neurons in	
	Olfactory bulb Mitral (neurons)	Lateral olfactory tract Pyramidal (neurons)
Ka parental	2000	>7000
Ka gp63$^-$	40	800
Ka gIII$^-$	3100	>7000

* Evaluation was performed in 15 and 10 sections of olfactory bulb and lateral olfactory tract respectively. Each number represents the average of 2 pigs

Both the pattern and the extent of infection with the Ka gIII$^-$ and Ka gp63$^-$ mutants were basically similar to those observed with the Ka parental strain. In the olfactory bulb (Table 2), the Ka strain had infected mitral cells both in a focal and in a scattered manner. The Ka gIII$^-$ mutant showed an infection pattern in the olfactory bulb similar to that of the parental strain but the mutant had infected 1.5 times more mitral neurons. The Ka gp63$^-$ mutant had infected 50 times less mitral neurons than the Ka parental strain. These neurons were infected mainly in a

scattered manner. In LOT (Table 2), many pyramidal neurons were infected with both the Ka strain and the Ka gIII$^-$ mutant. These neurons were infected both in a focal and scattered form. The infection pattern with the Ka gp63$^-$ mutant was similar to that observed with the Ka parental strain but the extent of the infection was reduced.

Discussion

Neurovirulence is an important characteristic of ADV both in pigs and in other animal species. Several virus functions have been suggested to influence neurovirulence in pigs such as the enzyme thymidine kinase (TK) and the envelope glycoproteins gI, gp63 and gIII (Kimman et al., 1992; Kit et al., 1985; McGrecor et al., 1985; Mettenleiter et al., 1987*b*, 1989). However, the exact role of these proteins in the viral neuropathogenesis is not fully understood. The most extensively studied protein is TK which is an enzyme necessary for the replication of the virus in nondividing cells such as cells of the nervous tissue (Jamieson et al., 1974). gI was suggested to play a role in determining the tropism of ADV for the nervous tissue since a gI$^-$ mutant can be recovered from several organs, but not from the CNS. The exact mechanism in which gI is involved is not known but it was postulated that gI facilitates the spread from neuron to neuron (Kimman et al., 1992). The mechanisms and the degree of involvement of gp63 and gIII in neurovirulence are not known yet. The present study showed that the Ka gp63$^-$ mutant invaded all the neuronal levels of the olfactory pathway but its replication was affected in the tissues of the CNS. These observations were confirmed by the ICC studies in which a defective spread of the Ka gp63$^-$ mutant in neural tissues was shown. These findings clearly suggest that gp63 plays a neurotropic role in ADV neuropathogenesis. The exact mechanism by which gp63 is involved in the neurotropism of ADV was not clear from the present study. It is known that gp63 and gI form a complex and that these two glycoproteins share similar functions, at least in cell cultures and in some animal species (Whealy et al., 1993; Zuckermann et al., 1988). Therefore, it is possible that the neurotropic role of gp63 can be explained in a similar way as that of gI, e.g. a role in transneuronal transport of the virus. The Ka gIII$^-$ mutant invaded all the neuronal levels of the olfactory pathway. The spread of this mutant was similar to that observed with the Ka parental strain.These findings indicate that gIII does not play a role in the neuropathogenesis of ADV for 1-week-old pigs. It should be noticed that, in the present study, lower titers were observed with the Ka gIII$^-$ mutant at all the neuronal levels compared to those observed with the Ka parental strain. It is known that gIII mutants, as compared to the parental strain, are detected less efficiently

in cell cultures and thus in lower titers due to a defective adsorption (Mettenleiter et al., 1988). Therefore, it is likely that the lower infectivity titers observed with the Ka gIII$^-$ mutant are not due to lower virus production in the examined neural tissues, but to a less efficient detection system. Our results with regard to the role of gIII in the neuropathogenesis of ADV are not fully consistent with those of Mettenleiter et al. (1989). The latter authors observed a reduced neurovirulence of the Ka gIII$^-$ mutant in 3-week-old pigs, a finding which suggests that gIII may be involved in the neuropathogenesis of ADV but that this involvement is age dependent in the pig. The investigation on the role that glycoproteins play in neuroinvasion is not only important for the better understanding of the pathogenesis of ADV, but also for the evaluation of vaccines. Mutants deleted in glycoprotein have recently been used for the purpose of vaccination. However, these mutants must lack neuroinvasiveness/neurovirulence. The present study indicates that, with regard to the tissue tropism for the CNS, gp63 mutants appear to be safer than gIII mutants.

Acknowledgements

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LATENT INFECTION IN A BOAR 6.5 YEARS AFTER EXPERIMENTAL INFECTION WITH AUJESZKY'S DISEASE VIRUS

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Latent Aujeszky's disease virus (ADV) infection in swine was described in the mid-seventies (Sabó and Rajčáni, 1976) and later was induced by experimental infection of pigs pretreated with an inactivated vaccine (e.g. Wittmann et al., 1983). Latent infections were also demonstrated in two experimentally infected pigs in our laboratory (Valíček et al., 1984). One of them was a non-vaccinated animal and ADV was detected in cultured fragments of the Gasserian ganglia (G.g.) three months after the infection. The other one was immunized with an inactivated vaccine and ADV was detected in the G.g. 186 days after challenge with the ADV strain DK-M. In the latter case, the infection was reactivated by immunosuppressive treatment with dexamethazone. The experiment presented here was aimed at the demonstration of prolonged or even lifelong latent Aujeszky's disease in pigs infected with the strain DK-M by means of cultured explants of the G.g.

Materials and methods

A male 2-month-old weanling, free of antibodies to ADV, was infected orally with 10^7 $TCID_{50}$ of the strain DK-M. One sentinel porker, free of antibodies to ADV, was brought into contact with the infected male 1 month after the infection, followed by another sentinel at 70 months. All three pigs (one infected and two sentinel controls) were examined for the presence of antibodies to ADV by neutralization test or ELISA throughout the whole experimental period. The infected male was slaughtered 6.5 years after the infection and explants of G.g. were maintained in Petri dishes in Eagle's MEM supplement with 10% of fetal bovine serum and 4.5 g of glucose per 1 l in a carbon dioxide incubator. The explants were maintained in the medium for 28 days. The medium was replaced 24 h after seeding and subsequently on alternate days. Cultures of RK-13 cells were inoculated with samples of the medium collected from the cultured explants. After 15 to 18 days of culture, explants positive for ADV by isolation in RK-13 were fixed in 3% glutaraldehyde and 1% osmium tetroxide and prepared for electron microscopic demonstration of the virus by the ultrathin sections technique.

Results

In the infected male, titres of antibodies neutralizing ADV varied between 64 and 256 during the 6.5 years after the infection, reaching the value of 128 at the time of slaughter.

Sentinel pigs remained free of antibodies to ADV throughout the whole observation period.

Of the 38 Petri dishes with cultured explants of the G.g. collected from the infected boar 6.5 years after the infection, ADV was detected in one at six days and in one after six, eight and eighteen days of culture, respectively.

ADV nucleocapsids and virions were demonstrated by electron microscopy in ultrathin sections of fragments of the Gasserian ganglion on culture days 15 and 18.

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DEVELOPMENT OF ACTIVE IMMUNITY IN NEWBORN PIGS WITH COLOSTRAL ANTIBODIES BY VACCINATION WITH gIII-DELETED PRV

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Maternal antibodies interfere with active immunization of swine by gI-deleted pseudorabies virus [(PRV); Aujeszky's disease virus] vaccines. To test the hypothesis that modified-live (MLV) vaccines retaining the PRV gI and with deletions in the PRV glycoprotein gIII and thymidine kinase (TK) genes might be efficacious in circumventing colostral antibody interference, the OMNI-MARK-PRV (gI^+ $gIII^-$ TK^-) vaccine was administered intramuscularly to 13 newborn pigs with colostral antibodies, while 10 pigs from the same litters served as nonvaccinated controls. At 49 days of age, when PRV virus neutralization (VN) antibodies were negative and all nonvaccinated pigs as well as 10 vaccinates were latex agglutination test (LAT) -negative, the pigs were challenged intranasally with the virulent PRV(SHOPE) strain. In support of the hypothesis, it was found that severe central nervous system and respiratory disease signs developed in 6 of 10 nonvaccinates, with one fatality, while 2 of 13 vaccinates showed only very mild and transient disease signs. Nonvaccinates lost weight until post challenge day (PCD) 6, did not regain prechallenge weight until PCD 8, and at PCD 11 had gained only 4.9 pounds/pig. Vaccinates gained weight after challenge and at PCD 11 showed a 9.4 pounds/pig weight gain. On PCD 11, the geometric mean titer (GMT) for VN antibodies of the nonvaccinates was 9.3, while the GMT of the vaccinates for VN antibodies was 49.0, showing that vaccinated pigs had been immunologically primed.

Key words: Pseudorabies, vaccination, gIII-deleted PRV, newborn pigs, maternal antibody interference

In regions where vaccination is widely practiced to control Aujeszky's disease, most newborn piglets have colostral antibodies against Aujeszky's disease virus [pseudorabies virus (PRV)]. These passively acquired antibodies reduce the incidence and severity of clinical disease and reduce shedding of virulent PRV once infection has occurred, but may not prevent the establishment of reactivateable latent infections and the carrier state. Furthermore, van Oirschot et al. (1991, 1992) have shown that pigs with high levels of maternal antibodies may not seroconvert to specific PRV marker antibodies after virulent virus infection, so that virulent PRV infection may not be detected. More important, maternal antibodies

can interfere with active immunization. As demonstrated by Kojnok many years ago, colostral antibodies in newborn pigs can show wide variation between littermates. A major impediment to active immunization of young pigs is the difficulty of forecasting the decline of colostral antibodies. The correct timing of active immunization is now an educated guess and is impacted by cost prohibitive laboratory analyses. Yet, correct timing is essential for optimizing subsequent vaccination schedules and minimizing the window of opportunity for virulent PRV infection. Thus, it is not surprising that vaccination schedules have varied considerably.

Table 1

Vaccination strategies using gI-deleted or gX-deleted PRV Vaccines

Vaccination of sows	Vaccination of growing pigs	Reference
Sows vaccinated biannually (gX-deleted vaccine)	IM vaccination at 4+10 weeks of age	Lehman et al., 1993
Sows vaccinated quarterly (gI-deleted vaccine)	IM vaccination at 8+12 weeks of age	Lehman et al., 1993
Sows vaccinated 3 times/year (gI-deleted vaccine)	IM or IN vaccination at 10 to 12+14 to 16 weeks of age	Kimman, 1992; de Smet et al., 1992; van Oirschot, 1991, 1992
Sows vaccinated twice before farrowing (gI-deleted or gX-deleted vaccines)	IM or IN vaccination at 4 to 6 weeks of age	Arellano et al., 1992

At the present time, the most widely used MLV PRV vaccines worldwide are gI-deleted vaccines. To induce colostral antibodies, many veterinarians vaccinate sows with these vaccines 3–4 times each year (Table 1). Other veterinarians utilizing gX-deleted PRV vaccines immunize sows biannually. The offspring of these vaccinated sows are usually vaccinated at least twice, beginning when the young pigs are either 4, 8, or 10 weeks of age (Table 1). This delay in the start of active immunization is necessary to allow for the decline in VN titers of interfering colostral antibodies. In addition, some veterinarians administer booster vaccinations to pregnant sows with inactivated PRV vaccines in mineral oil adjuvants to maximize colostral antibody titers and passive immunity, a procedure which tends to accentuate the maternal antibody interference problem. De Smet et al. (1992)

and Vannier (1985) have shown that interference by maternal antibodies with active immunization is more pronounced in pigs suckled by mothers vaccinated with inactivated and/or subunit vaccines which induce high levels of VN antibodies than in pigs suckled by mothers vaccinated with MLV vaccines, which induce relatively low levels of VN antibodies. However, the active protection obtained with a MLV vaccine was similar to that observed with an inactivated vaccine (Vannier, 1985).

Rationale for experiments

Recent molecular biology studies suggest that it might be worthwhile to re-examine current vaccination strategies, particularly in herds where the prevalence of PRV has been reduced. Three separate but related questions may be asked. First, is it really necessary to maximize colostral antibody titers by multiple vaccinations of sows? Second, can we prime piglets with colostral antibodies by actively immunizing them at birth? And third, is the vaccine marker relevant to the maternal antibody interference problem?

The rationale for asking these questions comes from recent tissue culture studies and from experiments in passively immunized mice (Zsák et al., 1992). Mice passively immunized with PRV antibodies were much better protected from lethal infection by gI-deleted PRV than from lethal infection by wild-type or gIII-deleted PRV. This is because gI-deleted PRV spreads mainly by a process in which viruses released from infected cells are absorbed to neighboring and to distant cells, a process relatively sensitive to gII, gIII, and gp50 antibodies. However, glycoprotein gIII-deleted PRV spreads mainly by a direct cell to cell fusion process which is relatively antibody-insensitive (Hanssens et al., 1993; Whealy et al., 1988; Zsák et al., 1989, 1992). Glycoprotein gI has an accessory role in promoting cell fusion and inhibiting the release of PRV from cells, while glycoprotein gIII promotes the release of PRV from infected cells and has an important role in the initial attachment of virus and virus-infected cells to heparan proteoglycan receptors on the surface of target cells.

We observed that the addition of PRV antisera to cultured cells at 4, 24, or 48 h after infection inhibited the cytopathic effects (CPE) produced by a gI-deleted PRV (Syntrovet PRV/Marker-Gold) much more than the CPE produced by wild-type PRV or by a gIII-deleted PRV. The gIII-deleted Omnimark-PRV virus (Table 2) used in these experiments is a syncytia-forming PRV strain, while the gI-deleted PRV induces typical herpesvirus Cowdry type A intranuclear inclusion bodies and a cell-rounding type of CPE. The studies cited and our own results moti-

vated us to extend Zsák's experiments (1992) on passively immune mice to piglets passively immunized with maternal antibodies.

Table 2

Effect of antisera* addition on the CPE produced by PRV deletion mutants on infected monolayer cultures

HR PI antisera added	HR PI cells strained	Syntrovet PRV/Marker Gold $gI^- gX^- gIII^+ TK^-$		OMNIMARK-PRV $gI^+ gX^+ gIII^- TK^-$		Wild type PRV $gI^+ gX^+ gIII^+ TK^+$	
		No antisera	Plus antisera	No antisera	Plus antisera	No antisera	Plus antisera
24	96	+++	<+	+++	++ to +++	++++	++++
48	96	++++	++	++++	+++	++++	++++
4	24	++	+	+++	++ to +++	++++	++++
4	48	++	+	+++	+++	++++	++++

* PRV field sera (VN=1:128) diluted 1:10 prior to application to cultured cells

Experimental design

The design of the experiments was as follows. Four multiparous pregnant sows were vaccinated with our OMNIMARK-PRV $gIII^- gI^+ TK^-$ MLV vaccine at 4 weeks and 2 weeks prior to farrowing. Then, 13 piglets with colostral antibodies, randomly chosen from each of the 4 litters were vaccinated intramuscularly (IM) at 1–3 days of age with the same OMNIMARK-PRV vaccine. Ten piglets from 3 of the litters served as nonvaccinated controls. At 49 days of age, when the VN titers of the maternal antibodies and the latex agglutination test (LAT) antibodies of the control pigs were negative, all control and vaccinated pigs were challenge exposed intranasally (IN) to the virulent SHOPE strain of PRV at a dose of 10^5 $TCID_{50}$, a challenge dose similar to that used by other investigators. The pigs were observed twice each day for clinical signs of disease. Weight changes were recorded on post challenge days (PCD) 3, 6, 9, and 11. Sera were collected prior to vaccination of the newborn pigs, prior to challenge, and on PCD 11, when the experiment was terminated. Three observations indicated that OMNIMARK-PRV vaccination of newborn pigs with colostral antibodies was indeed efficacious.

Table 3

Antibody titers of pigs from Ommimark vaccinated sows and challenged at 49 days of age

Group	Pig no.	Maternal antibodies at 1–3 days			49 days (0 PCD)			60 days (11 PCD)	
		1/VN	LAT	gIII	1/VN	LAT	gIII	1/VN	gIII
	1–1	<2	+*	–**	<2	–	–	16	+
V	1–2	4	+	–	<2	+	–	16	+
A	1–3	4	+	–	<2	–	–	16	+
C	2–1	4	+	–	<2	–	–	64	+
C	2–3	4	+	–	<2	–	–	128	+
I	2–4	<2	+	–	<2	+/–	–	32	+
N	2–5	<2	+	–	<2	–	–	256	+
A	2–6	<2	+	–	<2	–	–	256	+
T	3–1	8	+	–	<2	–	–	64	+
E	3–2	4	+	–	<2	–	–	64	+
S	3–3	4	+	–	<2	–	–	128	+
	3–7	8	+	–	<2	–	–	16	+
	4–1	8	+	–	<2	+	–	16	+
	1–4	4	+	–	<2	–	–	8	+
C	1–5	8	+	–	<2	–	–	4	+
O	1–6	4	+	–	<2	–	–	8	+
N	2–8	<2	+	–	<2	–	–	dead	NA
T	2–9	8	+	–	<2	–	–	8	+
R	2–10	8	+	–	<2	–	–	16	+
O	2–11	8	+	–	<2	–	–	16	+
L	2–12	<2	+	–	<2	–	–	16	+
S	3–5	4	+	–	<2	–	–	8	+
	3–6	4	+	–	<2	–	–	8	+

* Litters from sows no. 290, Y150, Y167, and Y160, respectively were designated 1–1 to 1–6, 2–1 to 2–12, 3–1 to 3–7, and 4–1; **(+)=positive; (–)=negative; NA=not available

Results

At birth, the colostral VN antibody titers of control and vaccinated pigs varied from <1:2 to 1:8 (Table 3). However, all of the newborn pigs were LAT-positive and, as expected, gIII ELISA-negative. The VN antibody titers are similar

to the titers found by others who vaccinated sows with MLV vaccines in the third trimester of pregnancy (Andries et al., 1978; Vannier, 1985; Wardley and Berlinski, 1988). Although low, these VN antibody titers can protect pigs from clinical signs of disease caused by field virus infection. Andries et al. (1978) found in their experiments that: "There was no definite correlation between the individual antibody titer of a pig and its clinical protection. Some seronegative pigs were also protected, possibly because they have antibody not detectable by SNT (or other factors)".

In our experiment, all pigs at 49 days of age were VN-negative and gIII-ELISA-negative, and all control pigs were LAT-negative. However, 2 vaccinates were LAT+ and one pig was LAT+/–, suggesting that low levels of PRV antibodies may have been induced by vaccination.

At 11 DPC, nonvaccinated pigs exhibited VN antibody titers of 1:8 to 1:16 and all nonvaccinated pigs seroconverted to gIII-ELISA antibodies. Vaccinated pigs were also gIII ELISA-positive. However, they exhibited VN antibody titers of 1:16 to 1:256. The geometric mean titer of VN antibodies of vaccinates was 49.0 compared with a GMT for controls of 9.3, showing that an anamnestic response had been induced in vaccinates. This secondary immune response demonstrates, first of all, that the vaccinates had been immunologically primed.

Second, 6 of 10 control pigs showed severe CNS signs and/or various degrees of respiratory disease signs with a fatal outcome in one pig. In contrast, only 2 of 13 vaccinated pigs showed very mild and transient CNS or respiratory disease signs and both pigs recovered rapidly. Vaccinated and control pigs experienced a similar rise in rectal temperature with bimodal spikes. Fall in temperature was similar between groups except that the temperature of vaccinates fell one day sooner than that of controls.

Third, differences were observed in the growth of vaccinates and controls. The importance of analyzing differences in weight during acute PRV infection has been previously emphasized by Stellman et al. (1989). As shown in Table 4 and Fig. 1, control pigs lost weight until 6 DPC and did not regain their prechallenge weight until PCD 8. Vaccinates gained weight after challenge. Differences in the weight gains between PCD 0 and 3 and between PCD 0 and 6 were very highly significant ($P<0.001$), while the weight differences from day 0–9 and 0–11 were highly significant ($P<0.01$). At PCD 6, vaccinates had gained an average of 2.1 pounds and controls had lost an average of 2.1 pounds. At PCD 11 vaccinates had mean weight gains nearly twice that of controls (9.4 vs 4.9 pounds).

Table 4

Weights of control and OMNIMARK-PRV vaccinated pigs after challenge with virulent PRV (Shope)

Group	Pig no.	Sex	Weight (pounds) on postchallenge day (PCD)				
			PCD 0	PCD 3	PCD 6	PCD 9	PCD 11
	1–1	M*	34.5	36.5	34.0	41.5	44.0
V	1–2	F	27.5	29.0	28.5	34.0	36.5
A	1–3	M	40.5	41.0	40.5	46.0	48.0
C	2–1	F	24.5	25.5	27.5	32.5	35.5
C	2–3	F	19.5	20.0	20.0	23.0	26.0
I	2–4	F	22.5	24.0	24.5	24.5	26.5
N	2–5	F	24.0	25.5	25.0	31.0	33.0
A	2–6	M	22.5	23.5	25.0	28.0	29.5
T	3–1	M	32.0	34.0	35.0	40.5	43.0
E	3–2	F	38.5	42.0	42.5	47.5	50.5
S	3–3	M	25.0	26.0	25.0	31.0	34.5
	3–7	M	26.5	28.0	28.0	32.0	35.0
	4–1	M	25.0	28.0	34.0	40.0	43.0
Mean±SD**			27.88±6.49	29.46±6.85	29.96±6.71	34.73±7.77	37.31±7.85
	1–4	M	34.5	35.5	34.5	39.5	44.0
C	1–5	M	40.0	41.0	40.0	47.0	50.5
O	1–6	M	40.5	39.0	38.5	43.0	46.0
N	2–8	M	28.5	26.5	21.5	Dead+	Dead
T	2–9	F	24.5	23.0	22.0	25.5	22.0
R	2–10	F	16.5	17.0	16.5	20.0	22.5
O	2–11	F	29.0	28.0	27.0	31.0	32.5
L	2–12	M	29.5	30.0	24.5	27.0	31.5
S	3–5	M	37.0	36.5	36.0	38.0	40.0
	3–6	F	28.0	27.0	26.0	30.0	32.5
Mean±SD**			30.80±7.4	30.35±7.57	28.65±8.06	32.50±8.92	35.72±10.08

*M=male, F=female; **Analysis of variance for OMNIMARK-PRV group vs. control group indicated that P values were <0.001 for the weight gains between day (6–0) and day (3–0) and were <0.01 for the weight gains between day (9–0) and day (11–0); +Estimated weight PCD 9 was 24 pounds

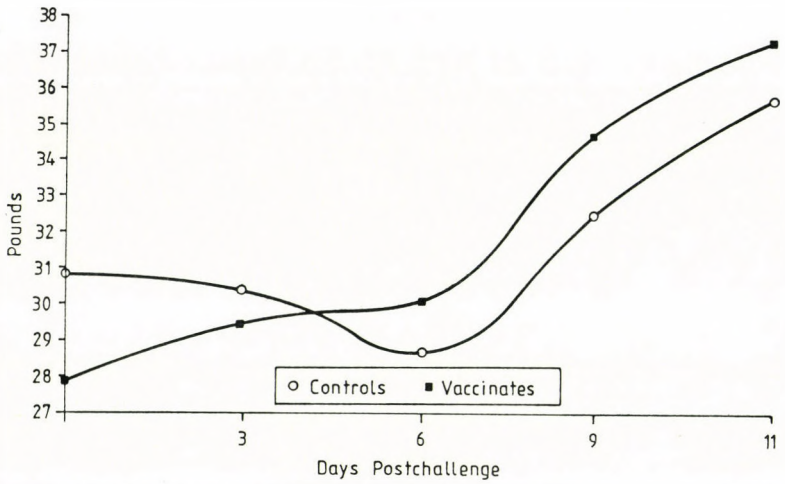

Fig. 1. Mean weight curves of control and OMNIMARK-PRV vaccinated pigs after challenge with virulent PRV (Shope)

Discussion and conclusions

The previous results were obtained with low levels of maternal antibodies. However, Shibata et al. (1993) have recently described experiments using control and OMNIMARK-PRV vaccinated pigs with higher maternal antibody titers (1:8 to 1:128). They reported that after virulent PRV challenge, the vaccinated pigs were clearly protected. The vaccinated pigs did not develop clinical signs of disease, while 2 of 5 nonvaccinated controls showed loss of appetite and extensive nasal discharge. The duration of virulent viral shedding of the vaccinated pigs was reduced compared with the nonvaccinated controls. At PCD 7, the mean weight gains of vaccinated pigs were significantly greater than that of nonvaccinates, and the VN titers of vaccinates had increased rapidly after challenge, indicating that an anamnestic response had been induced. The efficacy of vaccination tended to decrease at the highest maternal antibody titers.

In extending our own studies, experiments have been initiated on newborn pigs with moderate levels of colostral antibodies to compare the efficacy of vaccinating the newborns with the OMNIMARK-PRV ($gIII^- gI^+ gX^+ TK^-$) vaccine versus the Syntrovet PRV/Marker-Gold ($gIII^+ gI^- gX^- TK^-$) vaccine. The results of these experiments will be available in November of 1993.

To our knowledge, the OMNIMARK-PRV experiments cited and the experiments of McCaw and Xu (1993), which involved the use of $gX^- gI^+ gIII^+ TK^-$ PRV vaccine, are the only recent studies on the efficacy of actively immunizing

newborn pigs with maternal antibodies. McCaw and Xu (1993) intranasally vaccinated the offspring of 3 sows exhibiting PRV antibody titers of 1:8, 1:8, and 1:32, respectively, and challenge infected them at 15 weeks of age with 10^4 $TCID_{50}$ of virulent PRV (Iowa S62/26). The vaccinated pigs that suckled the PRV-immune sows exhibited fever and lost weight for 6 days after virulent PRV challenge, although the fever and weight loss were less than that of nonvaccinated controls. In the study by McCaw and Xu (1993), there appeared to be no difference in protection against weight loss after virulent PRV challenge of pigs suckling sows with serum VN antibodies of 1:8 compared with those suckling sows with serum VN antibodies of 1:32. Thus, the results obtained to date do suggest that it would be worthwhile to investigate more fully vaccination strategies which entail the active immunization of newborn pigs with colostral antibodies and the limits of their effectiveness.

Extensive laboratory experiments and field studies in the USA, New Zealand, and Japan have demonstrated that the gIII-deleted OMNIMARK-PRV vaccine is safe for newborn piglets and for pregnant sows in all stages of gestation (S. Kit and M. Kit, 1991). Indeed, the recent studies by Hanssens et al. (1993) suggest that gIII-deleted PRV is less likely than is gI-deleted PRV to infect the fetuses of pregnant pigs and to induce abortion. The shedding of the gIII-deleted OMNIMARK-PRV from IM-vaccinated pigs has not been detected. In a recent study, seronegative breeding gilts and boars were housed for 30 days in the same building with 6 pregnant sows that had been vaccinated IM with OMNIMARK-PRV. At the conclusion of the study, the 45 sentinel pigs were PRV antibody by LAT.

Many experiments have demonstrated the efficacy of the gIII-deleted OMNIMARK-PRV vaccine in newborn pigs, sheep, cattle, and mice. After 1- to 3-day-old seronegative piglets were immunized with OMNIMARK-PRV, the vaccinated pigs survived challenge infections administered at either 7, 10, or 14 days postvaccination, whereas none of the nonvaccinated controls survived (Sonoda et al., 1993). It may also be noted that anti-gIII differential ELISA test is extremely sensitive and specific and readily differentiated animals infected with virulent PRV from those treated with the gIII-deleted OMNIMARK-PRV vaccine (Jinyma et al., 1991; M. Kit and S. Kit, 1991; Schmitt et al., 1991).

The vaccination strategy recommended for use with the OMNIMARK-PRV ($gIII^-$ gI^+ TK^-) vaccine to control pseudorabies in infected herds consists of the following: (1) saturation vaccination of all pigs in the herd, regardless of their age, sex, serological status or use as breeders; (2) vaccination of all replacement breeders prior to their introduction into the herd; (3) at the discretion of the veterinarian, vaccination of breeding sows annually; (4) vaccination IM of all newborn pigs at 1 to 3 days of age, that is, at the time that they are injected with an iron

dextran supplement; and (5) revaccination of piglets at 8–12 weeks of age. Our vaccination strategy has been in use since July 1991 on some Japanese farms participating in the government's pseudorabies eradication program (Hirose, 1993). The following results have been obtained with the OMNIMARK-PRV vaccine from July 1991 through March 1992 (Sonoda et al., 1993). (1) A drastic reduction in the clinical cases of pseudorabies. (2) In finishing herds in PRV contaminated areas: (a) the mortality rate decreased from 5–6% before vaccination to 1–2% after vaccination; (b) after vaccination, the growth rate improved and pigs were sold about 10 days earlier than before. (3) In breeding herds producing feeder pigs in PRV contaminated areas: (a) before vaccination, cases of stillbirth and miscarriage were numerous probably because of PRV infection. After vaccination the number of stillbirths and miscarriages were greatly reduced; (b) the average litter size increased from 8 to 11 after vaccination. (4) In farrow to finish operations: (a) after vaccination, stillbirths, miscarriages, and deaths of piglets were reduced; (b) despite the reduced number of breeding sows, the number of piglets born increased by several percent compared to the previous year. The number of newborn piglets was determined based on the obligatory hog-cholera vaccination. (5) There was a reduction of respiratory diseases, especially pneumonia caused by *Actinobacillus pleuropneumoniae*. (6) Vaccinated piglets are sold at higher price than non-vaccinated piglets by 1,000 to 1,500 Yen ($8–$12).

In one example of the efficacy of the program, Hirose (1993) showed that on one farm with 93 sows, PRV was eliminated from the sows in 1.5 years, as evidenced by negative LAT and differential gIII-ELISA analyses. In contrast, on another farm in the same area where the OMNIMARK-PRV vaccination strategy of immunizing 1- to 3-day-old pigs was not employed, and the vaccination strategy was varied, many of the pigs remained serologically positive for PRV antibodies.

Finally, it is interesting to note that three kinds of gene-deleted MLV vaccines are currently being employed in the Japanese government's PRV control and eradication program, namely, OMNIMARK-PRV (BioCor) ($gIII^-$ gI^+ TK^-), NOBI-PORVAC (Intervet) (gI^- $gIII^+$ TK^-), and Suvaxyn (Solvay-Duphar) (gI^- $gp63^-$ $gIII^+$ TK^+). Only one kind of vaccine has been allowed in each of the 19 Japanese prefectures currently participating in the eradication program. In the Chiba prefecture, where OMNIMARK-PRV is used, piglets are vaccinated for the first time at 1 to 3 days of age and revaccinated at 8 to 10 weeks of age. In the Ibaraki prefecture where the Solvay-Duphar vaccine is used, piglets are vaccinated at 8 to 10 weeks of age only. In the Kanagawa prefecture where the Intervet vaccine is used, piglets are vaccinated once at 60 days of age and again at 90 days of age. Thus, a natural field experiment is in progress in Japan comparing vaccines and vaccination strategies. It will be interesting to see how each of the vac-

cines and vaccination strategies succeeds in eliminating pseudorabies from the Japanese breeding pigs.

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GENETIC AND STRESS-MEDIATED INFLUENCE ON AUJESZKY'S DISEASE VIRUS INDUCED INTERFERON-α PRODUCTION IN PORCINE LEUKOCYTES

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The ability to produce interferon-α (IFN-α) *in vitro* was measured in blood from 200 F_2-crosses between European wild boar and Swedish Yorkshire pigs, originating from a reference pedigree for gene mapping. A total of 200 pigs of 44 litters, descendent from 4 boars and 22 sows, were stressed by transportation together with non-littermates for 5 h. Blood samples were collected from each individual twice, i.e. immediately before transportation and the day after transportation. IFN-α production was induced in whole blood cultures by a monolayer of fixed, Aujeszky's disease virus infected, porcine kidney cells. In general, the amount of IFN-α produced was significantly lower ($p=0.02$) the day after transportation, although the ability to produce IFN-α showed a large individual variation ($p<0.001$). However, both the levels of IFN-α produced and the decrease after transportation varied between the four parental offspring groups. Also, indications of single genes with significant effects on the ability to produce IFN-α were found. These results confirm a genetic influence on the ability to produce IFN-α. In addition, stress, such as transportation and mixing, may decrease the level of IFN-α produced.

Key words: Interferon-α, pig, genetic influence, Aujeszky's disease virus

Aujeszky's disease virus (ADV) is one of the viruses currently used to study the interferon-α (IFN-α) system in the pig. For that purpose an *in vitro* IFN-α induction model, with ADV infected and glutaraldehyde fixed porcine kidney (PK-15) cells, was established (Artursson et al., 1989) and later modified by Wallgren et al. (submitted). Following exposure to ADV infected PK-15 cells *in vivo*, low but significant amounts of IFN-α are detectable in porcine serum 8 and 24 h post injection (Artursson et al., to be published).

The cell type believed to be responsible for the IFN-α production induced by ADV and some other viruses has been described as "the natural interferon producing cell", NIP-cell (Ito et al., 1981). This cell population has in humans been defined as an infrequent but highly productive mononuclear cell, not expressing any specific T-, B- or NK- cell antigens, but expressing e.g. MHC class I and

II antigens, CD4, CD11a/CD18 and CD36 (reviewed in Charley and Laude, 1992). The porcine NIP-cell has been characterized mainly by Nowacki and Charley (1993) as a non-adherent, non-phagocytic, non-T, non-B cell but expressing CD4. The porcine NIP-cell also expresses MHC-class-II molecules following virus induction. The frequency of NIP-cells in porcine blood was found to be extremely low; 1 out of 10^4 peripheral blood mononuclear cells (PBMC).

In an earlier study of genetic influence on the porcine immune capacity we have used the ADV-induced ability of porcine PBMC to produce IFN-α *in vitro* among other tests as a measure for "immune function" (Edfors-Lilja et al., in press). In this study the mean IFN-α producing capacity showed a large variation between paternal offspring groups, but the estimated heritability was low, $h^2=0.08$. The difference in IFN-α production between litters as well as between individual animals was high. Further results indicating breed differences both in the transmissible gastroenteritis virus-induced IFN yield per cell as well as in the frequency of NIP-cells have been described in the pig by Nowacki et al. (1993). They found that Chinese Meishan pigs of all age groups tested had significantly higher IFN yields per cell, as well as higher NIP-cell frequencies, than the European Large-White pigs.

The unique material provided by the Swedish part of the European PigMap project offered us the opportunity to further elucidate the genetic influence on the IFN-α production in the pig. The first preliminary data on the ADV-induced IFN-α production by PBMC obtained from 200 F_2 crosses (European wild boar x Swedish Yorkshire) are presented in this communication.

Materials and methods

Animals and blood sample collection. The animals used in the present study were the F_2 crosses originating from a reference pedigree for gene mapping (Johansson et al., 1992). They were developed by crossing two European wild boars with eight Swedish Yorkshire sows. Four F_1 boars were used for mating to 22 F_1 sows, generating 44 litters with a total of 200 F_2 individuals. A detailed genetic map comprising about 120 genetic markers have been established for this pedigree (Ellegren et al., submitted). In this preliminary study, 28 genetic markers distributed on 14 chromosomes have been tested for their influence on ADV-induced IFN-α production. At the age of approximately three months (20 kg body weight), the pigs were stressed by transportation together with non-littermates for 5 h and subsequently stalled together with non-littermates. Blood samples were collected from each individual on two occasions, i.e. immediately before transportation and the day after transportation. The blood samples were collected from the

cranial vena cava, using evacuated glass tubes (B-D Vacutainer, Meylan Cedex, France) with 143 USP units of heparin as additive.

IFN-α induction. The heparinized blood samples were diluted ten times in RPMI 1640 medium with 20 mM HEPES buffer, supplemented with glutamine (2mM), penicillin (200 IU/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (5 x 10^{-5} M) and 5% fetal calf serum (FCS; Myoclone, Gibco, Scotland). Two hundred μl volumes of the diluted blood were cultured in quadruplicate wells in microtiter plates with fixed, ADV-infected porcine kidney cells, according to Wallgren et al. (submitted). Wells with fixed, non-infected porcine kidney cells were used as negative controls. After 18 h of culture, 150 μl of the culture supernatant was collected and the IFN-α content was measured in a time-resolved fluoroimmuno-assay for porcine IFN-α (to be described by Artursson et al.).

Statistical analysis. The influence of genetic markers on the ADV-induced IFN-α production was analyzed using analysis of variance and a model including dam, parity of the sow, day of sampling and the effect of the marker. The analysis of the data was carried out with the Statistical Analysis System (SAS Institute Inc., 1985) using the GLM procedure. In addition the data were analyzed for differences between sample collection days, i.e. before and after transportation, using a Wilcoxon signed-rank X method from StatView™ II (©Abacus Concepts, Inc. 1987).

Results

Single gene effects on the ADV induced IFN-α production by porcine PBMC. In a preliminary analysis, significant effects ($p \leq 0.05$) on the levels of IFN-α produced were found for 6 of the 28 genetic markers tested (Table 1).

Stress-mediated effects on ADV-induced IFN-α production by porcine PBMC. In the present study, blood samples were collected before transportation and the day after transportation and installation of the pigs at new premises. In general, the amount of IFN-α produced by the F_2 crosses was significantly lower ($p=0.02$) the day after transportation/mixing (Fig. 1), although the ability to produce IFN-α still showed a large individual variation. However, both levels of IFN-α produced and the decrease after transportation/mixing varied between the four parental offspring groups.

Table 1

Single genes that may influence[+] the ADV-induced IFN-α production by porcine PBMC

Chromosome number	Marker loci
1	S0082, microsatellite
5	S0005, microsatellite
8	S0086, microsatellite
13	CP, ceruloplasmin[++]
15	S0088, microsatellite
16	S0061, microsatellite

[+] Significant effect, $p \leq 0.05$; [++] Ceruloplasmin: a plasma protein

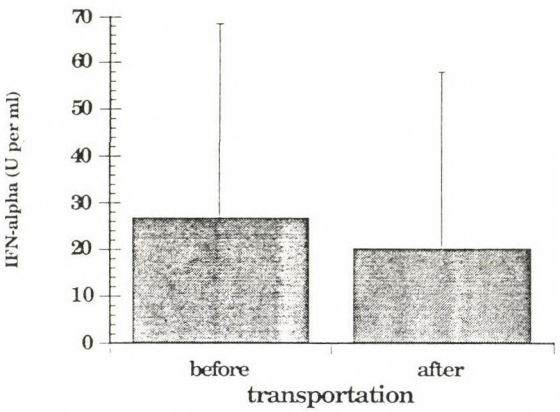

Fig. 1. Aujeszky's disease virus induced IFN-α production in porcine peripheral blood mononuclear cells. The blood was collected from 200 F_2 crosses (European wild boar × Swedish Yorkshire), before and after transportation/mixing. The values are mean values with one standard deviation

Discussion

In a previous study a low heritability for the ADV induced IFN-α production was demonstrated, although a significant difference between paternal offspring groups were found (Edfors-Lilja et al. in press). The present results indicate an influence of single genes on the IFN-α production by porcine PBMC. In mice and humans a 100-fold individual variation in virus induced IFN-α produc-

tion has been described. In the mouse, a number of genes that influence the amount of IFN-α produced per cell has been identified (De Maeyer and De Maeyer-Guignard, 1988). If this is the case also in the pig such gene effects could explain the large individual variation in the amounts of IFN-α produced. It is however, important to emphasize that the genetic markers found to influence the IFN-α response are not expected to have a direct influence on the IFN-α production, but should be regarded as representatives for a chromosomal region harboring one or more genes having an influence on this trait. Further studies of the influence of additional marker loci and chromosomal regions in the porcine genome are under progress, using a more powerful statistical method recently described by Haley et al. (1993).

In "natural stress situations", a decreased IFN-α producing capacity has been demonstrated in pigs eight days after allocation and installation in fattening farms (Wallgren et al., 1993). Similarly, a decrease in the levels of IFN-α produced was observed in piglets three days after weaning (Wattrang et al., 1992). In the present study a decrease in the ADV induced IFN-α production was observed as early as the day after transportation and allocation to new environments. This finding supports a stress-mediated suppression of the IFN-α producing capacity. However, the halothane gene (CRC-locus), classically associated with stress susceptibility in the pig, seemed to have very low, or no, influence on the IFN-α producing capacity.

Taken together, these preliminary data strengthen the earlier results on genetic differences in the IFN-α production in the pig. As the IFN-α producing capacity may play a major role in viral defence of the host, alterations in this capacity would be of importance for the immunity to infections.

Acknowledgements

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SEROLOGICAL DIAGNOSIS OF AUJESZKY'S DISEASE: A COMPARISON BETWEEN REACTIONS OF BLOOD SAMPLES FROM SOWS AND THEIR OFFSPRING

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Blood sera from sows and their piglets were compared for their suitability for the serological diagnosis of Aujeszky's disease. Within a few days after parturition, blood and colostrum samples were collected from a total of 104 sows from 8 different gI-vaccinated breeding herds. Three piglets of each litter were bled simultaneously with their mother and again 3 weeks later. All 416 sera reacted positively in the screening for vaccination-induced antibodies. Using the gI ELISA, the sera of 16 sows and their offspring reacted positively, while 86 sows and their piglets reacted negatively and 2 sows and their piglets showed reactions near the test-cutoff. By testing piglets from another 1,300 sows in 37 farms, the practicability of the proposed method was proved. Blood samples were taken at intervals of 4 to 6 weeks from piglets of all sows, which had farrowed in the meantime. Within a six to nine months period, the serological status of a breeding herd could be established.

Key words: Aujeszky's disease, serological diagnosis, gI ELISA, sow, piglet

In 1991 North Rhine Westphalia started an ADV eradication program. Two years of vaccination of all swine with gI-deleted vaccines are followed by serological testing and slaughter of field virus antibody carriers. Within the next 4 years approximately 3 million blood samples will have to be tested. Sampling blood from piglets is an alternative method for bleeding sows. The purpose of this study was to compare both methods in the serological diagnosis of gI antibodies.

Materials and methods

A. Within a few days after parturition, blood and colostrum samples were collected from a total of 104 sows from 8 different gI^- vaccinated breeding farms.

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Depending on their weight (light, medium, heavy), three piglets of each litter were chosen and bled simultaneously with their mother and again three weeks later. The sera were tested serologically for vaccination-induced antibodies using an ELISA (HerdChek PRV, IDEXX GmbH) and for gI antibodies using a blocking ELISA (HerdChek ADV gI, IDEXX GmbH) according to the manufacturer's instructions.

B. In a limited district 1,300 sows in 37 farms and nearly 20,000 fattening pigs were completely vaccinated with gI-deleted vaccine since November 1988. In August 1991 the serological screening of sows for gI antibodies started by bleeding piglets. Blood samples were taken at intervals of 4 to 6 weeks from piglets of sows which had farrowed in the meantime.

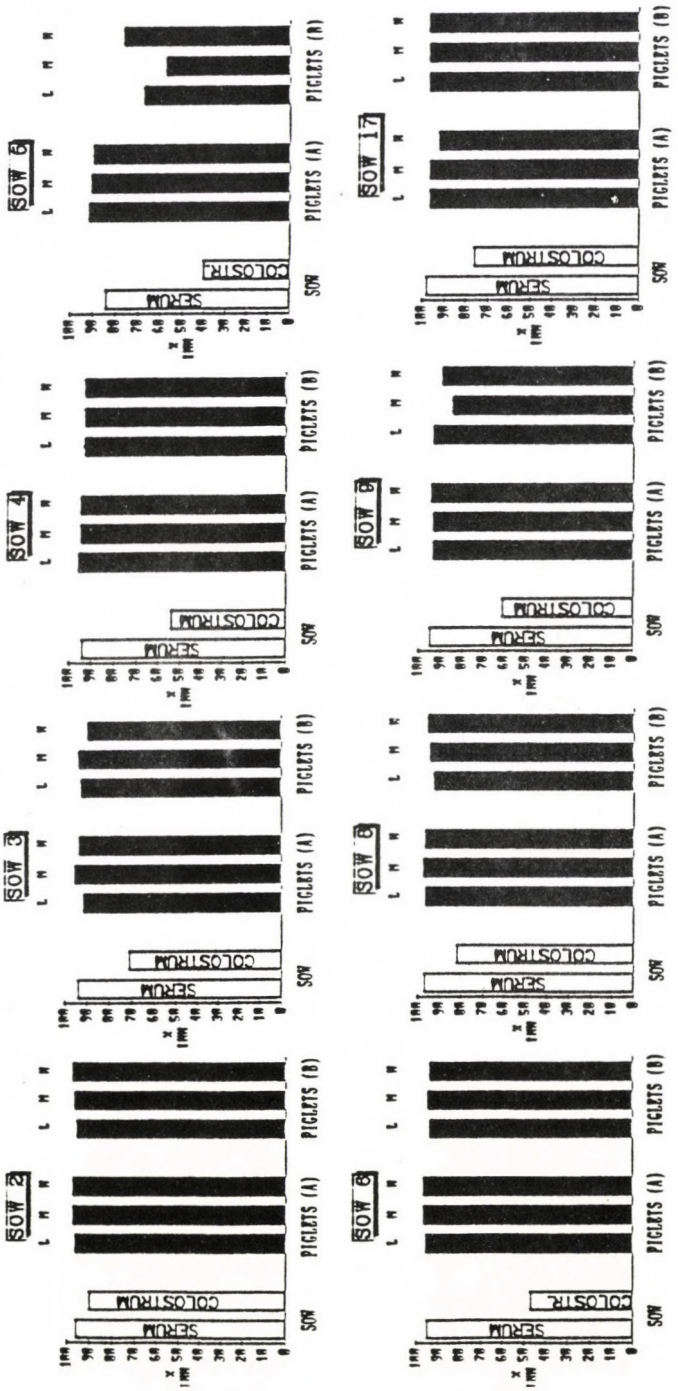
Results

A. In the screening for vaccination-induced antibodies all animals reacted positively. Using the gI ELISA, the sera of 16 sows and their offspring were also positive (Fig. 1), while 86 sows and their piglets reacted negatively and 2 sows (sow 15 and 39) and their piglets showed lower levels of antibodies (Fig. 1). The colostral gI antibodies reacted significantly lower in the gI ELISA compared to serum antibodies (Fig. 1). The different weight (light, medium, heavy) of the piglets had no obvious influence on their gI ELISA antibody titre. Later the maternal gI antibody levels of the piglets slightly decreased.

B. From August 1991 up to March 1993 1,797 blood samples were examined and 43 sows (2.3%) in four farms were found to be infected with ADV.

Discussion

In the first part of this field study the immunological status of individual sows was tested by bleeding the sows themselves, taking colostrum and bleeding one of their piglets. While colostrum samples had a lower ELISA reaction, this study shows that blood samples from sows and their offspring have corresponding antibody reactions in the ADV gI ELISA and the PRV ELISA over a period of at least 3 weeks after parturition. Similar results were published by van Oirschot et al. (1990) and Nienhoff (1989). Maternal antibodies in piglets from sows with a low gI ELISA reaction seem to persist for a shorter period only.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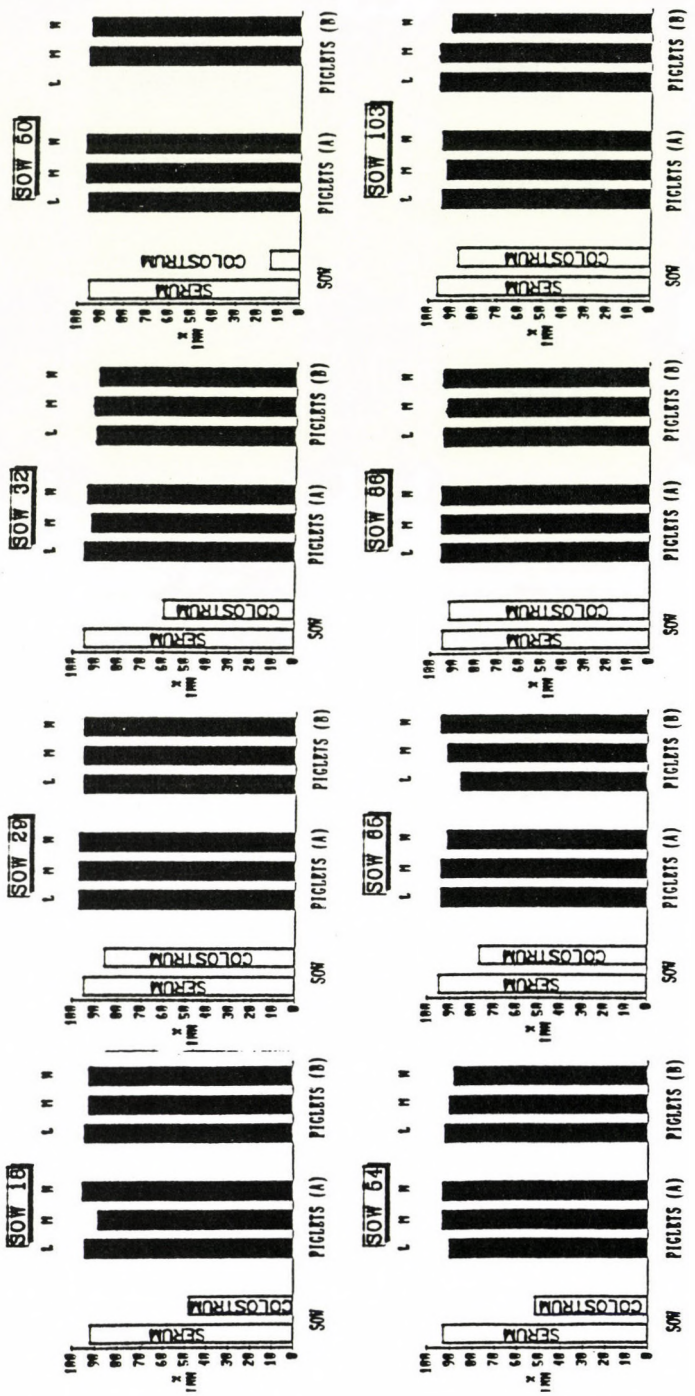
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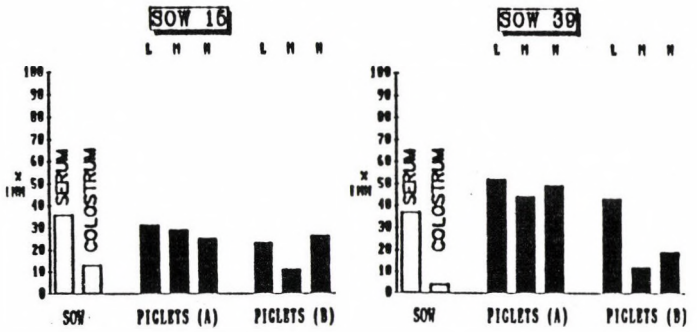

Fig. 1. gI ELISA antibody titres of sows and their offspring
The figure presents gI antibody titres of sows and their piglets. Blood and colostrum samples from sows were collected a short time after parturition. One light (L), medium (M) and heavy (H) piglet of each litter was bled simultaneously with its mother (A) and 3 weeks later (B). Blood and colostrum samples were tested serologically in a gI ELISA (HerdChek ADV gI, IDEXX GmbH). Evaluation: Inhibition (INH) <30% = negative; 30–40% = ???; >40% = positive

A practicable and simple method for the examination of a sow herd for ADV antibodies is taking blood samples from piglets of farrowing sows at intervals of 4 to 6 weeks. The serological status of a breeding herd can be established in a 6 to 9 months period.

Taking blood from piglets is an easier method bleeding sows and both farmers and veterinarians accepted it very well. High safety in laboratory diagnosis and good manual practicability are reasons to prefer this method in ADV eradication programs.

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DETERMINATION OF THE ANTIGENIC VARIABILITY OF PSEUDORABIES VIRUS FIELD ISOLATES WITH MONOCLONAL ANTIBODIES

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Five stable hybridoma cell lines secreting monoclonal antibodies (MAbs) were obtained by fusing spleen cells of pseudorabies virus (strain BUK) immunized BALB/c mice with mouse myeloma cell line SP2/0. Plate-trapped and antibody-trapped antigen ELISAs were done to compare the interaction of MAbs with TOP and "K" strains and 7 Slovak field isolates. Only 2 Slovak isolates reacted with all the MAbs, 3 MAbs reacted with TOP and "K" strains. One Slovak isolate gave no reaction with any MAbs.

Key words: Pseudorabies virus, antigenic variability, ELISA, monoclonal antibodies

Pseudorabies virus (PRV), a member of the Alphaherpesvirinae (herpesvirus suis 1), represents a major economic problem of the swine industry, causing Aujeszky's disease. The study of virus strain variability from the point of their virulence and immunological properties, as a result of their circulation under immune pressure in infected host animals, is of great value. The aim of the present study was to produce monoclonal antibodies (MAbs) to PRV for the analysis of the variability of Slovak PRV isolates from permanently infected breeding swine.

Materials and methods

Virus and cell culture. For MAb production PRV strain BUK (Škoda, 1958) was used. Strains TOP (Sabo, 1971) and "K" (Bartha, 1961) and 7 isolates of PRV from various locations in Slovakia [DK-P (Sabo, 1973), ČVOŠ (Sabo, 1970), Si 231, 185 RD Voderady, 18 Osytrov, 687 Šenkvice, 167 ŠM Čary (all Sabo, 1971)] were studied. PRV isolates were propagated in pig kidney (PK) cells and preserved in lyophilized form. Chicken embryo cells (CEC) were used for virus purification. Both types of cells were cultivated in Eagle's medium with 10% inactivated calf serum. Cells were infected with a multiplicity of infection = 0.3. After total cytopathic effect, the supernatants and cell debris (after disintegration) were used for isolation of virus by low-speed centrifugation. Virus was concentrated and partially purified by centrifugation at 22,000 × *g* for 1 h (Wölfer, 1990).

Production of MAbs. Two intraperitoneal injections were made 40 days apart on 6-week-old BALB/c mice, each with 10^6 $TCID_{50}$ of purified PRV BUK strain emulsified with complete Freund's adjuvant (Difco). Three weeks after the second injection the mice were injected with a final injection of 2 x 10^8 $TCID_{50}$ of purified virus without adjuvant in the tail vein. Three days later the spleen from a hyperimmunized mouse was harvested and the spleen cells were fused with SP2/0 myeloma cells at the ratio of 4:1 by a method of Lane et al. (1986) except that Dulbecco's modification of Eagle's medium (DMEM) was used. The fused cells were seeded in DMEM-2 x HAT($1x10^{-4}$ M hypoxantine, $4x10^{-7}$ M aminopterine, $1.6x10^{-5}$ M thymidine) supplemented with $1x10^{-3}$ M sodium pyruvate, $2.8x10^{-7}$ M 2-mercaptoethanol, 0.005% gentamycin, 2% human umbilicord serum, 15% horse serum mixed with the same volume of conditioned medium (SP2/0 culture supernatants). Hybridomas secreting specific antibody were selected from 10–14 days after fusion using plate-trapped antigen (PTA) indirect ELISA (Gallo and Matisova, 1993) and 10^6 $TCID_{50}$ of purified PRV as antigen. Selected colonies were controlled by antibody-trapped antigen (ATA) ELISA. Supernatants from uninfected PK, CEC and myeloma cells were used as negative controls. Antibody production was assayed with swine anti-mouse IgG-horseradish peroxidase conjugate using o-phenylenediamine as substrate. Antibody secreting hybridomas were cloned by the limiting dilution method and then used for ascitic fluid production.

Ascitic fluids were produced by intraperitoneal injection of 1 to $5x10^6$ hybrid cells into BALB/c mice which had been treated by 500 µl of incomplete Freund's adjuvant five days before.

Immunoglobulins from ascitic fluids were purified with ammonium sulphate presipitation and subsequent affinity chromatography on DEAE-AFFI-gel blue (Bio-Rad) or DEAE 52 cellulose (SERVA). Immunoglobulin concentrations were assayed spectrophotometrically (Gallo and Valenta, 1985). Immunoglobulin isotypes were determined with commercial reagents (Sigma Immunochemicals) using the procedure for indirect ELISA.

Results

Because of the great sensitivity of mice to pseudorabies virus that had resulted in a rapid death of mice, several immunization protocols were previously applied. We found that the most efficient immunization protocol was to inject mice with low doses of virulent strain BUK. We obtained a total of 40 hybridomas producing anti-PRV MAbs. Some of them lost their secretion or died. From the rest of the MAbs we have chosen 5 MAbs showing good activity in PTA ELISA and these were subjected to cloning. The four MAbs belonged to either the 1, 2b, or 3 subclass of IgG and 1 to IgM class. In two types of ELISA these MAbs re-

acted with strain BUK in different titres. MAb AD4 reacted with 9 and MAb AD1 with 4 strains or isolates. MAb AD5 distinguished only 2 isolates. We have not taken into account the results with titres under 5×10^2 because of the background in ELISAs.

Table 1

Titers and isotype characterisation of five monoclonal antibodies against pseudorabies viruses

MAb Isotype	AD 1 IgGM	AD 2 IgG1	AD 3 IgG3	AD 4 IgG2b	AD 5 IgG3
PRV					
1	$5x10^2$	$5x10^4$	$5x10^5$	$5x10^4$	$5x10^2$
2	$5x10^2$	$1x10^6$	$5x10^4$	$5x10^4$	$5x10^2$
3	$1x10^3$	$1x10^6$	$5x10^4$	$1x10^6$	$5x10^3$
4	$5x10^2$	$5x10^4$	$5x10^4$	$5x10^4$	$1x10^2$
5	$5x10^1$	$5x10^2$	$5x10^4$	$1x10^5$	$1x10^2$
6	–	$5x10^4$	$1x10^6$	$5x10^4$	–
7	–	$1x10^4$	$5x10^4$	$5x10^4$	$1x10^2$
8	$1x10^2$	$1x10^4$	$5x10^4$	$5x10^3$	$5x10^1$
9	$5x10^2$	$1x10^3$	$5x10^4$	$5x10^3$	–
10	–	–	–	$5x10^2$	–

No. 1 = BUK-immunizing strain, 2 = ČVOŠ, 3 = DK-P, 4 = 18 Ostrov, 5 = PRK "K", 6 = TOP, 7 = 167 ŠM Čary, 8 = 185 Voderady, 9 = Si 231, 10 = 687 Šenkvice; – = no reaction

ELISA results with 5 MAbs revealed significant differences among the PRV isolates tested. It seems that they can be divided into two groups: The ČVOŠ and DK-P reacted with all MAbs, as did strain BUK and 18 Ostrov with 4 MAbs, at relatively high titres. The other isolates reacted only with MAbs AD2, 3 and 4. An unresolved problem is the isolate 687 Šenkvice which reacted slightly only with MAb AD4. Further studies are necessary to find out against which type of glycoproteins these MAbs are directed.

Discussion

In this study we have described the production of monoclonal antibodies with a prospective use in the determination of the antigenic variability of PRV field isolates. From a panel of monoclonal antibodies, 5 MAbs were studied further. We obtained three MAbs of the IgG class (isotype IgG1, IgG3) and one of

the IgM class. Wathen et al. (1985) obtained only 4 MAbs that belonged to two isotypes of the IgG class (IgG 2a, IgG 2b) and one to the IgM class. All 30 MAbs obtained by Eloit et al. (1988) were of the IgG class (most of them represented the IgG 2a isotype). Fuchs et al. (1990) determined 13 MAbs: 5 IgG1, 4 IgG 2a and 3 IgG 2b. It is difficult to explain the Ig class or subclass variability of MAbs which we obtained. A partial explanation could be found in the different immunization protocols. It was possible to select two types of MAbs AD 2, AD 3, and AD 4 that reacted at relatively high titres with 8 resp. 9 isolates tested. On the contrary, MAbs AD 1 and AD 5 reacted at very low titres only with three or two isolates tested. Antigenic variation among PRV strains was also observed by Ben-Porat et al. (1986).

In the future we shall test the obtained MAbs against glycoproteins of described PRV viruses, especially gII, which play an important role in the protection from PRV infection.

Acknowledgements

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GLYCOPROTEIN gI OF PSEUDORABIES VIRUS: EPITOPE-SPECIFIC ANTIBODY RESPONSE IN MICE AND PIGS

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The detection of gI antibodies in infected pigs is crucial in monitoring the eradication or control of pseudorabies virus (PRV). Although tests for the detection of gI antibodies are based on various principles, most rely on the detection of antibodies directed against one or two epitopes on gI. Consequently it is crucial to know which epitopes on gI are recognized by the sera of pigs infected with PRV and whether factors such as the infecting PRV strain or the genetic background of the host can influence the epitope-specific antibody response.

Methods

The ES-IPMA. SK6 cells were seeded in a microtitre plate, infected with 10^4 PFU/ml of PRV and incubated for 15 h at 37 °C. Plates were washed with PBS, dried and stored at -20 °C. Before use the cells were fixed with an ice-cold solution of 4% paraformaldehyde during 15 min at RT. In the test 100 µl/well of diluted pig serum or mouse serum (1:10) was incubated during 1 h at 37 °C. The plates were washed and subsequently incubated with 100 µl of MAb (1h 37 °C). The optimal dilution of the monoclonal antibody (MAb) (or MAb-HRPO) used in the test was the highest dilution of MAb yielding a maximal absorbance at 450 nm. Maximal A_{450} values varied between 1.0 and 1.5. The detecting antibody was anti-mouse IgG conjugated to HRPO. After the incubation period, the plates were washed and a 100 µl/well substrate (0.1% 5-amino-hydroxybenzoic acid) was added and incubated overnight at RT. A serum was considered to block the binding of the MAb (or the MAb-HRPO) if the A_{450} value was decreased more than two times the SD of the mean A_{450} value of 8 control sera tested on each plate. For the testing of mouse sera MAb-HRPO conjugates were used.

Results

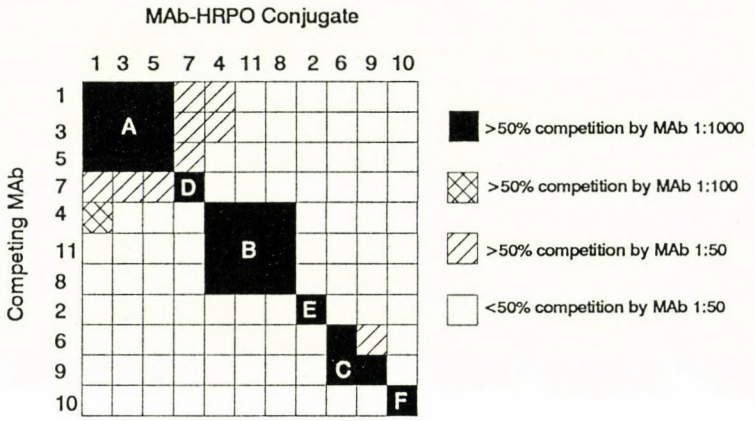

Fig. 1. Antigenic domains as described by Jacobs et al., 1990 (J. Gen. Virol. **71**, 881-887). Each MAb was diluted and allowed to compete for its epitope with each MAb-HRPO conjugate

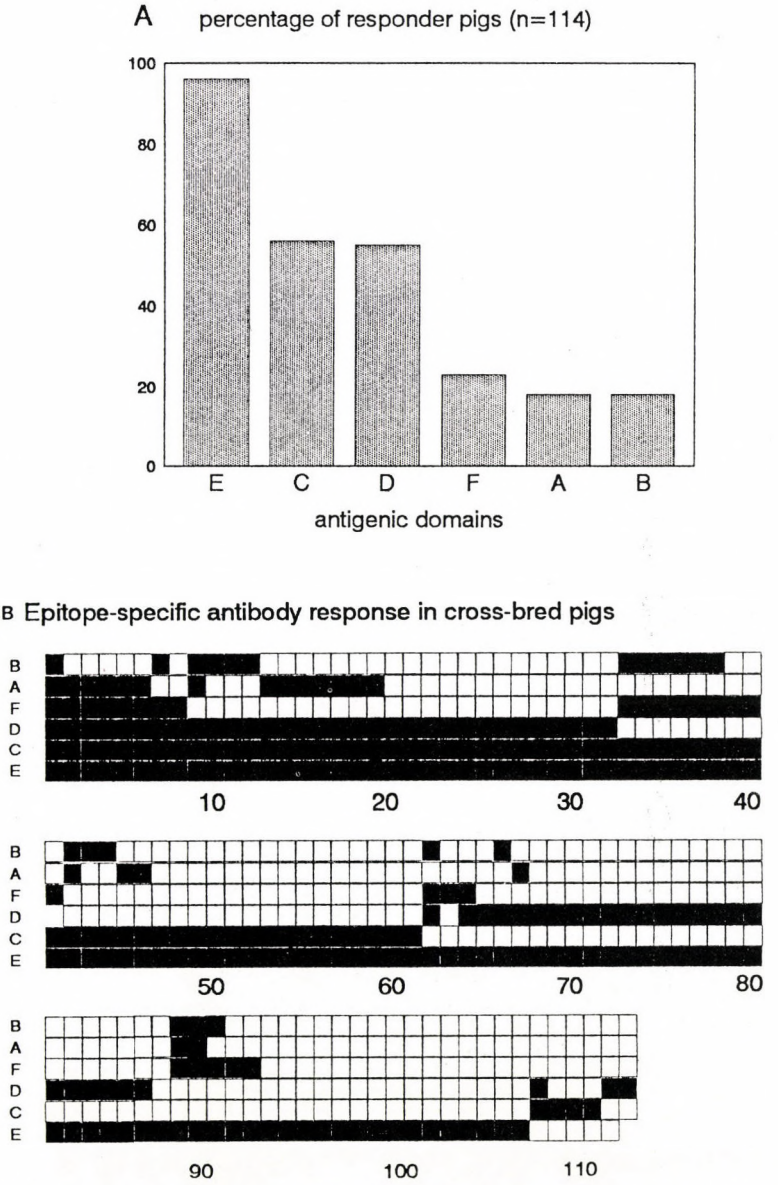

Fig. 2. 160 pig sera were obtained from pig farms in an area endemically infected with PRV. All pigs were vaccinated with a gI-negative vaccine strain. (A) The number of pigs positive in one or more of the ES-IPMAs was set at 100% (n=114). Each bar represents the percentage of pigs responding to the antigenic domain indicated at the bottom of the figure. (B) Each horizontal bar represents one pig. A black box indicates that the pigs had antibodies directed against the antigenic domain indicated at the left side of the figure

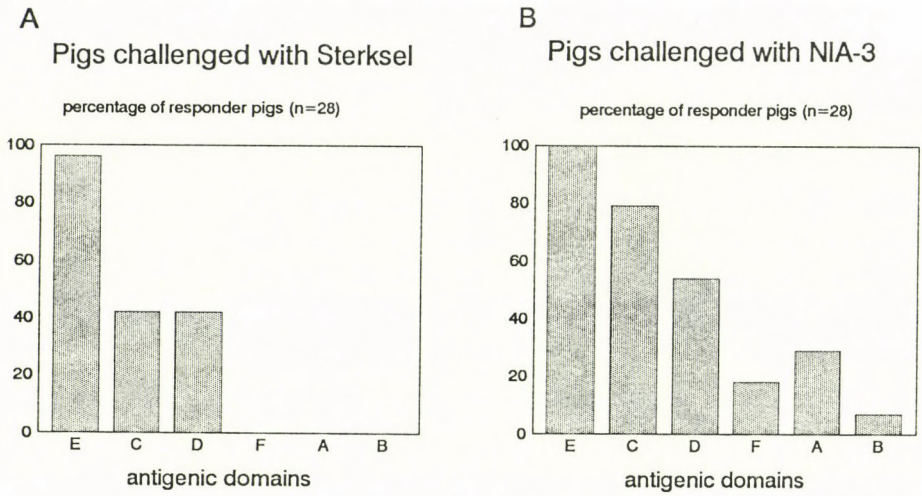

Fig. 3. The number of pigs investigated was set at 100%. Each bar represents the percentage of pigs with antibodies directed against the antigenic domain indicated at the bottom of the figure. (A) Challenged with the virulent NIA-3 strain (n=28); (B) challenged with the mildly virulent Sterksel strain (n=28)

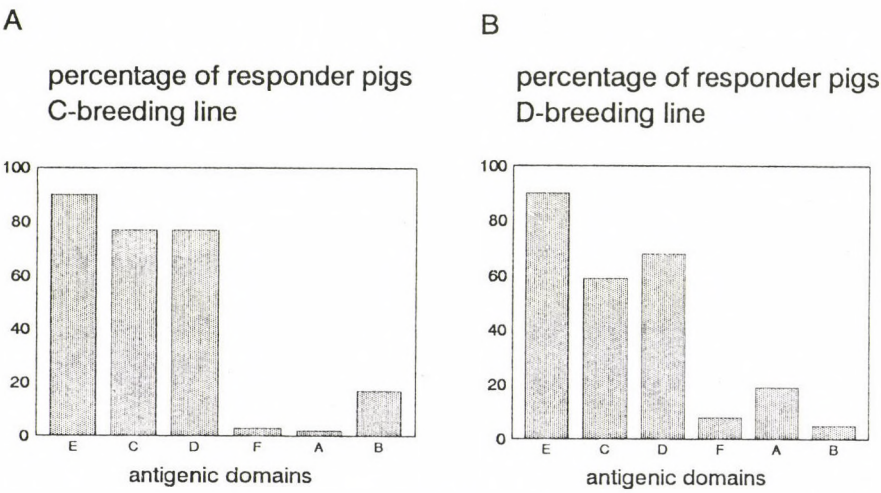

Fig. 4. One hundred pig sera from two different breeding lines were obtained from Eurobrid, Boxmeer. Fifty sera were from the D-breeding line; fifty sera were from the C-breeding line. All pigs were vaccinated with a gI-negative vaccine strain

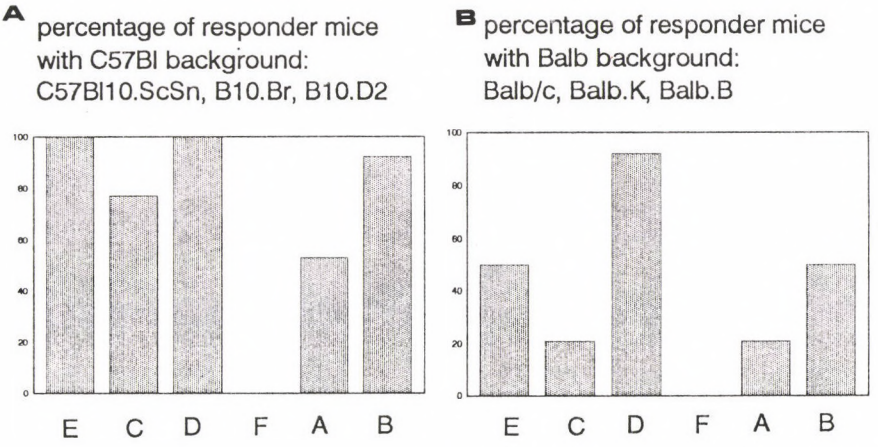

Fig. 5. Six inbred mouse strains of different genetic backgrounds were immunized intraperitoneally with 10^8 PFU of a thymidine kinase negative PRV strain 3 times. Blood was collected two weeks after the last immunization

Conclusions

(1) Most pigs responded to antigenic domain E, and to a lesser degree to the antigenic domains C and D. Only a few pigs responded to the antigenic domains F, A and B (Fig. 2A).

(2) In individual pigs the epitope-specific antibody response varied considerably (Fig. 2B).

(3) The infecting PRV strain influenced the epitope-specific antibody response (Fig. 3AB).

(4) Although no differences were observed in the epitope-specific antibody response between pigs of two different breeding lines (Fig. 4AB), the genetic background of the host can influence the epitope-specific antibody response, as demonstrated in mice (Fig. 5AB).

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TRANSGENIC MICE HARBORING AUJESZKY'S DISEASE VIRUS gD GENE

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Plasmid pMLP10 gp50.5 (gD) (Eloit et al., 1990) provided the sequence to integrate. The construct has been injected into the male pronucleus of one-cell mouse embryos (CBA/C5B1). A total of 1,567 microinjected embryos were transferred into the oviduct of pseudopregnant females (Hogan et al., 1986). PCR revealed that 3 out of 10 mice were born with an integrated construct; among them, 2 mice have integrated the construct in their gonads because 20 out of 107 (18.7%) of their offspring were also transgenic. This low percentage could be explained by germline mosaicism and/or differential mortality between transgenic and non-transgenic embryos.

Key words: Aujeszky's disease virus, gD gene, transgenic mice, PCR

The introduction of foreign DNA into the genome of mammals is one of the major recent technological advances in biology (Jaenisch, 1988). Transgenic animals have been instrumental in providing new information about mechanisms of development and developmental gene regulation, action of oncogenes, immune system, genetic diseases and study of pathogenesis.Most transgenic mice have been obtained by microinjecting foreign DNA into pronuclei of fertilized eggs. This technique involved transfer of microinjected embryos to pseudopregnant females and identification of transgenic animals among offspring.

It has been show that 3 glycoproteins, i.e., gB, gD, and gH are essential for Aujeszky's disease virus (ADV) entry (Peeters et al., 1992; Rauh et al., 1991).

Some studies have demonstrated that gD is required for primary infection but not for subsequent replication and viral spread *in vivo*. These results indicate that trans-synaptic transport of virus is independent of gD (Peeters et al., 1993; Heffner et al., 1993).

In vitro studies on transfected cells with the plasmid pMLP10 gp50.5 (gD) showed that they were resistant to infection with ADV (Eloit et al, 1990).

The aim of this study was to obtain a homozygous strain of transgenic mice harboring ADV gD gene for use in resistance and pathogenesis studies.

Materials and methods

pMLP10gp50.5 plasmid (Fig. 1)

The open reading frame coding for glycoprotein gD of NIA3 strain (Petrovskis et al., 1986) is under the control of the Major Late Promoter (MLP) of Adenovirus 2 (Ad2) (Eloit et al., 1990).

The promoter is surrounded by the leftmost 455bp of Ad5 viral genome (ITR) and by the Tripartite Leader Sequences (TLS) minus splicing donor site located at the end of the third leader (Ballay et al., 1987).

TLS are 3 short (40 to 90 bp) regions located upstream of the Ad structural genes and required for efficient translation (Thummel et al., 1983) and accumulation of primary transcripts.

The SV40 polyadenylation signal follows the gD coding sequence without any splicing signal (Eloit et al., 1990). In these surroundings, MLP is a strong promoter (Ballay et al., 1987), which may be ubiquitous.

Preparation of microinjected DNA

Plasmid pMLP10gp50.5 (gD) is amplified in 500 ml of a DH5α culture (NZY medium + 50 μg/ml ampicilline), extracted on a column (Quiagen plasmid maxi kit, Quiagen), precipitated with isopropanol and resuspended in sterile Tris-Cl 10 mM (pH7.6) EDTA 0.1 mM.

*Eco*R1-Sph1 fragment, containing the sequence to integrate, is cleared out of the plasmidic sequences by electrophoresis on 1% agarose gel and a "glass powder" method (Gene Clean II Kit, Bio 101).

Egg manipulation

Gonadotropins are administered to females prior to mating to induce superovulation; pregnant mare's serum (PMS) (5UI, IP) is used to mimic follicle-stimulating hormone (FSH) and after 42–48 h, human chorionic gonadotropin (hCG) (5 UI, IP) is used to mimic luteinizing hormone (LH).

Fertilized mouse eggs were collected from superovulated CBA/C57B1 F1 females approximately 20 h after hCG injection (Hogan et al., 1986). They were flushed from oviduct to PB1 medium (Whittingham, 1971). Cumulus cells were removed from the eggs with hyaluronidase (340 U/ml) and the eggs were washed free of debris and enzyme. Eggs were transferred to a microdrop containing culture medium (Kasaï, 1978) and cultured at 37 °C in 5% CO_2 in air for 3 h.

For micromanipulation, an inverted microscope Diaphot-TMD Nikon was used. Embryos were held in place by a blunt pipette (outside diameter of 30 μm)

while the tip of the injector pipette was inserted through the zona pellucida and vitellus into the male pronuclei (micromanipulator Narishige).

Plasmid pMLP10 gp50.5 (gD) in the injector pipette was slowly discharged (2 pl, approximately 500 copies/embryo) by using microinjector 5242 Eppendorf. After injection, one-cell eggs were transferred into the oviducts of pseudopregnant foster females (8 eggs/oviduct) (Hogan, 1986).

Extraction of genomic DNA

For routine detection of mouse with integrated DNA, tails from 2 week old mice were cut and lysed in 0.7 ml of 50 mM Tris-HCl (pH8), 100 mM EDTA, 0.5% SDS, 0.05% proteinase K (55 °C, overnight with shaking). Lysates were submitted to two extractions: first with phenol-chloroform and then with chloroform only.

They were precipitated in 2 volumes of ethanol at room temperature with 1/10 NaAc 3M (pH6) and rinsed in 70% ethanol. The pellet was dissolved in 100 µl of sterile water. This method provided about 20 to 250 µg of genomic DNA (measured by spectrophotometry at 260 nm).

Polymerase Chain Reaction (PCR) analysis of genomic DNA

The final amplification conditions were 10 mM Tris-HCl pH 8.5, 50 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin, 0.2 mM dNTP (Pharmacia), 0.2 µM of each primer, 20 U/ml Amplitaq* DNA polymerase (Perkin Elmer Cetus), and the template DNA was estimated at 5 ngr/µl.

Samples were submitted to 1 cycle of 3 min 94 °C, followed by 40 cycles of 30 sec 94 °C, 1 min 65 °C, 30 sec 72 °C on a TC480 DNA thermal cycler.

Samples were analyzed on 3% agarose gel. The sequence selected for amplification consisted of 222 base pairs covering nucleotides 272 to 534 of the gene coding for glycoprotein gD of ADV (Jestin et al., 1990).

Results

From 1,567 micromanipulated and transferred embryos, 10 mice were born. The percentage of birth was rather low (0.64%). PCR revealed that 3 out of 10 mice (2 males and 1 female) were born with integrated construct (Fig. 2). Two out of these 3 transgenic mice had transgenic descendants (20/107) (18.7%).

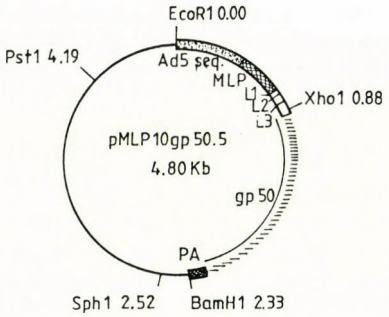

Fig. 1. Plasmid pMLP10 gp50.5

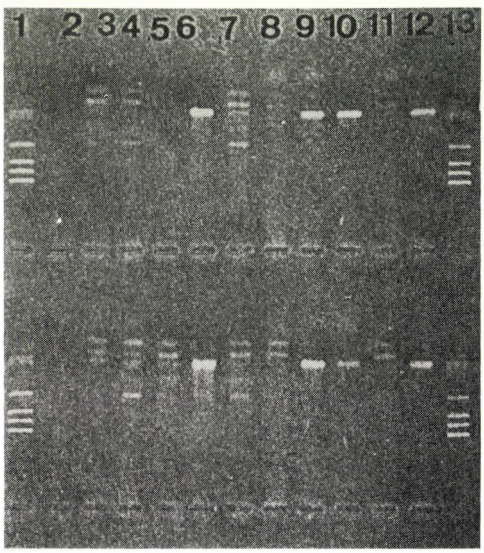

Fig. 2. Lanes 1 and 13: phage ϕX; lane 2: buffer; lane 3: DNA of normal mouse; lane 4: DNA of mouse no. 3; lane 5: DNA of mouse no. 4; lane 6: plasmid pMLP10 gp50.5 (gD); lane 7: DNA of mouse no. 5; lane 8: DNA of mouse no. 6; lane 9: DNA of mouse no. 7 (transgenic); lane 10: DNA of mouse no. 9 (transgenic); lane 11: DNA of mouse no. 8; lane 12: DNA of mouse no. 10 (transgenic)

Discussion

Important parameters that influence successful integration of foreign DNA into mouse chromosomes are the effects of DNA concentration, the form and size of DNA, the buffer composition, the site of injection on the frequency of integra-

tion. It must also include factors such as the ease of obtaining fertilized eggs, ease of microinjection, survival of the eggs after microinjection, ability of the eggs to continue development after transfer to pseudopregnant mothers. The overall efficiency also depends on the choice of mouse strains (Brinster et al., 1985).

Usually, the percentage of birth of micromanipulated embryos reaches about 10%. In this experiment, the percentage has been rather low (0.64%). As the above parameters have been validated, this low percentage could depend on the nature of the injected gene or integration site.

Two out of 3 parental mice have integrated the construct in their gonads as 18.7% of their offspring were transgenic ($p<0.01$). Theoretically, all micromanipulated one-cell eggs should integrate the construct but mosaicism appears in 15% of cases (Babinet et al., 1989). Experimental indication of mosaicism is the reduced frequency of genetic transmission to successive generations below Mendelian levels (normally, we expected about 50% of heterozygotes).

One possible explanation is that stable integration occurred late, after the one-cell stage. Brinster et al. (1985) showed that the amount of injected DNA does not change following a 24-h incubation, the sequences are replicated and maintained as free elements during early embryonic development.

Another explanation for the lack of integration is that the DNA molecules microinjected into the egg were preserved in an unintegrated form and partitioned into cells of the developing mouse. It is also possible that embryonic viability could be reduced because of inappropriate integration site or unfavourable effects of the transgene.

Acknowledgement

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AUJESZKY'S DISEASE ELISA USING BACULOVIRUS EXPRESSED GLYCOPROTEINS

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The genes coding for two Aujeszky's disease virus glycoproteins, gp50 and gIII, were amplified by PCR and inserted into a baculovirus transfer vector. Following co-transfection of insect cells with a modified wild type baculovirus, recombinant virus clones expressing the inserted glycoproteins were detected by ELISA. These clones were then amplified and the expressed products used as the antigen for an ELISA to detect antibodies to Aujeszky's disease virus in pig sera.

Key words: Aujeszky's disease virus, ELISA, glycoprotein, baculovirus vector

With the advent of gene-deleted vaccines for the control of Aujeszky's disease (AD) came the potential for serological discrimination between field virus positive pigs and vaccinated pigs (Van Oirschot and De Waal, 1987). This discrimination is central to the AD eradication efforts now being made in Europe, the USA and elsewhere. So far, a number of non-essential glycoprotein genes have been deleted in the construction of these vaccines including gI, gIII, gX and gp63. The serological discrimination between vaccinates and field virus infected pigs is usually made by the use of an ELISA kit complementary to the deleted glycoprotein.

The baculovirus expression system has been used to produce high yields of authentically processed, antigenically active, foreign glycoproteins (Smith et al., 1983). AD glycoproteins gp50 and gIII have been expressed in the baculovirus system and shown to be immunologically active (Thomsen et al., 1990; Inumara and Yamada, 1991). It is a logical step in an environment of gene-deleted vaccines to use these expressed glycoproteins in a serological assay, with the ultimate aim of providing discrimination between vaccinates and field virus infected pigs. In addition the need to culture large volumes of high titre Aujeszky's disease virus (ADV) would be removed by the use of an expressed recombinant antigen. This work describes the rapid construction, selection and application to the AD ELISA of recombinant baculovirus clones expressing gp50 or gIII.

Materials and methods

Construction of transfer vectors

The PCR (Saiki et al., 1985) was used to amplify the full length genes of gp50 and gIII. The primers for the amplification were designed using the published sequences of gp50 (Petrovskis et al., 1986) and gIII (Robbins et al., 1986). The reaction conditions and cycling parameters were identical to those described previously (Banks, 1993). ADV infected tissue culture supernatant (1 µl) was used as the template for each reaction.

gp50 primer 1: *Bam*HI cloning site plus bases +1 to +24
$^{5'}$TTTGGATCCATGCTGCTCGCAGCGCTATTGGCG$^{3'}$ –33mer
gp50 primer 2: *Bam*HI cloning site plus bases +1212 to +1233
$^{5'}$TTTGGATCCCATCATCGACGCCGGTACTGCGGA$^{3'}$ –33mer
gIII primer 1: *Bgl*II cloning site plus bases –3 to +18
$^{5'}$TTTAGATCTGCCATGGCCTCGCTCGCGCGT$^{3'}$ –30mer
gIII primer 2: *Eco*RI cloning site plus bases +1474 to +1494
$^{5'}$TTTGAATTCGGGTCGGACTCGCTGTCGTTT$^{3'}$ –30mer

The gp50 primers were designed with *Bam*H1 cloning sites at their 5' ends. The gIII primers had a *Bgl*II cloning site and an *Eco*R1 cloning site at the 5' ends of primers 1 and 2 respectively.

Amplified DNA preparations were subjected to electrophoresis at 1.4 v/cm in a 1.5% agarose gel and the DNA fragments excised following visualization with ethidium bromide over a UV light source. The DNA was recovered using Prep-a-gene (Bio-Rad, UK), and subjected to digestion with the appropriate restriction endonucleases in order to prepare the termini for ligation. The preparations were then ligated with the transfer vector pBacPAK1 (Clontech, USA) previously cut with *Bam*H1 (gp50) or *Bam*H1/*Eco*R1 (gIII).

Following transformation of *E. coli* (DH5α, Gibco-BRL, U.K.), recombinant clones were selected and confirmed by PCR, restriction digestion and Southern hybridization (Southern, 1975). The recombinant plasmids were purified in a CsCl gradient and designated pBgp50 and pBgIII respectively.

Co-transfections and recombinant virus selection

The baculovirus AcNPV (BacPAK6, Clontech, USA) was linearized with the restriction endonuclease *Bsu*36I (Kitts and Possee, in press). Co-transfection of *Spodoptera frugiperda* (Sf21) cells with the linearized BacPAK6 and pBgp50 or pBgIII was carried out using Lipofection (Gibco-BRL) according to the manu-

facturers' instructions. After 48 h the co-transfections were subjected to a single round of plaque assay incorporating Bluo-Gal (Sigma, U.K.) to detect non-recombinant plaques, according to standard protocols (King and Possee, 1992). The plaque assays were stained with neutral red after four days, and clear plaques picked from dishes which contained no blue plaques. Virus was eluted from the agarose plugs in 500 µl of TC100 medium (Gibco-BRL, UK), and 5 µl of each applied to a microplate well, previously seeded with Sf21 cells at 1.5 x 10^6 cells/ml. After three days incubation, 50 µl of cell medium was removed from each microplate well, clarified at 13,000 *g* for 3 min, and added to 50 µl of 0.5 M bicarbonate buffer, pH 9.6, in the corresponding well of an ELISA plate (Maxisorp, Nunc, UK).

Assessment of recombinant protein expression by ELISA

The ELISA plate was incubated for a minimum of 16 h at 4 °C before being subjected to the ELISA protocol. Control wells included were supernatants from non-infected Sf21 cells, BacPAK6 infected Sf21 cells and a whole AD virus ELISA antigen. A single positive polyclonal antiserum diluted 1:200 in 0.01 M PBS, 0.05% Tween 20, pH 7.2 (PBST) was used for all wells.

ELISA-protocol (all volumes 100 µl per well unless stated). Discard bicarbonate/clarified supernatant solutions. Wash once with PBST. Add diluted serum, incubate for 30 min at 37 °C. Discard serum, wash 6 times with PBST. Add Protein A-peroxidase conjugate diluted 1:15,000 in PBST, incubate for 30 min at 37 °C. Discard conjugate, wash 6 times with PBST. Add substrate, tetramethylbenzidine (TMB), incubate 10 min at room temperature. Stop reaction with 25 µl 2.5 M H_2SO_4. The optical densities of the wells were determined using a plate reader (Anthos Labtech HTII, Austria).

Validation of recombinant gp50 and gIII ELISAs

The gp50 and gIII recombinant baculovirus clones which gave the highest optical density by ELISA were amplified in tissue culture. An initial expression kinetics study was performed to determine the optimal time for harvesting the cultures post-infection. The clarified cell supernatant was used to optimise and validate the ELISA for each of the glycoproteins. The optimal antigen and conjugate dilutions were determined against four standardized sera; a control negative, a high reading (non-specific) negative, a strong positive and a control positive equivalent to the EC1 reference serum diluted 1:2. One hundred and twenty AD antibody negative field sera were assessed using a non-infected cell supernatant as a negative control with each serum. A panel of EC reference sera and ten known positive field sera were similarly tested for sensitivity and specificity assessment.

Characterization of recombinant clones

The ELISA positive clones were subjected to PCR amplification with the gp50 or gIII primers and protocols used for the initial subcloning. ELISA negative clones, BacPAK6 and non-infected Sf21 cell supernatant were included as controls.

The demonstration of the recombinant glycoproteins by PAGE and Western blotting, and the junction sequencing will be presented elsewhere.

Results

Co-transfections and recombinant selection by ELISA

The use of the *Bsu*36I linearized BacPAK6 baculovirus produced recombination frequencies with the transfer plasmids pBgp50 and pBgIII of 92% and 98% respectively on the first plaque assay following co-transfection. All plaques picked exhibited a good cytopathic effect (CPE) following inoculation onto Sf21 cells in the microplate wells. The ELISA screening of plaques for expression of the respective glycoproteins produced a high preponderance of positive clones (Table 1).

Table 1

ELISA screening of recombinant clones

	Positive	Negative	Positive, %
gp50	24	5	82.8
gIII	20	4	83.3

Negative clones were checked in culture for the presence of non-recombinant virus; no "wild-type" virus was detected.

Validation of recombinant gp50 and gIII ELISAs

The results of the expression kinetics study showed that ELISA reactivity was optimal between 3–5 days post infection for each of the glycoproteins. After more than five days following infection of Sf21 cells, the signal to noise ratio declined.

The optimal antigen and conjugate dilutions were established at 1:10 and 1:20,000 respectively for both the gp50 and gIII preparations. The optical densities of many negative sera were very high with each of the glycoprotein antigens. The use of the control antigen at an appropriate dilution cancelled out this reaction, and only the positive sera gave a net positive result above 0.050. This did result in highly coloured ELISA plates, a problem which was not resolved by antigen-conjugate chequerboard titration. However, 104 of the 120 known negative sera produced a net negative response and none produced a net positive response greater than 0.050. For this assessment a serum was scored positive if it produced a net positive result of twice the highest reading negative serum. All ten known positive sera produced a net positive response (range 0.503–1.347). The EC reference sera were interpreted correctly, with EC1 being found positive at an initial dilution of 1:8 with gp50 and 1:4 with gIII (Table 2). The gp50 positive serum results were approximately 10% higher than those of the gIII (mean 9.55%).

Table 2

ELISA sensitivity assessment

Serum	Test result	
	gp50	gIII
EC Ref. Serum A	+	+
EC Ref. Serum B	+	+
EC Ref. Serum C	+	+
EC Ref. Serum D	+	+
EC Ref. Serum E	+	+
EC Ref. Serum F	+	+
EC1 Undiluted	+	+
EC1 Diluted 1:2	+	+
EC1 Diluted 1:4	+	+
EC1 Diluted 1:8	+	–
EC1 Diluted 1:16	–	–
EC1 Diluted 1:32	–	–

Characterization of recombinant clones

ELISA positive and negative clones were assayed by PCR for the presence of the respective inserted gene. Amplification of the full length gene was seen only with the ELISA-positive clones with the gp50 recombinants (Fig. 1). The gIII ELISA positive and negative clones were both amplified by PCR (Fig. 2). The identity of the amplified DNA was confirmed by Southern hybridization with a probe made from the PCR amplified DNA used for the original transfer vector ligation (data not shown).

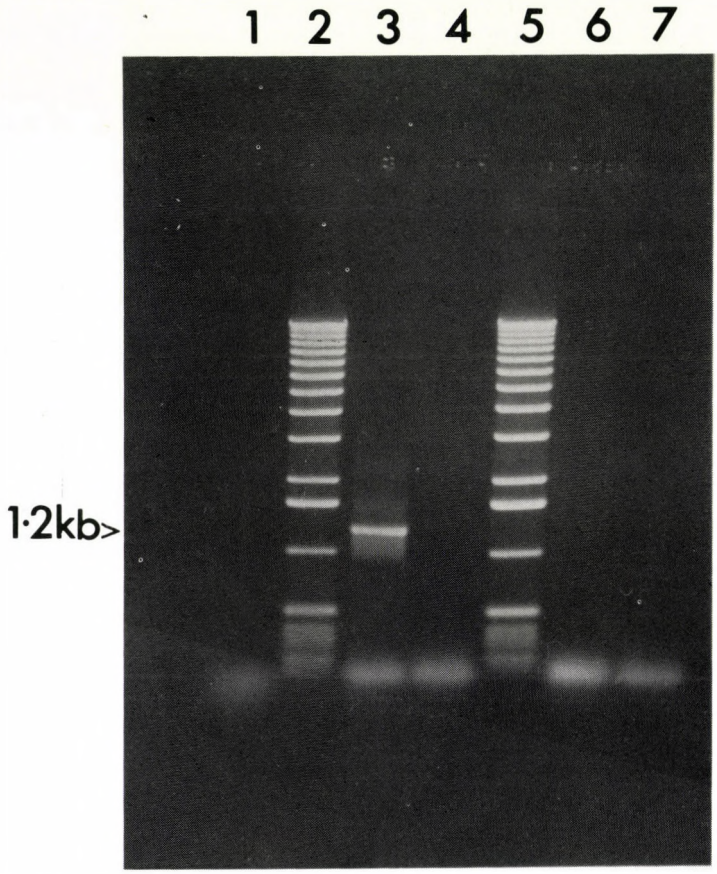

Fig. 1. PCR amplification of gp50 recombinant clones. Track 1: reagent control; track 3: ELISA-positive clone; track 4: uninfected Sf21 cell control; track 6: BacPAK6 infected cell control; track 7: ELISA-negative clone; tracks 2 and 5: 1kb ladder molecular weight marker

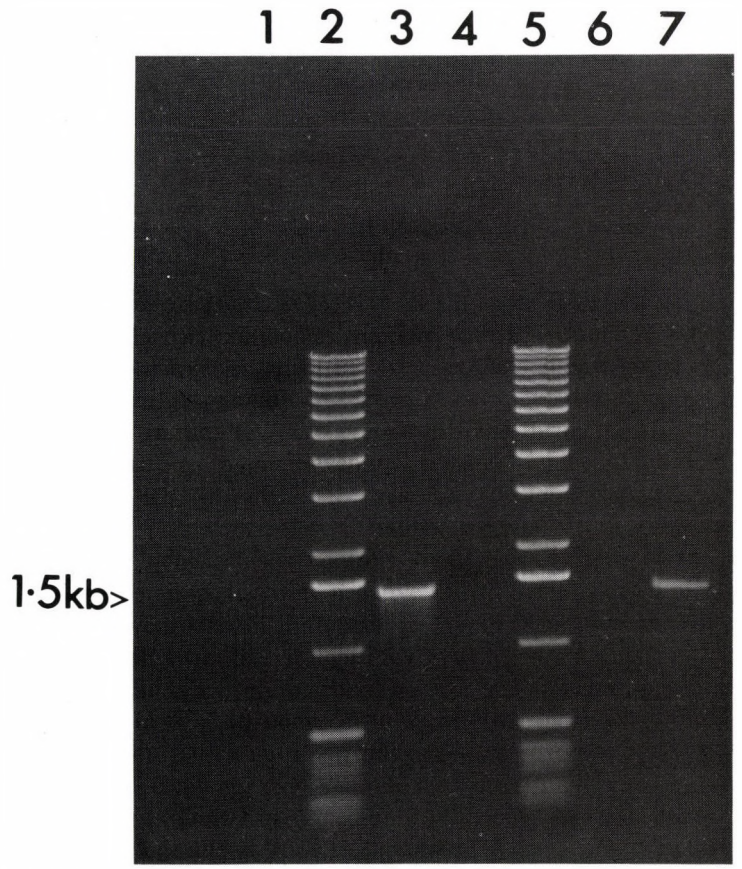

Fig. 2. PCR amplification of gIII recombinant clones. Track 1: reagent control; track 3: ELISA-positive clone; track 4: uninfected Sf21 cell control; track 6: BacPAK6 infected cell control; track 7: ELISA-negative clone; tracks 2 and 5: 1kb ladder molecular weight marker

Discussion

The use of the PCR to isolate the ADV genes for cloning into the baculovirus transfer vector imparted a twofold benefit. Firstly, the process was much more rapid and simple than conventional cutting and subcloning techniques. Secondly, the termini of the cloned gene could be more precisely tailored and did not depend on the availability of nearby non-coding restriction sites. This was of particular importance for the 5' end of the gene, since GC rich non-coding 5' overlaps can cause problems with expression in the baculovirus system (King and Possee,

1992). The PCR also facilitates precise manipulation of the translation initiation context, which may be a factor in expression levels (Kozak, 1986).

It is worthy of note that a higher percentage of recombinant *E. coli* colonies were obtained using the directed cloning of the gIII construct compared to the gp50 construct, both primers of which contained *Bam*HI sites. This latter approach could not be used with the gIII construct due to the presence of an internal *Bam*HI site near the 3' terminal of this gene. The additional advantage of directed cloning was that orientation checks were unneccessary. The manipulation of the *Ac*NPV genome by Kitts and Possee (in press) to produce the BacPAK6 virus significantly reduces the time required to isolate cloned, recombinant baculovirus. The loss of the essential gene, ORF1629 on linearization with *Bsu*36I enables rescue only by homologous recombination with the transfer vector carrying a copy of ORF1629. The effect of this is to produce almost 100% recombinant colonies following co-transfection with virus and vector.

That the recombinant glycoproteins were present in their respective infected cell supernatants was confirmed by ELISA screening. Since the ELISA was to be the ultimate use of the recombinant proteins this seemed to be a particularly appropriate way to screen for expression. With each glycoprotein construct, a very high proportion of recombinant clones expressed ELISA reactive protein, indicating that it is probably not neccessary to screen large numbers of putative recombinant plaques. The infected tissue culture cell pellet was assessed by ELISA in some of the early trials. This source of antigen had a much lower signal to noise ratio than the clarified supernatant and so was not assessed further.

An interesting difference between the ELISA-negative "recombinant" clones of the two glycoproteins was seen with PCR. The genetic basis for this disparity in PCR results will be investigated by junction sequencing.

The ELISA described herein is still only a prototype and will require a more comprehensive optimisation and validation before being phased into routine AD serological monitoring. The high background colour may be reduced by further purification of what is a very crude extract. An alternative is the use of blocking agents. A preliminary investigation into the inclusion of an insect cell preparation in the serum diluent has shown good results. Early results with Sf9 cells show a reduction in background levels when compared to the Sf21 cells used for this work.

The sensitivity of the ELISA was satisfactory by contemporary standards and is better than the "in-house" assay currently being used in this laboratory (Banks, 1983). Although the EC reference antisera were primarily selected for use with gI ELISA kits, the EC1 reference serum represents a thorough test of sensitivity. A more flexible range of ELISAs may have been possible with the use of an ADV gI recombinant glycoprotein. Attempts to express this glycoprotein in the

baculovirus system have so far failed; a recombinant gI expressed in *E. coli* is currently being assessed for use with the ELISA.

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DIFFERENTIATION OF AUJESZKY'S DISEASE VIRUS STRAINS ISOLATED IN POLAND USING DNA BIOTINYLATED PROBES

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The aim of this study was to compare 17 different Aujeszky's disease virus (ADV) isolates from clinical outbreaks of AD by using DNA biotinylated probes. All isolates were collected in Poland between 1984 and 1991. The restriction fragment pattern (RFP) analysis done by hybridization to NIA-3 DNA biotinylated probe indicated that all Polish ADV field strains can be classified as type I of Suid herpesvoirus 1. Hybridization with *Bam*HI fragment 7 and gI gene biotinylated probes revealed an unusual heterogeneity of *Bam*HI fragment 7 in almost 50% of strains isolated in Poland. The nature of the molecular changes in this fragment will be discussed.

Key words: Aujeszky's disease virus, clinical outbreak, isolate, differentiation, DNA probe, restriction fragment analysis

Aujeszky's disease (AD) is caused by a typical representative of the family Herpesviridae, subfamily Alphaherpesvirinae, genus Varicellovirus — Herpesvirus suis 1 [Suid herpesvirus 1 (Brown, 1989)]. In the epidemiology of AD, swine have the greatest significance. These animals become infected regardless of their age and immunological status. The simultaneous occurrence of latency implies that swine become permanent carriers and shedders of AD virus (ADV) and its only reservoir (Ohlinger et al., 1991; Sabo, 1981; Ursache et al., 1984; Vannier, 1984; Wittmann, 1982; Wittmann, 1984).

A rapid increase in the number of AD outbreaks and, connected with this, considerable losses in pig husbandry in many countries in the early 'seventies (Van Oirschot, 1991) forced the investigation of this disease and ADV itself. The restriction fragment pattern (RFP) analysis of viral DNA (Gielkens and Berns, 1982; Knowles and Gorham, 1990; Ludwig et al., 1982; Paul et al., 1982) is one of the fundamental techniques in these studies. RFP analysis was also used in epidemiological studies on AD and it was found to be very efficient in monitoring the spread of ADV (Andersen et al., 1989; Cristensen et al., 1992; Christensen et al., 1990; Christensen and Sørensen, 1988; Christensen and Sørensen, 1991; Erickson et al., 1986; Espuna et al., 1991; Jestin et al., 1990).

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The sensitivity of RFP analysis was considerably increased by means of nucleic acid hybridization (Gielkens and Berns, 1982; Knowles and Gorham, 1990; Pirtle et al., 1984). Nucleic acid probes used in these investigations labelled with isotopes are increasingly replaced by non-radioactive probes because of their stability and safety. Among them biotin-labelled probes are of increased interest (Knowles and Gorham, 1990; Maes et al., 1988; Norval and Bingham, 1987; Paul, 1990; Wilchek and Bayer, 1988).

The aim of this work was to analyze and compare different field strains of ADV isolated in Poland using DNA biotinylated probes.

Materials and methods

Virus isolates. In the studies 17 field isolates of ADV from clinical outbreaks in different regions of Poland were used (Table 1). ADV strains with numbers 1–10, 12–14 and 17 were isolated in the years 1984–1991 and isolates 11, 15 and 18 in the mid-'70s. With the exception of strains 1, 11 and 16 isolated from breeding foxes and 9 isolated from a dog all others were of pig origin. Strain NIA-3 was included as a reference for genomic characterization.

Table 1

Description of the ADV field strains used in the studies

Strain no.	Description	Origin	Year of isolation
1	Au 12	breeding fox	1984
2	Au 11	pig	1990
3	Au 9	pig	1990
4	Au 6	pig	1990
5	Au 5	pig	1990
6	18/06	pig	1989
7	3149–3150	pig	1990
8	39/87	pig	1987
9	03/91	dog	1991
10	02/91	pig	1991
11	PR-75	breeding fox	1975
12	343/90	pig	1990
13	9/89	pig	1989
14	330–332A	pig	1989
15	Kz	pig	1976
16	P-74	breeding fox	1974
17	Au 7(10)	pig	1990

Purification of viral DNA. Viral strains were grown on RK 13 rabbit kidney cell line (Christofinis and Beale, 1968) until the cytopathic effect was well advanced, approximately 48 h post infection. Cells were spun down and the viral DNA was purified by a short procedure which included disruption of cells, treatment of the supernatant with RNase and DNase, phenol/chloroform extraction and ethanol precipitation.

Preparation of biotinylated DNA probes. Three biotinylated probes were prepared from the NIA-3 DNA, i.e. a full length DNA probe, *Bam*HI fragment 7 probe and gI gene probe. For labelling NIA-3 DNA or its fragments photobiotin was used according to the method described by Forster et al. (1985). Briefly, it was as follows: mixing of the DNA with photobiotin, irradiation with visible light and finally ethanol precipitation of biotinylated DNA.

Restriction fragment pattern analysis. DNA of 17 ADV field isolates was digested with *Bam*HI restriction endonuclease (New England Biolabs, Inc.) and subjected to agarose gel electrophoresis (Maniatis et al., 1982).

Southern blot hybridization. Restriction fragment patterns were transferred to nitrocellulose membrane (Schleicher-Schuell, BAS 85, 0.45 μm) as described by Maniatis et al. (1982). Hybridization with biotinylated DNA probes and visualization using streptavidin-alkaline phosphate conjugate and NBT/BCIP dyes was done according to Maniatis et al. (1982).

Results

The fast and simple method of viral DNA purification which we have developed yields nucleic acids of sufficient purity both for preparing DNA biotinylated probe and for RFP analysis of ADV isolates. The biotinylated probes were found to be very specific, sensitive and stable.

*Bam*HI generated fragment patterns of purified viral DNAs and the corresponding Southern blot hybridizations with biotinylated full length DNA probe are shown in Fig. 1. The difference in mobilities of some of the bands as compared to those of NIA-3 DNA can in most cases be attributed to changes in the inverted repeat regions of the genome or in the regions spanning the junction between the inverted repeats and U_S/U_L, i.e. fragments 5, 8, 8', 10, 12 and 13. These fragments are known to be highly variable.

However, the most significant feature of almost 50% of the isolates was the lack, or at least mobility changes, of *Bam*HI fragment 7 from its original position in comparison with NIA-3. This refers to the strains no. 1, 2, 3, 4, 5, 6, ,7, 8 (Fig. 1A) and 17 (Fig. 1B). In the case of strains 3 and 4 *Bam*HI fragment 7 was hardly visible (Fig. 1A).

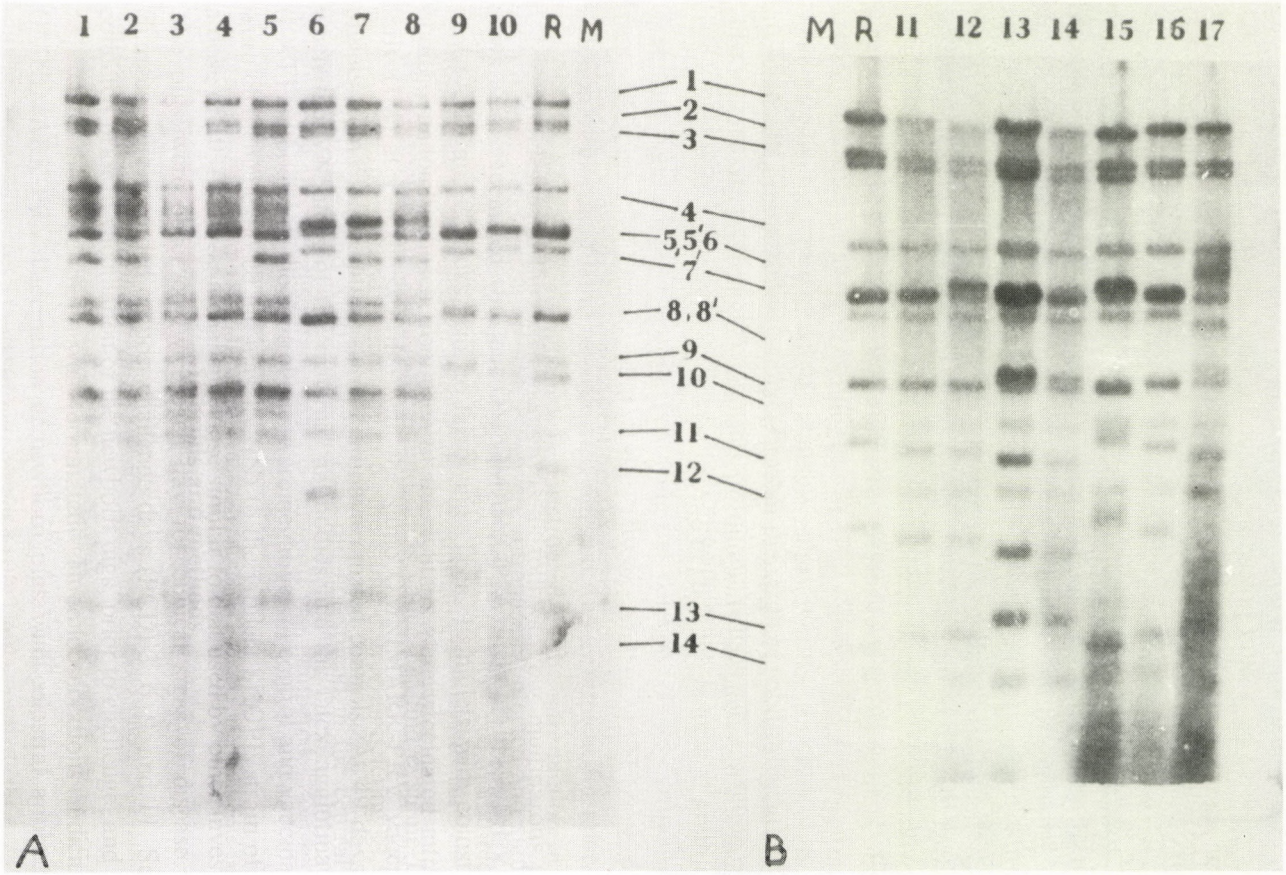

*Fig. 1. Bam*HI restriction profiles of ADV DNA isolates collected in Poland after hybridization with NIA-3 DNA biotinylated probe. Lanes 1–10 (A) and 11–17 (B): field isolates; lanes R (A and B): reference NIA-3 strain; lanes M (A and B): molecular weight standard (phage λ DNA digested with *Hind*III)

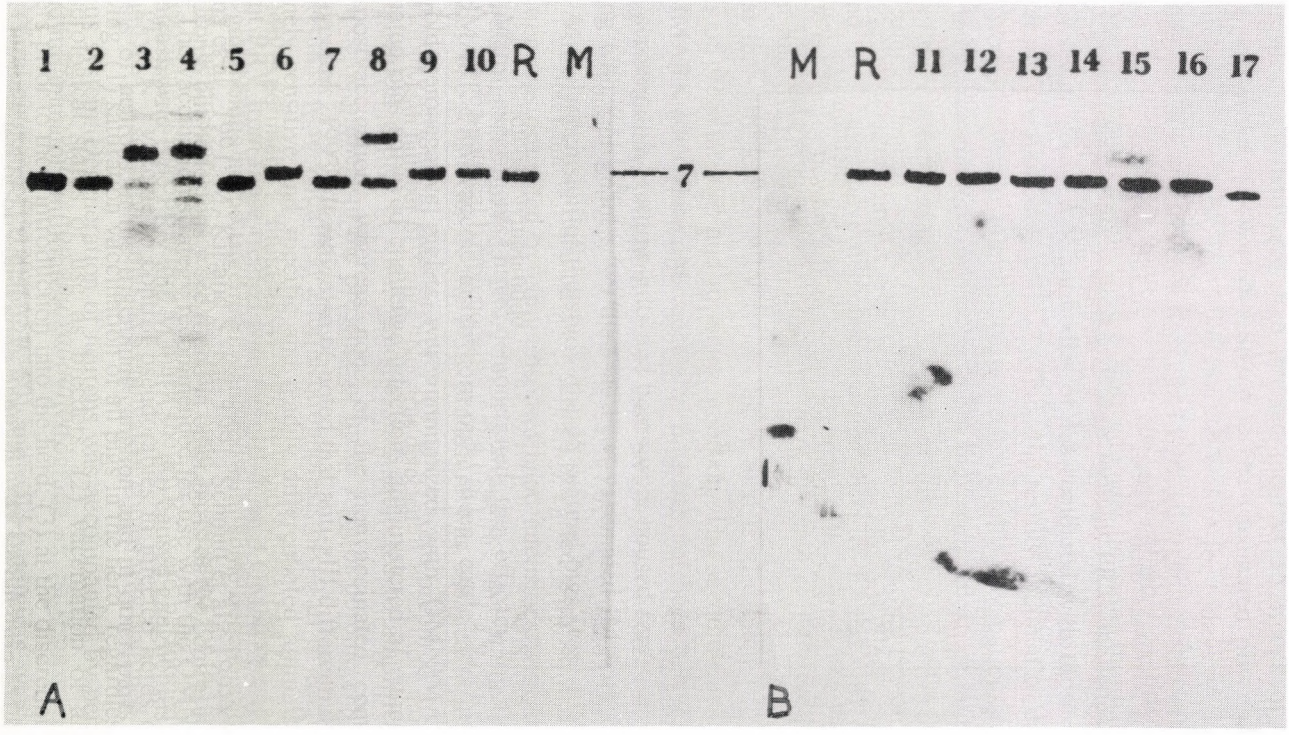

Fig. 2. Southern blot containing *Bam*HI generated fragment patterns of ADV DNA isolates to which biotinylated *Bam*HI fragment 7 probe was hybridized. Legend: see Fig. 1

Figure 2 shows Southern blot containing *Bam*HI digests of the DNAs of the ADV field isolates to which biotinylated *Bam*HI fragment 7 probes was hybridized. The unusual RFP behaviour of fragment 7 of the above-mentioned strains was confirmed. In the case of strains no. 3 and 4 hybridization with fragment 7 probe reveals the presence of several bands and in the case of strain 8 two bands in *Bam*HI digest are visible (Fig. 2A). Strains 1, 2, 5, 7 (Fig. 2A) and 17 (Fig. 2B) show a slightly truncated *Bam*HI fragment 7.

Hybridization with biotinylated gI gene probe (data not shown) gave the same profile as with *Bam*HI fragment 7 probe.

Discussion

On the basis of the RFP analysis and Southern blot hybridizations with biotin-labelled NIA-3 DNA it can be concluded that all the ADV field isolates can be classified as type I of Suid herpesvirus 1. This finding is inconsistent with that of Christensen and Sørensen (1988) who stated that in Central Europe type II of Suid herpesvirus 1 is common. However, they did not mention which particular country in Central Europe they had in mind.

The observed diversity of the tested ADV isolates is typical of wild type strains and is used not only for monitoring the spread of ADV but also for subgrouping of the isolates (references: see Introduction). What we found interesting in 8 out of 17 ADV isolates tested, however, were the changes in electrophoretic mobilities of *Bam*HI fragment 7 which is conserved and codes for proteins responsible for virulence. Similar results were obtained by Christensen et al. (1992). They tested 7 of our isolates (i.e. strains no. 1, 3, 4, 5, 15, 16, 17) and additionally BUK-TK 900 used in Poland as a live vaccine. After RFP analysis and subsequent hybridization with *Bam*HI fragment 7 probe they found by comparison with BUK-TK 900 that some of the Polish field isolates are derivatives of this vaccine strain. In the same work Christensen et al. (1992) revealed that 3 out of 5 Hungarian isolates tested have been derived from the Bartha K-61 strain.

Taking into account the above-mentioned results it was interesting to characterize further the isolates of interest. Four ADV field strains (no. 1, 4, 6, 8) out of 17 were chosen and BUK-TK 900 and NIA-3 were used as reference strains. By comparative RFP analysis and hybridization with *Bam*HI fragment 7 and gI gene probes it was found that strains no. 1, 4 and 8 seemed to be derivatives of the BUK-TK 900 vaccine strain. This result is partly in accordance with that reported by Christensen et al. (1992). To investigate in which part of *Bam*HI fragment 7 the changes are located, DNAs from all the tested ADV strains were double-digested with both *Bam*HI and *Sal*I endonucleases. As a result 2 subfragments of *Bam*HI fragment 7 were obtained: *Sal* 7A (2000 base pairs in the case of reference NIA-3 strain) and *Sal* 7B (4800 base pairs in the case of reference NIA-3

strain). Fragment *Sal* 7A contains genes for glycoprotein gX and a part of gp50 and fragment *Sal* 7B contains gene for the rest of gp50 and genes for gp63, gI, 11K and a part of 28K (Kost et al., 1989; Mettenleiter, 1991). After hybridization with *Bam*HI fragment 7 biotinylated probe it was found that fragment *Sal* 7A was of the same size in all tested ADV stains including NIA-3 and BUK-TK 900. The differences were seen in fragment *Sal* 7B spanning genes for gp50 (a part of), gp63, gI, 11K and a part of 28K.

The remaining ADV isolates are currently being tested. As a continuation of this work, we will concentrate on the detailed mapping of the mutations observed in *Sal* 7B fragment.

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SEROLOGICAL STUDIES IN AUSTRIAN FATTENING PIGS WITH RESPIRATORY DISORDERS

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Serum samples of 253 fattening pigs out of 44 different herds with respiratory signs were examined for antibodies to Aujeszky's disease virus by ELISA, swine influenza virus (HI test), porcine respiratory coronavirus (ELISA) and porcine reproductive and respiratory syndrome virus (IPMA). One single case of Aujeszky's disease was detected at slaughter. On the other hand 24.5% of the animals proved to be positive for swine influenza, although no vaccine is licensed in Austria, and 63.6% reacted positively to porcine respiratory coronavirus. None of the sera showed antibodies to porcine reproductive and respiratory syndrome virus, which has not as yet been identified in the Austrian swine population.

Key words: Respiratory diseases, serology, pig, Austria

Viral infections play a major role in porcine respiratory disorders. The aim of this study was to determine the seroprevalence of 4 different respiratory viruses in Austrian fattening pigs with a history of respiratory disease.

Materials and methods

A total of 253 serum samples of fattening pigs out of 44 different herds were investigated: 175 sera (out of 35 herds) were taken at slaughter, and 78 sera (9 herds) at the farms. Antibodies to Aujeszky's disease virus (ADV) were examined by ELISA (Chekit® Aujeszkytest, Dr. Bommeli AG, Bern), swine influenza virus (SIV) A/H1N1, strain SW/Germany/2/81 by HI test (Palmer et al., 1975), porcine respiratory coronavirus (PRCV) by ELISA (Svanova Biotech, Uppsala) and porcine reproductive and respiratory syndrome virus (PRRSV) by indirect immunoperoxidase monolayer assay (IPMA; Ohlinger et al., 1991; Wensvoort et al., 1991).

Results

The results are summarized in Tables 1 and 2.

Table 1

Percentage of fattening pigs with antibodies to

Aujeszky's disease virus (ADV)	0.03%[1]	
Swine Influenza Virus (SIV)	24.5%	19.0% of them SIV + PRCV positive
Porcine Respiratory Coronavirus (PRCV)	63.6%	
Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)	0.0%	

[1]One single case detected at slaughter

Table 2

Percentage of herds with positive animals to

Aujeszky's disease virus (ADV)	[1]	
Swine Influenza Virus (SIV)	40.9%	36.4% of them SIV + PRCV positive
Porcine Respiratory Coronavirus (PRCV)	81.8%	
Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)	0.0%	

[1]One single case detected at slaughter

Discussion

The serum of one fattening pig collected at slaughter proved to be positive for antibodies to ADV. The examination of the herd revealed no further cases of AD, possibly because all the animals of that group had been slaughtered. In Austria AD is controlled by eradication. The last 2 positive cases were reported from April 1992 on the occasion of a serological survey (Amtliche Veterinärnachrichten, 1992). At present it seems that Austria is free of AD.

The clinical syndrome of swine influenza occurred in Austria for the first time from April 1983 to January 1984 (Bürki et al., 1985). In 1992/93 24.5% seropositive animals and 40.9% positive herds, respectively, indicate a relatively high prevalence of infection with SIV A/H1N1. In contrast to the first enzootic in

1983/84, the recent course of infection was mild in the majority of cases, nevertheless almost all of the animals in a herd seroconverted.

Since the introduction of PRCV into the Austrian swine population in 1988/89 (Möstl et al., 1989) a high seroprevalence of 63.6% positive animals and 81.8% positive herds, respectively, has been revealed. PRCV is thought to be a cofactor in respiratory disease, which was supported by our results (19.0% pigs and 36.4% herds, respectively, showed antibodies both to SIV and PRCV).

Although some neighbouring countries report a high seroprevalence, PRRS has not yet been observed in the Austrian swine population, either clinically or serologically. In addition to our negative results 800 serum samples tested in the Bundesanstalt für Viruskrankheiten bei Haustieren (Vienna) proved negative as well (Renate Krassnig, pers. commun.).

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CHEMILUMINESCENT DETECTION OF AMPLIFIED PSEUDORABIES VIRUS gp50 DNA WITH IMMOBILIZED PROBES ON MICROTITER WELLS

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A highly specific and sensitive PCR assay for the envelope glycoprotein gp50 has been developed for the detection of PRV-DNA sequences. Primer pairs from PRV gp50 gene were used with the enzyme uracil N-glycosylase and dUTP instead dTTP to prevent contamination due to PCR product carry-over. Biotinylated PCR products were captured in microtiter wells by specific oligonucleotide covalently linked to the polystyrene wells. After recognition of the biotinylated PCR product with a streptavidin phosphatase conjugate, a chemiluminescent detection was realised. Different factors influencing the binding, the hybridization and the detection efficiency have been tested.

Key words: Aujeszky's disease, PCR diagnosis, microtiter wells, chemiluminescence

Several methods for detecting PRV-DNA sequences by PCR have been described (Belák et al., 1989; Jestin et al., 1990). Generally, PCR-amplified DNA is identified by gel electrophoresis and ethidium bromide staining or hybridization. These methods are not suitable for routine clinical diagnosis. Diagnostic laboratories use the 96-well format for immunological techniques. Therefore, we developed a chemiluminescence-based detection of PCR products on microtiter wells which were coated with an oligonucleotide.

The power of the PCR is a source of false positive results. The products of previous PCR reactions may contaminate pre-PCR reaction mixes. Physical separation of pre- and post-PCR work areas, the use of positive displacement pipettes and aliquoted reagents are not completely efficient. We chose to synthesize PCR products with dUTP instead of dTTP. The enzyme uracyl N-glycosylase (UNG) destroys U-containing DNA which can be a target of PCR reactions (Longo et al., 1990).

The present paper describes a new method to detect PRV DNA sequences on a 96-well format. This method includes the coating of microplates, the addition of an internal control and a chemiluminescent detection.

Materials and methods

Amplification of PRV gp50 gene sequence. PCR mixtures (20 µl) for amplification of gp50 sequence from PRV DNA contained 67 mM Tris-HCl, pH 8.8; 16.6 mM $(NH_4)_2SO_4$; 1.5 mM $MgCl_2$; 0.1% Tween 20; 150 µM dATP, dCTP, dGTP; 300 µM dUTP; 10% DMSO; 1U Taq polymerase; 8 pmole of oligonucleotide primers:

498: 5' Biotin-CCTGGTGTCCGTCGACGGCGTGAAC 3'

669: 5' CCGGTGCGGGGGCTGCTGGTAGAAC 3'

Reactions were cycled: 50 °C 15'; 95 °C 10'; 5 times 94 °C 30", 66 °C 30", 72 °C 30" and 35 times 94 °C 30", 64 °C 30", 72 °C 30".

Construction of an internal control. An internal control (IC) was constructed by introducing a few base pair modifications in the target viral DNA. This IC was added to the sample (Boutin et al., submitted).

Covalent binding of oligonucleotide capture to microwell plates. Phosphorylated oligonucleotides were covalently bound to the polystyrene at their 5' end by a phosphoramidate bond according to Rasmussen et al. (1991). One was specific to PCR-PRV (GAAGTCGGTG) and the second to the internal control amplified DNA (Boutin et al., submitted).

Hybridization. The wells were rinsed with hybridization buffer (6× SSC, 5× Denhardt, 100 mg/ml herring sperm DNA). Hybridization was performed for 2 hours at 37 °C with 100 µl of buffer in presence of 25 µl of biotinylated PCR products. After hybridization, plates were washed at 37 °C, once with 2× SSC, 0.1% Tween-20 and twice with 0.1× SSC, 0.1% Tween-20. 100 µl of streptavidin-alkaline phosphatase conjugate (1:500) were added per well and incubated for 30' at 37 °C. The conjugate buffer was: 0.3M NaCl; 0.1M Tris-HCl pH 7.5; 2mM $MgCl_2$; 0.05% Triton ×100. Then the wells were washed 3 times with the conjugate buffer.

Colorimetric detection (according to Kawai et al., 1993). 100 µl of 1M diethanolamine buffer (pH 9.8) containing 0.5 mM $MgCl_2$ and 10 mM p-nitrophenyl phosphate (PNPP) were added. The colorimetric reaction was performed at 37 °C for 30'. The enzyme reaction was stopped with 5 µl of 10 mM NaOH and the OD was read at 405 nm.

Chemiluminescent detection. 100 µl of Lumi-Phos 530 solution (Boehringer Mannheim, Germany) was added per well. This solution contains: 0.33mM 4-methoxy - 4-(3-phosphatephenyl)-spiro-(1,2-dioxetane-3,2'-adamantane), disodium salt; 750 mM 2-amino-2-methyl-1-propanol; 0.88 mM $MgCl_2$; 1.13mM cetyltrimethylammonium bromide; 0.035 mM, pH 9.6, fluorescein surfactant. The plates were incubated for 15' at 37 °C. The light released was read using a luminometer (Berthold, Germany).

Results

Amplification. The PCR system chosen could detect between 1 to 5 copies of gp50 gene in presence of 1 μg of porcine DNA (data not shown). Two PCR products from PRV DNA were produced using biotinylated primer 498 and primer 669. The first corresponded to the native sequence of gp50 and was named PCR-PRV. The second corresponded to the internal control (IC) and differed from the PCR-PRV sequence by a few base pairs. Hybridization was performed in microtiter wells 2 hours at 37 °C. Dilutions of each denatured PCR products were added in wells.

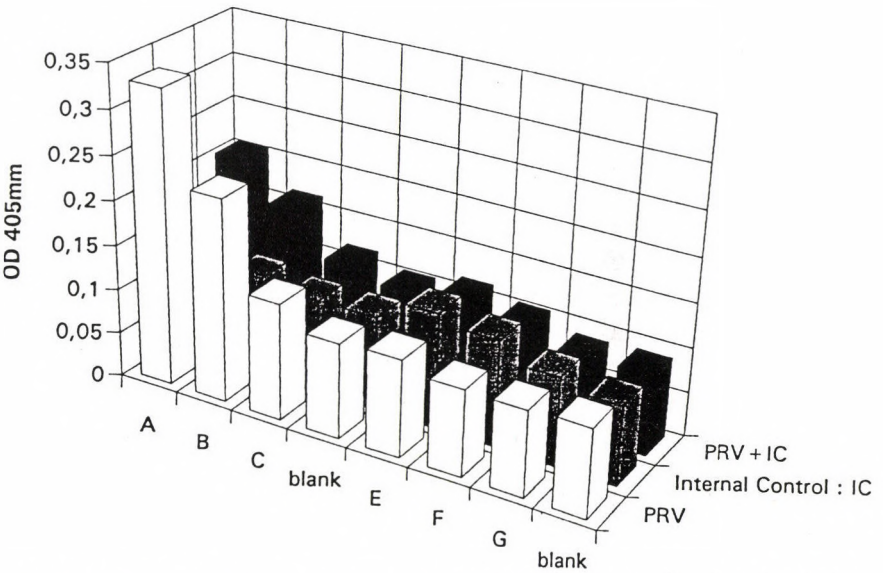

Fig. 1. Colorimetric detection test. Lanes ABCD: Wells coated with PCR-PRV oligonucleotide capture. Lanes EFGH: Wells coated with IC (internal control) oligonucleotide capture. PCR products added in wells: A: PCR, dilution 1; B: PCR, dilution 1/2: C: PCR, dilution 1/20; D: negative control (no DNA); E: IC, dilution 1; F: IC, dilution 1/2; G: IC, dilution 1/20; H: negative control (no DNA)

UNG system. dUTP was used instead dTTP. dUTP was two times more concentrated than the other dNTPs. Enzymatic degradation of PCR contaminants (containing dUTP) was achieved by pre-incubating each PCR mixture at 50 °C for 15 min. The enzyme was heat-denatured to avoid any activity during the next steps.

Binding of capture oligonucleotide. Different concentrations of oligonucleotides coated were tested (7.5 to 60 ng/µl). The optimum of hybridization was realised at 30 ng/µl.

Hybridization. Temperature and buffer of hybridization were tested for their effects on the capture efficiency. A better signal (OD or RLU) was obtained at 37 °C in presence of 6× SSC, 5× Denhardt's and 100 µg/ml herring sperm DNA.

Colorimetric and chemiluminescent detection. Hybridized PCR products were detected with alkaline phosphatase-conjugated streptavidin and a chromogenic or a chemiluminescent substrate.

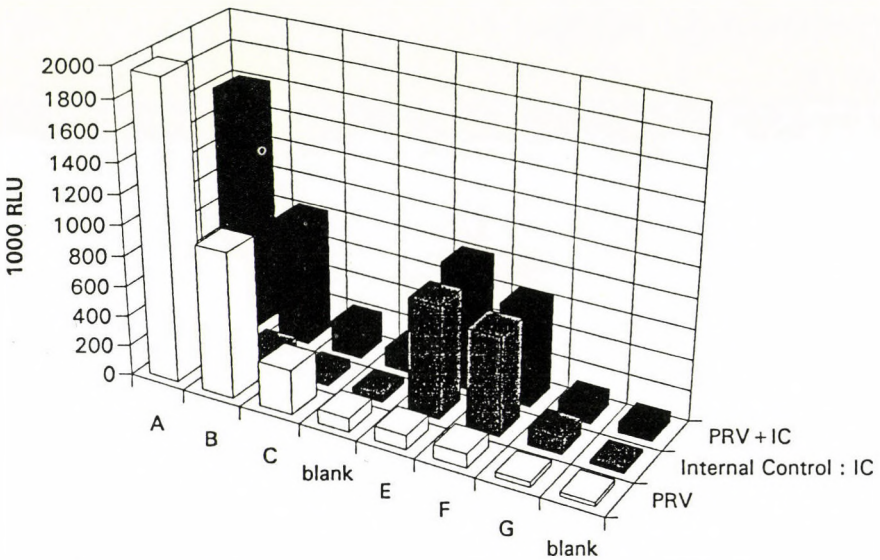

Fig. 2. Chemiluminescent detection test. Lanes ABCD: Wells coated with PCR-PRV oligonucleotide capture. Lanes EFGH: Wells coated with IC (internal control) oligonucleotide capture. PCR products added in wells: A: PCR, dilution 1; B: PCR, dilution 1/2; C: PCR, dilution 1/20; D: negative control (no DNA); E: IC, dilution 1; F: IC, dilution 1/2; G: IC, dilution 1/20; H: negative control (no DNA);
RLU: Relative Light Unit

The results of the hybridization are represented in Figs 1 and 2. The reaction was specific. Each capture recognized only its own PCR product. For the colorimetric detection (Fig. 1), the signal (OD) was positive only for the dilutions 1 and 1/2. The background level was too high. When we used a chemiluminescent detection this background was less significant and the dilution 1/20 became positive. Chemiluminescence thus increased the sensitivity.

Discussion

Using the UNG system (Longo et al., 1990), we decreased the problem of false positive results. The pre- and post-PCR areas were separated with their own pipettes and aliquoted reagents (Kwok et al., 1989).

Sometimes in biological samples the problem of Taq polymerase inhibition can induce false negative results. In each sample, we add the internal control which is co-amplified with the target DNA (Cone et al., 1992; Zazzi et al., 1992).

The advantages of microtiter format is that it is known and practical in routine diagnosis (Whetsell et al., 1992; Bretagne et al., 1993). The power of chemiluminescence (Suzuki et al., 1992) allows a very sensitive detection of PRV DNA sequences.

The microtiter format allows automation (Harrison et al., 1993). The purpose of our work is to develop a rapid and safe diagnostic method for PRV DNA sequences and other important pig pathogens.

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SPATIAL AND BIOLOGICAL CHARACTERISTICS OF BETWEEN-HERD TRANSMISSION OF AUJESZKY'S DISEASE VIRUS

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The eradication of Aujeszky's disease virus (ADV) in Pennsylvania has suffered because the modes of viral transmission between herds are unknown. Our objective was to identify the major risk factors involved in viral transmission by comparing proportions of operation type, density, quarantine level, and vaccination status of neighboring herds for infected case and uninfected control herds. Multivariate analysis demonstrated an elevated risk of infection with local increases in the proportion of finishing floors. A local increase in the proportion of ADV-quarantined herds was shown to be a significant risk factor, as was an increase in the proportion of vaccinating herds. A reduced risk of infection was found with local increases in the number of feeder pig producer operations.

Key words: Aujeszky's disease virus, between-herd virus transmission, risk factors

The program to eradicate Aujeszky's disease virus (ADV) in Pennsylvania has existed for approximately ten years. While this program has helped eliminate ADV from individual herds, it has not succeeded in lowering the herd prevalence of ADV. Newly infected herds continually outnumber or equal those herds removed from quarantine. Eradication efforts are hindered by unidentified sources of herd infection. The two most common sources of infection cited are "unknown", and "area spread", which are, in effect, synonymous. Analysis of the dynamic pattern of ADV infection in Pennsylvania is now possible due to the incorporation of an ADV-Geographic Information System (ADV-GIS) into the eradication program. The ADV-GIS contains temporal and spatial locations of infected and uninfected swine herds and geologic, epidemiologic, and ADV strain data. The aim of this study was to identify area risk factors for new herd infections. The information gained from this study will be used to help identify modes of between-herd transmission of ADV. Because most of the infected farms are located in and immediately around Lancaster County, PA, we have centered our research on this region.

Materials and methods

Location, herd attributes (biosecurity, herd management, and demographics), and temporal vaccination and quarantine data for swine herds in Lancaster County were obtained from producer questionnaires and Pennsylvania state records. Data were organized using the spreadsheet programs Lotus 1-2-3 and Paradox, and transferred to the ADV-GIS, which utilizes Arc-Info software. The ADV-GIS also contains the road and stream networks and county boundary data received from the Pennsylvania Department of Transportation. Control herds were selected using a random number table. These were group-matched to case herds by year of case quarantine. Study herds were placed at the center of a 7.5 km^2 area and proportions of operation types, density, quarantine level, and vaccination status of neighboring herds were compared using SAS (SAS Institute Inc., 1989).

Results

Study herds. Ninety-nine ADV-quarantined herds infected between 1986 and 1993 were identified as cases and an equal number of uninfected herds were randomly chosen as controls. Sixty-three percent of the 99 case herds in the study were farrow-to-finish operations. The remainder of the cases were comprised of feeder pig finisher (32%) and feeder pig producer operations (5%). Of the 99 uninfected control herds, approximately 41% were feeder pig finisher operations. Farrow-to-finish (31%), feeder pig producer (19%), and miscellaneous operations (show animals, pets) constituted the rest of the control herds.

Case herds were categorized by date of first quarantine order. Thirty-seven percent of the case herds in the study were quarantined in 1992, while in other years, the number of herds quarantined remained relatively constant, ranging from five to twelve percent.

Multivariate analysis. Statistical analysis using a logistic regression model was used to evaluate the probability of herds becoming infected conditional on spatial risk factors. An elevated risk of infection was seen with a local increase in the proportion of finishing floors. A reduced risk of infection was found with local increases in the number of feeder pig producer operations. Increased swine herd density in a 7.5 km^2 area surrounding study herds was not found to elevate the risk of infection. However, a local increase in the proportion of herds quarantined was found to be a significant risk factor, as was an increase in the proportion of ADV-vaccinated herds.

Discussion

The two main risk factors of ADV transmission identified in this study were area quarantine and vaccination densities. Austin and Weigel (1992) also found that increased regional ADV density was a risk factor for ADV infection. We did not find swine density to be a risk factor for a herd becoming infected. This differs from the results of Marsh et al. (1991) who identified regional density of swine herds as a risk factor. An unusual finding in our study was that as the proportion of herds vaccinating for ADV increased, the risk of infection for neighboring herds also increased. One reason for this may be that producers were not vaccinating their entire herd, and therefore the area was only partially protected. It is also possible that producers were using vaccination as a substitute for implementing adequate biosecurity measures.

Acknowledgements

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EFFECT OF VACCINATION ON SHEDDING OF AUJESZKY'S DISEASE VIRUS IN EXPERIMENTALLY INFECTED PIGS AND ROLE OF VACCINATION IN HERD HEALTH PROGRAMMES

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The effect of vaccination on the shedding of Aujeszky's disease virus (ADV) was investigated in three experiments:

Experiment I (see Table 1). Twelve miniature pigs aged 5–6 months were divided into groups A, B, C and K. Group A (n=2) was treated with two doses, and group B (n=2) with three doses of a commercial inactivated vaccine. Group C (n=3) received two doses of the subunit ADV antigen with a lipoid adjuvant. The antigen was obtained from an infected cell culture, treated with the detergent NP-40 and ultracentrifuged. The interval between the 1st and the 2nd dose was 21 days and that between the 2nd and the 3rd dose was 40 days. Group K was left unvaccinated to serve as control. Group A and C were infected 47 days and group B 7 days following the 2nd and 3rd vaccination, respectively.

Experiment II (see Table 2). Fifteen Large White (LW) weaners aged 5–6 weeks were divided into groups A, B and K with 5 animals each. Group A received two doses and group B three doses of a commercial inactivated vaccine, the intervals being 21 days between the 1st and the 2nd dose, and 38 days between the 2nd and the 3rd dose. Group K was left unvaccinated to serve as control. The interval between the last vaccination and the infection was 45 and 7 days in groups A and B, respectively, except for pig no. 1 where the interval was only 17 days.

Experiment III (see Table 3). Fourteen LW weaners aged 5–6 weeks were divided into groups A and B with 7 animals each. Both groups received three doses of a commercial inactivated vaccine, the intervals between the 1st and 2nd, and the 2nd and 3rd dose being 21 and 42 days, respectively, and the vaccination-to-infection inteval being 7 and 28 days in groups A and B, respectively.

Conclusions

1. A high degree of humoral immunity developed in animals after three treatments with the commercial inactivated vaccine.

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Table 1

The effect of vaccination on the excretion of ADV following intranasal infection (10^6 $TCID_{50}$ per animal; Experiment I)

Number of treatments Vaccine type	Group	Pig no.	Int	Pre-infection titre (ELISA)	Virus detection (dpi)											
					1	2	3	4	5	6	7	8	9	10	11	12
2x	A	1	47	2,700	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	–	–	–	–	–	–
inactived		2	47	2,700	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	–	–	–	–	–	–
3x	B	3	7	72,900	–	T+	–	–	–	–	–	–	–	–	–	–
inactived		4	7	24,300	–	–	–	–	T+	–	–	–	–	–	–	–
2x		5	47	8,100	T+	T+	T,N+	T+	T+	T+	T+	–	–	–	–	–
	C	6	47	2,700	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	–	–	–	–	–
subunit		7	47	2,700	T+	T,N+	T,N+	T,N+	T,N+	T,N+	–	T,N+	T+	–	–	–
		8	0	negative	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	T+	T,N+	T,N+	T+	–	–
non-		9	0	negative	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	died	died	died	died	died
vaccinated	K	10	0	negative	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	N+	–	–
		11	0	negative	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	N+	N+	–	N+	–	–
		12	0	negative	T+	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	–	–	–

dpi = days post infection; Group A received two doses; Group B received three doses; Group C received two doses; int = last vaccination-to-infection interval (days); T,N+ = tonsils and nasal cavity positive; T+ = tonsils only positive; N+ = nasal cavity only positive; – = virus not detectable

Table 2

The effect of vaccination on the excretion of ADV following intranasal infection (10^6 $TCID_{50}$ per animal; Experiment II)

Number of treatments Vaccine type	Group	Pig no.	Int	Pre-infection titre (ELISA)	Virus detection (dpi)													
					1	2	3	4	5	6	7	8	9	10	11	12	13	14
		1	17	2,700	–	–	–	–	–	–	–	–	–	T+	–	–	–	–
T,N+		2	45	2,700	T+	T+	T,N+	T,N+	T,N+	T,N+	T,N+	–	–	T+	–	–	–	–
	A	3	45	2,700	–	–	T+	T+	T,N+	T+	T+	T+	–	T+	–	–	–	–
inactived		4	45	2,700	–	–	–	N+	N+	N+	–	–	–	–	–	–	–	–
		5	45	900	N+	N+	T+	T,N+	T,N+	T+	T+	T+	T+	–	–	–	–	–
		6	7	24,300	–	–	–	T+	–	T+	–	–	–	–	–	–	–	–
3x		7	7	8,100	–	–	–	–	–	T+	–	–	–	–	–	–	–	–
	B	8	7	8,100	–	T+	–	–	T+	–	T+	T+	T+	T+	–	–	–	–
inactived		9	7	8,100	–	–	–	–	T+	–	–	–	–	–	–	–	–	–
		10	7	24,300	–	–	–	–	–	–	–	–	–	–	–	N+	–	–
		11	0	negative	–	T+	T+	T,N+	T,N+	T,N+	T,N+	N+	N+	T,N+	N+	N+	N+	N+
non-		12	0	negative	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	T+	T,N+	T,N+	N+	–
vaccinated	K	13	0	negative	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	T+	T+	T,N+	T+	–	T+	T+	died
		14	0	negative	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	T+	T+	T+	–	–	–
		15	0	negative	T,N+	T,N+	T,N+	T,N+	T,N+	T,N+	died	died	died	died	died	died	died	died

dpi = days post infection; Group A received two doses; Group B received three doses; int = last vaccination-to-infection interval (days); T,N+ = tonsils and nasal cavity positive; T+ = tonsils only positive; N+ = nasal cavity only positive; – = virus not detectable

Table 3

The effect of three doses of an inactivated vaccine on the excretion of ADV following intranasal infection (10^6 $TCID_{50}$ per animal; Experiment III)

Group	Pig no.	Int	Neutralizing antibody		Virus detection (dpi)													
			pre-infection	14 dpi	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	1	8	2,048	512	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	2	8	1,024	1,024	–	–	–	–	–	–	T+	–	–	–	–	–	–	–
	3	8	512	nd	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	4	8	128	256	–	–	–	T+	–	–	–	–	T+	–	–	–	–	–
	5	8	512	256	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	6	8	512	256	–	–	–	–	–	–	–	–	T+	–	–	–	–	–
	7	8	512	512	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B	8	28	128	1,024	T+	–	–	–	T+	T+	–	–	–	–	–	–	–	–
	9	28	256	1,024	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	10	28	256	1,024	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	11	28	128	1,024	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	12	28	64	2,048	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	13	28	512	256	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	14	28	512	512	–	–	–	–	–	–	–	–	–	–	–	–	–	–

dpi = days post infection; Group A received three doses; Group B received three doses; int = last vaccination-to-infection interval (days); nd = not done; T+ = tonsils only positive; – = virus not detectable

2. Virus shedding was strongly reduced and limited mostly to tonsils in the vaccinates after three treatments with the inactivated vaccine. The titre of the shed virus never exceeded 10^2 $TCID_{50}$/ml.

3. Treble vaccination allowed us to eliminate ADV from large swine herds without any operational interruptions.

The disease control in the herd proceeded in three stages without any operational interruptions as described below:

Stage 1:

- All breeder sows and boars were vaccinated repeatedly at three-month intervals.
- Purchased gilts and porkers were included in the herd only after double vaccination. The third vaccination was done thereafter (six month after the second one).
- Piglets and porkers born in the farm were vaccinated three times, namely at the age of 8, 13 and 19 weeks.
- Serologically negative sentinel gilts and porkers were introduced into the herd after a one-year period of vaccination of the whole herd. The sentinel animals were examined by ELISA one month after their transfer to the herd and then repeatedly at two-month intervals.
- The progeny of the sentinel gilts was examined serologically at the age of 3 to 4 weeks.

Stage 1 was finished as soon as negative results were obtained from all the serological examinations.

Stage 2:

No animals except the breeder sows vaccinated already during Stage 1 were vaccinated any more. Revaccination of the breeder sows was repeated at 6-month intervals until culling. Serological examinations were conducted in: (a) non-vaccinated sows at weaning; (b) breeder gilts born in the farm; (c) purchased gilts two months after their introduction into the herd; (d) aborting sows; (e) progeny of non-vaccinated sows (random tests); (f) ten per cent of fatteners at slaughter.

- Virological examinations (virus isolation attempts) were conducted in all aborted fetuses and in selected dying and emergency-slaughtered animals.
- The first extensive serological examination of all breeder gilts with a liveweight higher than 100 kg was done after the last vaccinated animal had been culled or otherwise removed from the herd and Stage 2 was concluded by disinfection and rat control.

Stage 3 (one-year observation period):

Two serological examinations of all the animals were conducted at a six-month interval during Stage 3. The herd was declared free of AD when all results

of the repeated serological tests were negative. Stage 3 was concluded by disinfection and rat control.

Conclusion

Prolonged and repeated vaccination allowed us to eliminate AD from the last positive large pig herds in the Czech Republic. Currently the Czech Republic is free of AD.

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FIELD STUDY FOR REDUCTION OF SPREAD OF AUJESZKY'S DISEASE VIRUS IN FINISHING PIGS WITH DIFFERENT VACCINATION REGIMES

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In a comparative field trial in feeder-finishing pigs on 8 farms with a history of Aujeszky's disease (AD) the incidence and spread of Aujeszky's disease virus (ADV) and serological effects of vaccination were investigated. Nobi®-Porvac Aujeszky live (strain Begonia) was used either dissolved in the aqueous adjuvant Diluvac Forte (DF) or in Unisorve (watery solvent) to vaccinate whole feeder-finishing units one or two times. Incidence of ADV measured by gI ELISA within infected finishing units was significantly reduced by the use of the adjuvant solvent DF. Furthermore the use of the adjuvant DF improved the efficiency of the vaccination as was shown by significantly higher virus-neutralising (VN) titres at slaughter time. The number of samples without detectable VN antibodies at the end of the finishing period was significantly lower in the finishing units vaccinated once with DF. The latter effect, though not statistically significant, was also seen after double vaccination in combination with the adjuvant. The investigation of VN antibody titres at the moment of the first vaccination (around 10 weeks of age) suggests that on problem farms the spread of infection can start earlier than that time.

Key words: Aujeszky's disease virus, spread, finishing pigs, vaccination regime, serology

An aqueous adjuvant (Diluvac Forte, DF) has been developed for use as a solvent for live and inactivated vaccines. Immunization and challenge experiments in pigs indicated that the use of this adjuvant in combination with the live Aujeszky's (Pseudorabies) vaccine strain Begonia improved the antibody response and the degree of protection as measured by body temperature, growth, virus excretion and clinical signs after challenge (Pensaert, et al., 1990; Vannier et al., 1991).

It has been demonstrated that with an intensive vaccination scheme it is possible to eliminate the field virus on a herd basis (Visser et al., 1991).

This trial was designed to study the increase of efficiency by the use of the adjuvant DF as a solvent for Nobi®-Porvac Aujeszky live (strain Begonia) in feeder-finishing pigs on problem farms under field conditions including single (late) and double (early) vaccination.

Materials and methods

Farms, pigs. The trial was performed between April 1991 and July 1992 on 3 closed-cycle and 5 finishing farms located in the east of Noord-Brabant, The Netherlands, an area with a very high pig density. In cooperation with local practitioners these farms were selected because they had a history of PRV infections. All farms had finishing units where all-in all-out was a standard routine. The characteristics of the individual farms participating in the field trial are described in Table 1.

Table 1

Characteristics of the farms

Farm	Size of farm	n of pigs per unit
1	800 finishing pigs + 180 sows	72
2	600 finishing pigs + 120 sows	60
3	5300 finishing pigs	120
4	1040 finishing pigs + 180 sows	80–160
5	1600 finishing pigs	84–180
6	3400 finishing pigs	80–120
7	880 finishing pigs	90
8	2000 finishing pigs	88–110

Vaccine. The vaccine used in this trial was Nobi®-Porvac Aujeszky live (strain Begonia gI^-, TK^-). As solvent the standard diluent Unisolve and the aqueous adjuvant Diluvac Forte were used. All Begonia, Unisolve and DF batches used were of commercial origin.

Vaccinations. Vaccinations were performed by the local practitioners. On farms 1 and 2 pigs were vaccinated once as on farm 3 which switched later on to double vaccination. On farms 4 to 8, all pigs were vaccinated twice. In case of a single vaccination, this was done at the age of 13–15 weeks of age. If two vaccinations were given, the first vaccination was done at the age of 10–12 weeks and the second vaccination four weeks later (14–16 weeks). Farm finishing units were randomly assigned to the two solvent groups.

Table 2 shows the number of finishing units vaccinated once or twice, with either Unisolve or with the adjuvant DF per farm.

Serology. Blood samples from ±10% of the animals were taken at first (or only) vaccination and approximately one week before slaughter. Serum samples were prepared and tested for the presence of VN and gI antibody. The VN test was performed to investigate the serological status at the moment of the (first) vaccination and to show the persistence of VN antibody induced by vaccination at the

end of the finishing period in non-infected units. The gI test was performed for monitoring field infection.

Table 2

Number of finishing units per farm

Farm	Number of finishing units included in the trial			
	1 vaccination		2 vaccinations	
	Unisolve	Diluvac Forte	Unisolve	Diluvac Forte
1	15	15		
2	8	7		
3	10	10	5	5
4			14	15
5			16	17
6			15	16
7			10	13
8			11	10
Total	33	32	71	76

Variables and statistics. VN titres at the two sampling moments were investigated in the two sample *t*-test. In the large sample sets a normal distribution was assumed. The x^2 test was used for the evaluation of categorical data. Statistical analysis was performed on a personal computer using Statistix 3.5.

Results

General aspects

During the trial period of one year 212 finishing units (in total 21, 173 pigs of different breeds) were vaccinated once or twice either with the adjuvant Diluvac Forte or Unisolve. No adverse reactions, either local or systemic, were observed.

On farm 5 there was a very high mortality (7.3–21.9%) during the last third of the trial. This was caused by respiratory disease, diagnosed as *Actinobacillus pleuropneumoniae* infection. Aujeszky's disease did not occur in these finishing units, since they all were negative for gI antibodies at the end of the finishing period.

On farm 1 (1 vaccination) and farm 4 (2 vaccinations) only one single out of approximately 240 samples at slaughter age were found to be positive for gI antibodies. Since the VN titres of these samples were relatively low, these samples were considered to be false gI positive. Data of the two finishing units in which one false positive sample was found were excluded from the calculations.

Serology

Serological results are summarized in Table 3 for the units which were vaccinated once and in Table 4 for units vaccinated two times.

Table 3

Serological results of feeder finishing units vaccinated once at 13–15 weeks of age

Solvent	gI negative at slaughter		gI positive at slaughter	
	Unisolve	Diluvac Forte	Unisolve	Diluvac Forte
n of units	24	26	7	4
Average VN titre at vaccination $\log_2$ (S.D.)	1.7 (1.3)	1.7 (1.1)	3.1 (2.2)	4.4 (1.8)
Average VN titre at slaughter $\log_2$ (S.D.)	4.4 (1.7)[a,b]	6.1 (1.6)[a,c]	10.1 (1.1)[b]	10.3 (0.8)[c]
n (%) of samples with VN titre at slaughter (>1$\log_2$)	158/174[d] (90.8)	194/196[d] (99.0)		

Groups in rows with identical letters (a)–(c) were tested in the two sample *t*-test and (d) in the χ^2-test: $^{a-c}p \leq 0.001$; ^{d}p (Pearson) ≤ 0.0003

Table 4

Serological results of feeder finishing units vaccinated two times with an interval of 4 weeks beginning at an age of 10–12 weeks

Solvent	gI negative at slaughter		gI positive at slaughter	
	Unisolve	Diluvac Forte	Unisolve	Diluvac Forte
n of units	42	50	29	24
Average VN titre at 1st vaccination $\log_2$ (S.D.)	3.3 (1.5)	3.2 (1.7)	4.8 (1.8)	4.4 (1.3)
Average VN titre at slaughter $\log_2$ (S.D.)	4.5 (1.4)[a,b]	5.7 (1.7)[a,c]	7.8 (2.4)[b]	7.5 (3.0)[c]
n (%) of samples with VN titre at slaughter (>1$\log_2$)	331/345[d] (95.9)	433/441[d] (98.2)		

Groups in rows with identical letters (a)–(c) were tested in the two sample *t*-test and (d) in the χ^2-test: $^{a}p \leq 0.0003$; $^{b}p \leq 0.000$; $^{c}p \leq 0.002$; ^{d}p (Pearson) ≤ 0.058

VN titre in units gI negative at slaughter age which were vaccinated once were 1.7 $\log_2$ (SD=1.3/1.1) at the moment of vaccination (13–15 weeks of age) in both solvent groups. At the end of the finishing period in units which remained negative in the gI ELISA the VN titre was 4.4 $\log_2$ (SD=1.7) for Unisolve vaccinated units and 6.1 $\log_2$ for DF (SD=1.6), which is a significant difference (*t*-test $p \leq 0.001$).

For the units with double vaccination (starting at 10–12 weeks of age) the average titre in the units turning out as gI negative at slaughter age was 3.3 $\log_2$ (SD=1.5) for Unisolve and 3.2 $\log_2$ (SD=1.7) for DF. The VN titre of gI negative units at slaughter age was 4.5 $\log_2$ (SD=1.4) for Unisolve and 5.7 $\log_2$ (SD=1.7) for DF. Again this difference is significant (*t*-test $p \leq 0.0003$).

The difference in VN titre measured at the moment of (first) vaccination in gI negative remained units until slaughter represents the decrease of maternal immunity during roughly one month.

As expected, infected finishing units showed significantly higher VN titres at slaughter age compared to the vaccination titres in units remaining gI negative (Tables 3 and 4).

Table 5

Differences in starting VN titres between units which were gI negative or gI positive at the end of the finishing period

Vaccination	VN ($\log_2$) titre (S.D.) in gI negative units	VN ($\log_2$) titre (S.D.) in gI positive units
1x	1.7 (1.2)[a]	3.4 (2.1)[a]
2x	3.3 (1.6)[b]	4.5 (1.7)[b]

Groups in rows with identical letters (a) and (b) were tested in the two sample *t*-test: $^{a}p \leq 0.020$; $^{b}p \leq 0.000$

Table 5 shows VN titres from units which remained gI negative until slaughter and from units which had gI positive sampling results at slaughter age. The presented VN titres are from the first blood sample at the moment of the (first) vaccination without separating units for the solvent used. In both vaccination schemes the gI negative units at slaughter age had significantly lower VN titres at the first or only vaccination compared to units which became gI positive by slaughter.

Table 6 shows the number of finishing units which showed a significant increase of gI positive samples at slaughter age compared to the moment of sampling at (first) vaccination, indicating that field infection had either just started or occurred after (first) vaccination.

Table 6

Occurrence of field infection in vaccinated finishing units

Vaccine group	Number/total of finishing units with a significant increase of gI positive samples at slaughter age
Unisolve 1x	5/31
Diluvac Forte 1x	1/30
Unisolve 2x	16/71
Diluvac Forte 2x	9/74

Combining the gI positive units from Table 6 for the two solvents revealed a significant positive effect on the reduction of field infections in favor of the use of the adjuvant DF [p (Pearson) ≤ 0.03].

Discussion

This trial is the first one to show serological results with the aqueous adjuvant Diluvac Forte from the field. The use of the adjuvant Diluvac Forte together with Nobi®-Porvac Aujeszky live (strain Begonia) clearly enhanced the immune response. An important parameter for vaccination efficiency is the percentage of animals without a detectable VN titre at the end of the finishing period. These animals form a very susceptible population during virus circulation. After the use of the adjuvant as solvent this was less than 2% and after the use of Unisolve this was less than 10% which is in obvious contrast to the findings of Pensaert et al. (1990), who found 28% and 45% of seronegative samples, respectively, after a double vaccination in feeder-finishing farms with the Bartha strain dissolved in PBS and in o/w emulsion. However, the method of VN testing might explain at least part of the difference.

The addition of the adjuvant DF significantly reduced the number of field infections. A conclusion concerning the single and double vaccination could not be drawn due to the fact that it was not possible to find problem farms where a random assignment of single or double vaccination schemes was acceptable. Obviously if a suitable farm belongs to the problem herd category there is little chance that the farmer is willing to go for a single vaccination.

The evaluation of the VN titre at the moment of admission/vaccination showed significant differences between units which were gI positive at the end of the finishing period and the ones which remained gI negative. Several factors can

play a role to explain this finding. On one hand maternal immunity is quite high at 10 weeks of age, making it difficult to raise an active immune response after vaccination. On the other hand, field virus could be circulating among these young pigs already at the moment of the first vaccination. Our results, together with results in sow populations with rearing (de Jong, 1992), suggest that virus circulation indeed might occur already before vaccination starts around 10 weeks of age.

Considering this finding in eradication programmes it is advisable to have double vaccination instead of single vaccination because the later vaccination takes place the less it will be possible to reduce virus spread in general. Moreover farms where early virus spread occurs could be a serious hazard for the success of an eradication programme.

The observation period of one year was too short to come to a balance between present field virus and herd immunity in order to be able to stop virus circulation. It would be necessary to include the sow population on the closed cycle farms and the supplier herds if eradication of the field virus from these feeder-finishing units/farms should be achieved.

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ECONOMIC ASPECTS OF THE CONTROL OF AUJESZKY'S DISEASE IN THE EUROPEAN COMMUNITY

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Aujeszky's disease (AD) is as much an economic problem as an epidemiological one. Several strategies are available for controlling the disease, and it is the task of the economist to compare all control options; the "best" strategy may differ depending on whether it is defined at the farm, regional, national or European Community (EC) level. After taking into account both the production losses due to the impact of the AD virus, and the expenditures for the control programme, the strategy which is most efficient from an economic point of view can be determined. However, the most efficient control strategy in one region cannot be chosen solely on the basis of the characteristics of that region, and circumstances in neighbouring regions have to be taken into account as well. Therefore, linkages between neighbouring EC regions are built into the economic model being developed in the EC-funded research project on Aujeszky's disease.

Key words: Aujeszky's disease, control, economic aspects, European Community

Introduction

In recent decades there has been considerable effort aimed at controlling and eliminating Aujeszky's disease in numerous countries of the world. In the EC, Denmark and Great Britain succeeded in eliminating the AD virus during the 1980's by so-called "eradication programmes", while other countries have started to control the disease by some sort of vaccination strategy. Denmark and Britain are still the only EC countries free of the disease, and in recent years political pressure has risen to eliminate the virus in other countries as well. However, it is unclear which strategy should be adopted. In particular, the development of the genetically engineered vaccines, which allow vaccinated pigs to be differentiated from those which are infected, have resulted in new options to control and maybe eliminate the AD virus; however, research has not yet demonstrated that elimination of the virus is feasible in areas of high pig density. Moreover, from an economic point of view the question is whether eliminating the virus by heavily vaccinating is the most efficient option of controlling the disease, or whether other strategies are more cost-effective for the EC as a whole. The analytical framework

for "the economics of livestock diseases" is therefore essential in deciding which control strategy is most efficient whether at the farm, regional, national or the EC level. Based on assessments of the costs and benefits of each strategy in different regions, the least cost strategy can be determined.

In an EC-funded research project the economics of the control of AD are presently being studied by the Agricultural Economics Unit at the University of Exeter. The project is in collaboration with veterinary epidemiologists at the Royal Veterinary and Agricultural University in Copenhagen, the Federation of Danish Pig Producers and Slaughterhouses, the Free University of Berlin, and the Animal Health Service in the Southern Netherlands. In Germany and The Netherlands field data are being collected in areas of high pig density where strict vaccination programmes are carried out.

In this paper attention is focused on some economic aspects of controlling Aujeszky's disease in the EC.

Terminology

Before getting into the economics of AD control, clarification of the terminology used in the veterinary epidemiology and economics is needed to avoid confusion. First, the words "control" and "eradication" have to be defined more precisely. Eradication is one way of controlling the disease. In this paper we define five main AD *control* strategies: (1) doing nothing; (2) depopulation-repopulation; (3) test and removal of positive animals; (4) offspring segregation, and (5) vaccination. The second, third and fourth control strategy were identified as "*eradication* strategies" by Thawley et al. (1987), but since the development of genetically engineered vaccines, allowing the differentiation of vaccinated from infected animals, a stringent vaccination strategy could result in the eradication of AD as well. Certain *vaccination* strategies can therefore be called *eradication* strategies too. However, not all vaccination strategies result in eradication, and moreover the effects of the vaccination programmes depend highly on other epidemiological determinants in the region such as pig density, level of management, and meteorological conditions.

Obviously, eradicating the AD virus is one form of *controlling* the disease, and hence the phrase "control and eradication" is saying something double. Therefore, it is proposed in this paper to use "control strategies" for what previously were called "eradication strategies".

Furthermore, in this paper the word *region* is used for an area within the EC where one AD control policy can be identified. Hence, regions can be equal to countries, or can be part of a country. For example Denmark or Portugal may well be one region respectively, whereas Brittany in France and Schleswig-

Holstein in Germany may both be defined as regions within their respectively countries.

Economic modelling of Aujeszky's disease

The total costs of Aujeszky's disease consists of two components: (1) the production losses due to the effects of the disease (such as reduced growth and increased mortality), and (2) the expenditures for controlling the disease (e.g. vaccination, disinfection, and quarantine measures) (McInerney et al., 1992). In summary: C = L + E; where C = total costs of the disease; L = financial losses due to changes in production; E = expenditures for the control programme.

The total costs of the disease can be established for different control strategies at the farm, regional and EC level. It must be emphasized, however, that the regional economic costs due to AD are not simply the average costs at the farm level multiplying the number of affected farms in the region. At the national and EC level other factors, such as subsidies, market effects, etc. play an important role in the estimation of the total economic costs. Besides, it is not only producers but also consumers and taxpayers who are to be affected by changes in control policies (see Howe, 1992). In the research project a computer model is under development which can identify the best control strategy for the EC as a whole and for regions separately.

It is extremely important to realise that the AD virus can easily be transmitted from one farm, or from one country, to another. The virus can obviously be transported by live animals and other vehicles which can, in principle, be freely moved throughout the EC since 1 January 1993, but AD is also an airborne transmitted disease. Evidently, the most efficient control strategy in one region cannot be chosen solely on the basis of the characteristics of that region, and circumstances in neighbouring regions have to be taken into account as well. Therefore, linkages between neighbouring EC regions are being built into the economic model.

Data collection

Obviously input data have to be collected for the economic model. The first task is to collect regional data on pig production and Aujeszky's disease effects. This data set contains information about (1) swine population, (2) production, (3) prevalence/incidence of Aujeszky's disease, (4) AD control programmes and their costs, (5) expenditures in AD research, (6) the production structure of the pig sector, and (7) international trade of live and carcase pigs.

Data are collected at the farm level, the aim being to assess the average economic costs due to Aujeszky's disease faced by the producer applying different control and eradication strategies. This data collection is carried out in project areas in Germany and The Netherlands, but other data sets collected in other parts of the EC might also be used if they can add additional information to the economic model.

Conclusions and discussion

Different strategies can presently be employed to control Aujeszky's disease. Economic analysis is essential to decide which control strategy is the best. On the basis of estimating the total costs associated with alternative strategies for the disease (consisting of production losses and control expenditures) the most cost-effective strategy can be identified. Those analyses can be applied at the farm, regional, national and the EC level.

EC countries (or regions within countries) can have separate AD control strategies, but the economic costs of an AD control programme in one region depend on the control programme in the neighbouring regions. The best policy cannot therefore be determined in isolation. EC regions have to cooperate to achieve the overall most efficient control of AD.

The outcomes of the final economic model are heavily dependent on the input to the model. As in any area of applied economic analysis, a model focused on the economic aspects of livestock diseases is, among other things, inevitably based on the underlying technical information — in this case the epidemiological input data. Some of the data required will be derived from the EC project areas while more will be collected from sources in other Community regions. Additionally, literature and expert knowledge will need to be built into the model as well.

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CONSTANCY OF AUJESZKY'S FIELD VIRUS ANTIBODY TITRES IN SOWS REPEATEDLY VACCINATED WITH A gI-NEGATIVE VACCINE

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A breeding herd repeatedly vaccinated with a gI-deleted vaccine was regulary tested serologically for Aujeszky's field virus antibodies over a period of up to 35 months. Of the 100 sows tested, 18 animals reacted positively with constant titers during the whole period of the investigation. On the other hand, there was no serological evidence that any of the contact animals contracted field virus infection.

Key words: Aujeszky's disease virus, antibody titre, sow, gI-negative vaccine

gI-deleted vaccines are now available, which allow a distinction between infected and vaccinated animals using the gI-ELISA. In a district of the Veterinary Office Soest all swine have been vaccinated with a gI-deleted vaccine since 1988. Two years later the serological screening of all sows for gI antibodies was started. The purpose of this study was to demonstrate the gI titre of several sows from a selected breeding herd over a period of nearly 3 years.

Materials and methods

Pigs. The breeding herd consisted of 100 sows. In an initial serological screening 35 sows showed antibodies against Aujeszky's disease virus (ADV). These animals were purchased from a multiplier herd with an unrecognized ADV infection. Every 4–5 months the pigs were vaccinated with a gI-deleted vaccine.

Samples. By taking blood samples at intervals of 4 to 6 weeks from piglets of all sows which had farrowed in the meantime, the serological status of each individual sow was tested at 5-month intervals. At the end of the investigation of 18 gI-positive sows, 9 of them had been checked four times, 3 animals five times, 5 animals six times and 1 animal eight times.

Serological testing. Blood samples were tested for gI antibodies using a blocking ELISA (HerdChek ADV gI, Idexx GmbH). Test procedure and calculations were done corresponding to the manufacturer's instructions.

Results

A breeding herd with 100 sows, 18 of them with antibodies against Aujeszky's disease virus, was repeatedly tested serologically over a period of up to 35 month. The animals with a gI antibody titre reacted positively in the ELISA during the whole period of the investigation. The gI antibody titres of sows which were examined for at least 6 times are demonstrated in Fig. 1. A decline of the ELISA titre could not be estimated. Some animals even showed an increase in gI antibodies. The remaining contact animals without gI antibodies revealed no serological evidence of contracting field virus infection over a period of nearly 3 years.

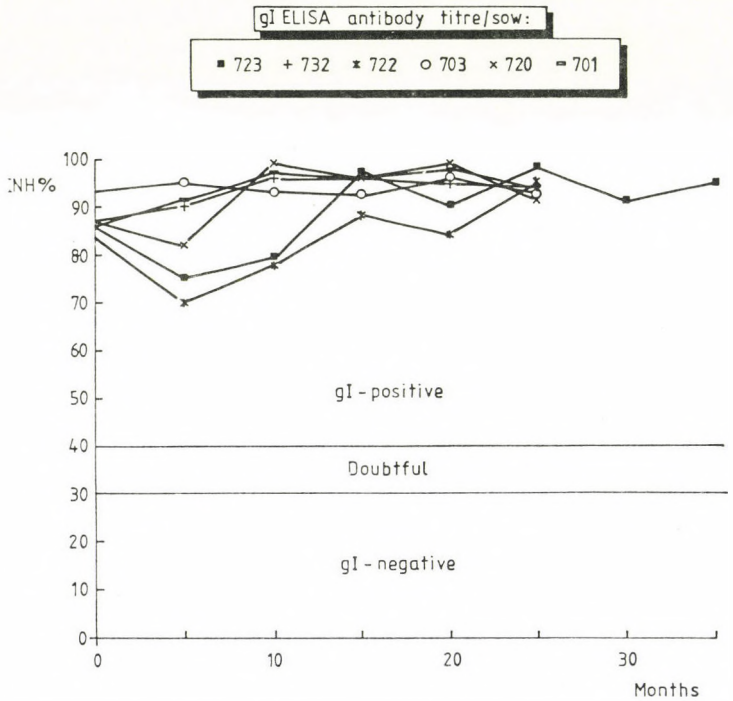

Fig. 1. Blood samples from 6 field virus infected sows, repeatedly vaccinated with a gI-negative vaccine, were examined at 5-month intervals in a gI-ELISA (HerChek ADV gI, IDEXX GmbH) for Aujeszky's field virus antibodies. Five sows were tested 6 times over a period of 25 months, sow 723 was tested 8 times over a period of 35 months. Evaluation: Inhibition (INH) <30% = negative; 30–40% = ???; >40% = positive

Discussion

The present study shows the persistence of gI antibodies over a period of nearly 3 years. No decrease of high level gI antibody ELISA titres was observed. This supposes an even longer period of detectable gI antibodies in ADV-infected sows.

Van Oirschot et al. (1988, 1989, 1990) found constant gI angibody titres in vaccinated and unvaccinated sows over a period of 32 to 75 weeks. Chaudhuri (1992) detected gI antibodies 105–112 days after a field-virus infection.

There was no serological evidence that animals of the gI-negative contact population had been infected over the whole period of investigation. Observations of Van Oirschot et al. (1990) showed that repented vaccination minimizes the risk of spreading virus in a herd. Challenge experiments done by Pensaert et al. (1990) and Vannier et al. (1991) demonstrated a reduction of the level and the duration of virus excretion in gI^--vaccinated animals.

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INTRADERMAL APPLICATION OF AUJESZKY'S DISEASE VIRUS STRAIN BEGONIA WITH TOCOPHEROL-BASED ADJUVANT AND A NOVEL DESIGN INJECTION DEVICE

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Initially the use of intradermal application of Aujeszky's disease vaccines was shown to be very effective. However, for thus far unknown reasons the gI-deleted vaccines were much less efficacious by using this route of vaccination as compared to gI-positive vaccines. By the use of a tocopherol-based adjuvant and an improved design of the intradermal injection device it now appeared feasible to obtain the same efficacy both in specific pathogen free pigs and in pigs with material antibodies as found before when intramuscular administration was performed. With respect to safety we found a complete lack of skin lesions, no adverse systemic reactions (e.g. body temperatures) and no effect on growth rates. Last but not least, the easiness of intradermal injections is of great advantage in large-scale vaccination programs.

Key words: Aujeszky's disease, vaccine, intradermal application, safety

The intradermal (I.D.) route of vaccination has been shown to be an effective and easy-to-apply method (Vannier and Cariolet, 1991): For thus far unknown reasons gI-negative modified live vaccines were less effective by the intradermal route than gI-positive vaccines.

To date more and more countries are allowing only the use of gI-negative marker vaccines.

Here we report that with the use of a tocopherol-based adjuvant and a newly designed intradermal application device, safe and effective (I.D.) vaccinations with gI-negative vaccines are possible.

Materials and methods

Viruses. Throughout all the experiments modified live vaccine virus strain Begonia (gI^-, TK^-), batch 01714D and 09702 at a titre of 10^6 $TCID_{50}$ per dose was used, with Diluvac Forte (tocopherol-based adjuvant, batch 16.652) as solvent. Challenge infections were performed intranasally using the 75V19 strain obtained from Prof. Pensaert (Gent, Belgium), given as two or three doses of 10^5 $TCID_{50}$ within 24 hours.

Pigs. SPF pigs were obtained from Dalland Meuse, France whereas maternally-derived antibody positive (MDA^+) animals were obtained locally after a check for the absence of gI antibodies using the Intertest Aujeszky gI Elisa.

I.D. Device. A newly designed applicator (I.D.A.L., pronounce "ideal") is used. For the mechanical design by Weston Design Engineers a patent application (WO9303779) has been filed.

Results and discussion

Safety. No adverse local or systemic reactions were observed. However, I.D. vaccination gives a small pale lump (3–4 mm) indicating a proper injection. The papule disappeared after a few days and at slaughter time we were not able to trace back the original injection sites. First a double dose injection followed 2 weeks later by a single dose given to 10-week-old pigs and pregnant sows revealed no abnormalities in recorded body temperatures (Fig. 1). The weight gains of the feeder pigs in this safety test showed a complete absence of adverse effects (Fig. 2).

Efficacy. Comparison of I.D. and intramuscular (I.M.) vaccination in SPF pigs showed a similar effectivity of both vaccination routes as judged from, the relative weight gain after challenge on one hand and the calculation of the relative average daily weight gain (DG) according to the Ph. Eur. (Fig. 3) on the other. The DG values being 1.9 for the I.D. and 2.1 for the I.M. route easily comply with the required Ph. Eur. value of 1.5.

In addition, the reduction of challenge virus excretion was monitored: again both routes were equally effective (Fig.4). In contrast to the controls, from day 6 p.c. virus was no longer recovered from the vaccinated pigs, whereas at day 1 and 3 p.c. much lower amounts of virus were recovered from the vaccinates.

In a second trial, MDA^+ pigs were vaccinated twice at 10 and 14 weeks of age and challenged at slaughter weight (24 weeks old). The relative weight gain after challenge was similar and after calculation of DG both routes scored a value of 1.6 (Fig. 5). With these values the required of 1.5 of the Ph. Eur. is passed, even though the level is set for SPF animals.

The overall serological response was almost identical for the I.M. and I.D. route of injection (Fig. 6).

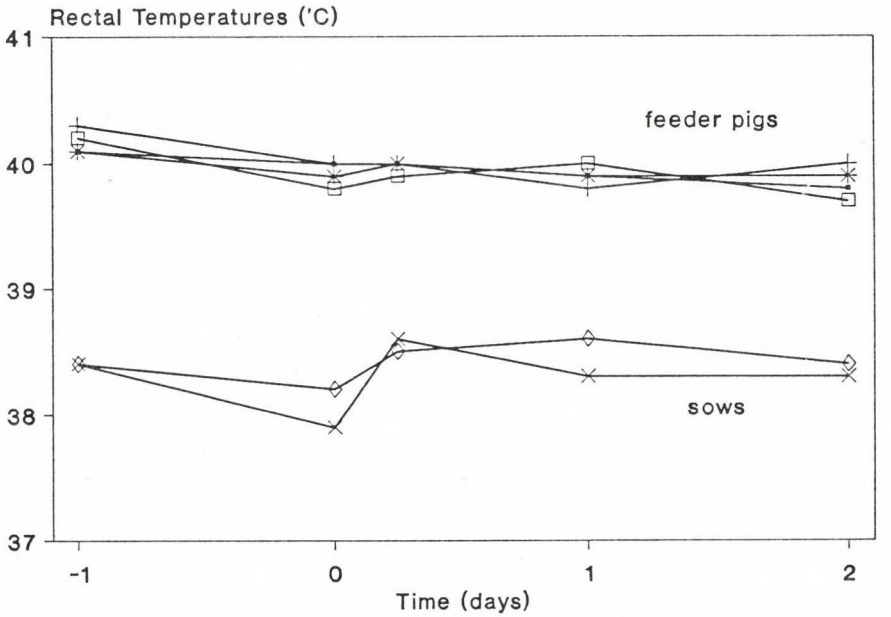

Fig. 1. Double dose vaccinations and a single dose 2 weeks later in pregnant sows (n=10) and 10-week-old feeder-finishing pigs (n=10). Rectal temperatures (°C) were recorded the day before and at 0, 7, 24 and 48 hours after I.D. inoculation. (□ , 10-w-old pigs, double dose; +, 10-w-old non-vaccinated controls; *, 12-w-old pigs, single dose booster; •, 10-w-old non-vaccinated controls; x, pregnant sows, double dose; ◊, pregnant sows, single dose booster, 2 weeks later)

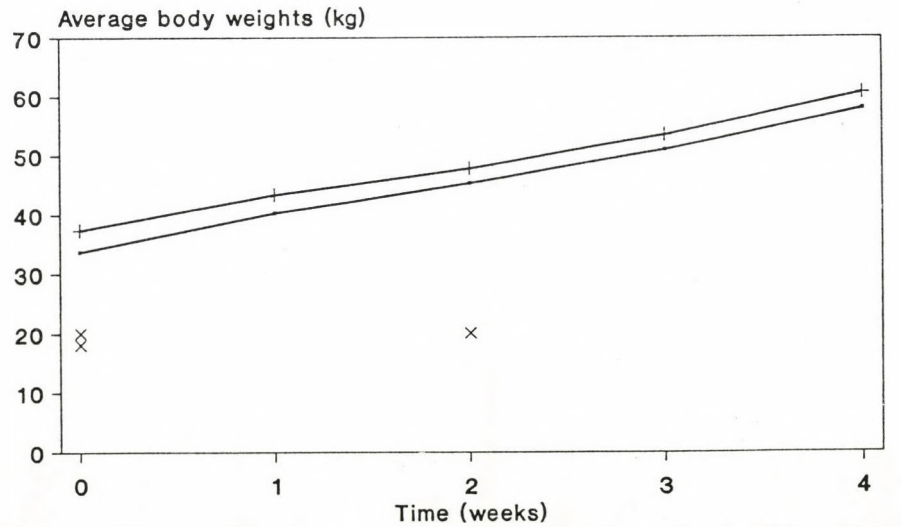

Fig. 2. Effect of an intradermal double dose and a single dose 2 weeks later on the weight gain of feeder-finishing pigs (xx, double dose; x, single dose)

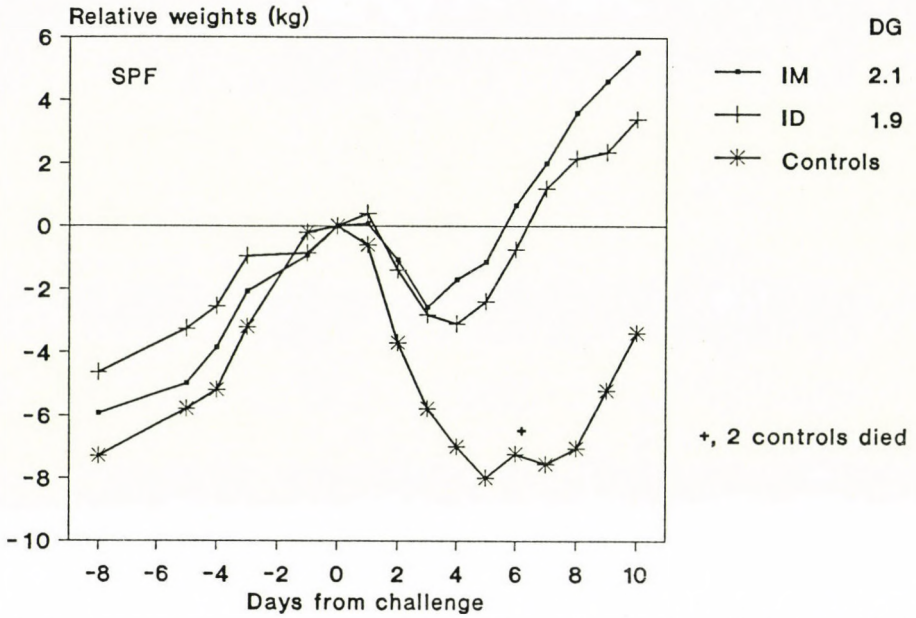

Fig. 3. One vaccination at 14 weeks of age and challenge 4 weeks later with strain 75V19 (3x). The relative weight gain is shown together with the DG calculation according to the Ph. Eur. The requirement of the Ph. Eur. of a minimal level of 1.5 is easily passed. The severity of the challenge complies with the Ph. Eur. The death of two controls also indicated a severe challenge

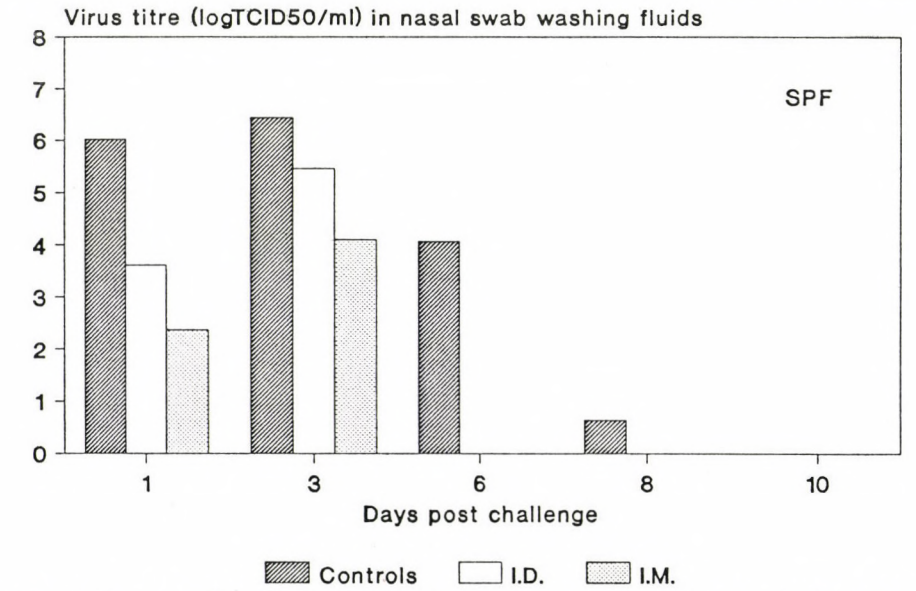

Fig. 4. Virus excretion after challenge is shown from the same experiment as in Fig. 3. With respect to the controls both routes I.D. and I.M. reduced to a similar extent the amount of virus in nasal swabs and the duration of excretion

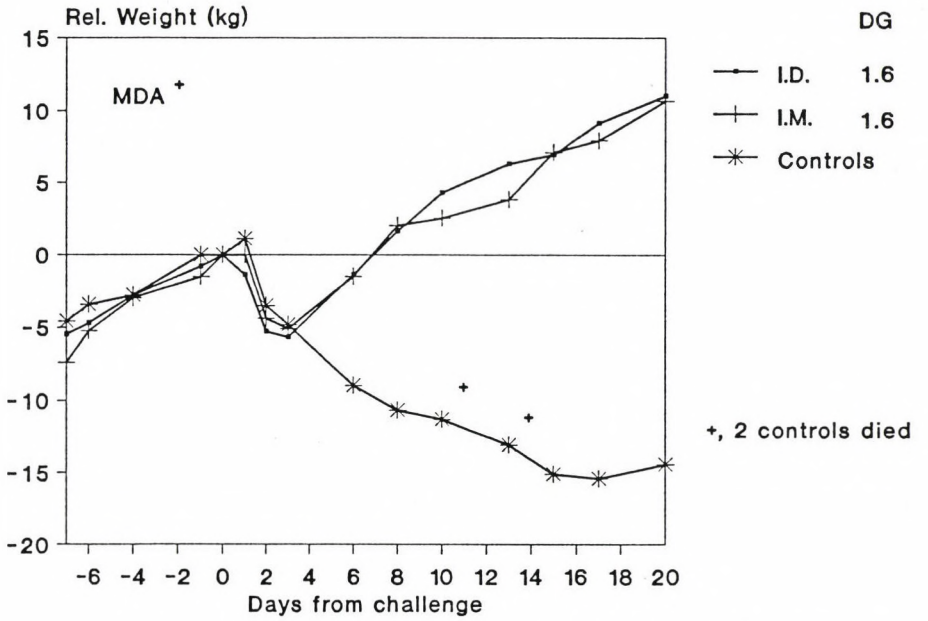

Fig. 5. Commercial MDA$^+$ pigs were vaccinated at 10 and 14 weeks of age and challenged at slaughter weight (24 weeks old) with strain 75V19 (2x). The effect on the relative weight gain and a calculation of DG according to the Ph. Eur. is given. Both routes passed the Ph. Eur., even though the requirement is set for SPF animals

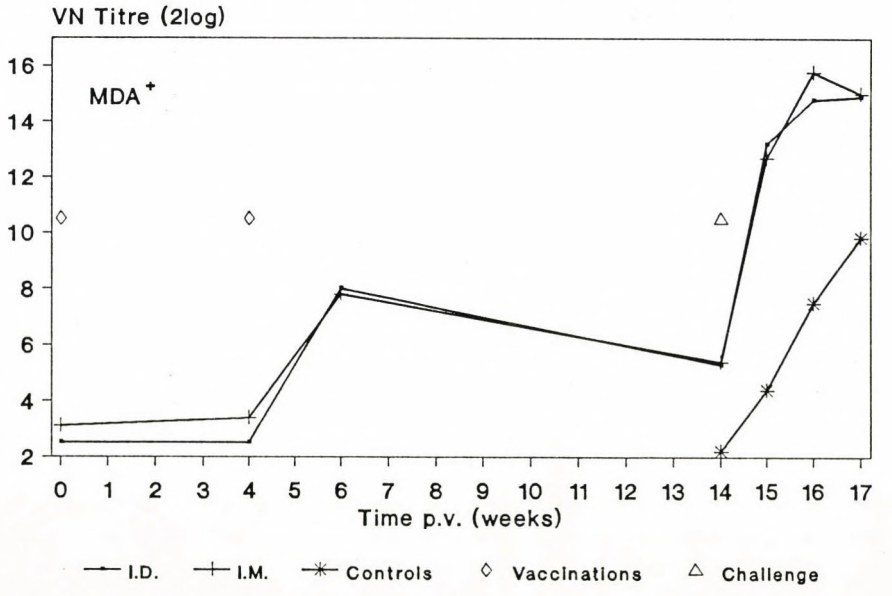

Fig. 6. Serological results are shown of the same pig experiment as in Fig. 5

Conclusion

The intradermal route of vaccination can be applied with the same safety and efficacy as the current intramuscular route using strain Begonia with a tocopherol-based adjuvant and a newly-designed intradermal injection device.

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Vannier, P. and Cariolet, R. (1991): Vaccination of pigs against Aujeszky's disease by the intradermal (I.D.) route using live attenuated and inactivated virus vaccines. Vet. Microbiol. **26**, 11–23.

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CONTENTS

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ERADICATION OF BOVINE LEUKOSIS FROM A HEAVILY INFECTED HERD BY THE USE OF OWN OFFSPRING

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(Received April 22, 1994)

In a Holstein-Friesian dairy herd (n = 1,248) kept in loose housing system and showing 62% prevalence of infection with bovine leukosis virus (BLV), the newborn calves were separated from their dams and placed into individual pens. They were given freeze-stored colostrum derived from a BLV-free farm and then a calf starter diet. Strict hygienic measures of leukosis control were observed. Serum samples were taken from the calves and tested by agar gel immunodiffusion (AGID) before and after colostrum uptake, at 2.5 and 6 months of age, and then at the heifer-rearing farm at 2-month intervals. Animals giving a positive reaction were culled. No AGID positivity occurred before the uptake of colostrum. Subsequently, six times as many calves became positive among the offspring of infected cows than among calves born to negative animals. As no separate barn was available, the infected farm was divided into a "black" and a "white" part with a foil to prevent aerogenic infection. The negative heifers being in the 7th month of pregnancy were placed into the "white" part. A separate calving house was established for them, as the joint calving house proved to be a source of infection. By this method a leukosis-free progeny herd was obtained from 90% of the heifer calves of the cow population showing a high prevalence of infection. In this way the original infected herd was gradually replaced.

Key words: AGID, bovine leukosis, leukosis control, sources of infection, routes of infection, eradication

Freedom from bovine leukosis virus (BLV) on herd level has by now become a precondition of the sale of breeding cattle. Eradication has been given an impetus by the possibility of identifying infected animals by the agar gel immunodiffusion (AGID) test or by ELISA. Serological tests are carried out several times every year, the positive reactors are removed from the herd and slaughtered soon afterwards. Numerous European countries and partly Canada (Roberts et al., 1982; Mammerickx, 1984; Shettigara et al., 1986; Wittmann and Gold, 1987) have eradicated bovine leukosis from their herds by this "test and slaughter" method (Brenner et al., 1988).

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It has been demonstrated that BLV does not infect embryos through the genetic route (Callahan et al., 1976; Kettman et al., 1978; Eaglesome et al., 1982). Many authors have documented that intrauterine infection is not common (Kaaden et al., 1978; Van Der Maaten et al., 1981; Jacobsen et al., 1982; Thurmond and Burridge, 1982; Liebermann et al., 1983; Simoyan et al., 1984; Mewes and Weege, 1985; Crespeau et al., 1986; Shishkov et al., 1988; Lassauzet, 1988). That finding served as a basis for the "test and isolate" method of eradication (Brenner et al., 1988). The essence of this method is that the identified positive animals are isolated and from their calves a BLV-free herd is developed by rearing under strict hygienic measures. This method is used for eradication primarily in countries having cow populations of large size (Liebermann et al., 1983; Simoyan et al., 1984; Mészáros et al., 1986; Tekes, 1986; Müller et al., 1987; Wittmann and Gold, 1987; Antal et al., 1988*a,b,c*; Deuter et al., 1988; Shishkov et al., 1988; Shettigara et al., 1989; Detilleux et al., 1991; Losieczka and Klimentowski, 1992).

This paper reports on the elimination of the routes of infection spread in a Holstein-Friesian herd (n = 1,248) showing 60% prevalence of infection and kept in the loose housing system, and describes eradication experience gained in a 8-year period.

Materials and methods

Herd and management. A purebred and crossbred herd developed from Holstein-Friesian heifers imported from the U.S.A., kept under loose housing conditions on a farm with a spacing capacity for 1,248 cows was used in the study. Already before the start of eradication the calves were immediately isolated from their dams and placed into a prophylactorium (individual pens) for 48 h. Subsequently they were kept in Stejman pens erected outdoors and reared on calf starter diet. Then the calves were kept in the large-group post-rearing unit of the farm up to 6 months of age (under the pressure of necessity the calves from positive and those from negative cows were in the same air-space!). From there the animals were transferred to the heifer-rearing farm where they remained up to the 7th month of their pregnancy, when they were retransferred to the infected dairy unit. Strict hygienic measures of leukosis control were observed (use of disposable or thoroughly disinfected equipment, obstetric gloves, etc.).

Method of eradication. In the absence of an isolating barn, the cow population already showing 62% positivity was divided into a positive and "negative" group on the farm using loose housing conditions. Of necessity, the negative heifers being at the 7th month of gestation, arriving from the heifer-rearing farm, were initially assigned to the latter group. In the first year the cows of the two groups calved in the same calving house. In the second year of eradication all cows pre-

sent on the farm were considered suspicious of infection and were not tested further. To prevent the transmission of nasal and pharyngeal secretion, the place where they were staying (the "black" side) was separated from the "white" side with a partition wall consisting of foil and a 12-m wide unpopulated demarcation zone. From that time on, the negative pregnant heifers were housed on the "white" side and they calved in a separate calving barn. No further animals were housed on the "black" side; thus, as a result of "normal" culling the area of the "black" side gradually decreased, while that of the "white" side increased by changing the place of the separating foil.

Colostrum supply. The calves were not allowed to suckle their dams, they received colostrum from a bottle. At the beginning of eradication only the calves of seronegative cows received their own mothers' colostrum, and other calves were given colostrum from a permanently leukosis-free herd and stored frozen before use. Because of organization difficulties, from the second year of eradication all calves received colostrum from a leukosis-free herd. These calves were transferred to the heifer-rearing unit from the second year of eradication.

Antigens. The AGID tests were performed with the standardized antigen containing the gp58 capsular antigen of BLV, prepared in our institute (Harrach et al., 1984).

Serological tests. The calves were first tested before colostrum uptake (at "0 day" old). The lack of colostral immunoglobulin transfer was checked by the method of Pfeiffer and McGurie (1977) for each individual. After colostrum uptake, at 24 hours of age a second test was done to assess whether the calves had received anti-BLV antibodies with the colostrum. That test also served for the qualification of colostrum. The third test was performed at 2.5 months of age before transfer to the post-rearing unit, while the fourth one at 6 months of age before transfer to the heifer-rearing unit. On the heifer-rearing farm each animal was tested first at 2-month, then at 3-month intervals, and in the last years of eradication every 6 months. The last test was carried out in the 7th month of pregnancy, before transfer to the dairy unit. In the dairy unit serum from each cow was tested by AGID first at 3-month and subsequently at 6-month intervals.

Results

The serologic survey of cow herds established from tens of thousands of heifers imported in the second half of the 'seventies became possible only in 1982. By testing a total of 44,749 cows in 93 such dairies it was found (Tekes et al., 1984) that all farms keeping Holstein-Friesian cows were infected. Of the crossbred populations and Hungarian Fleckvieh herds that had been free until then, positive individuals occurred only in those using natural service. The herds in-

seminated with Holstein-Friesian semen remained free, as did the beef cattle populations.

In the Holstein-Friesian herd of 1,248 cows used in this study, the prevalence of BLV infection rose from 20% found at the first test to approx. 62% in three years.

The immunoglobulin content of the precolostral serum samples of calves demonstrated that the calves had not had access to colostrum. Sera from more than one thousand such calves were consistently negative by the AGID test, despite the fact that the calves had been born in a cow herd showing a high rate of seropositivity. The serological results obtained for calves born on the farm were divided into four groups based upon the serological qualification of the calves' dams and of the colostrum received. The correlations thus revealed are presented in Table 1. The positivity rate was the highest among calves which had been born to positive dams and which had consumed their mother's colostrum for two days at the beginning of eradication (though also these calves had been separated from their mothers after birth!).

Table 1

Changes in the positivity rate of calves born on the farm between birth and 6 months of age

Serological qualification		No. of animals	Age on the day of blood sampling			
dam	colostrum		0 h	24 h	2.5 months	6 months
negative	negative	398	0	0	0	4(1.0)*
negative	positive	44	0	44 (100)	5(11.4)	4(9.1)
positive	negative	234	0	0	15(6.4)	21(9.0)
positive	positive	206	0	206 (100)	102(49.5)	46(22.3)
Total		882				

*number of animals (positivity rate, %)

The heifers that had given a negative result at 6 months of age were tested further on the heifer-rearing farm. Some of the results of these tests are summarized in Table 2. It can be observed that the positivity rate was (6 times!) higher among calves derived from positive dams than among calves born to seronegative dams on the same farm. This tendency could be observed not only in calves less than 6 months old but also among those older than that.

Table 2

Qualification of heifers derived from positive and from negative cows, up to the 7th month of pregnancy (the heifers were negative at 6 months of age)

Qualification of cows	No. of cows	Qualification of heifers			
		negative	%	positive	%
negative	523	510	97.5	13	2.5
positive	212	180	84.9	32	15.1
Total	735	690	93.9	45	6.1

At the time of the first positive reaction the age of seronegative calves born to heavily infected cows and transferred to the heifer-rearing farm at 6 months of age was 7–10 months in 40.6%, 11–14 months in 36.3% and 15–19 months in 18.7% of the heifers. Only 4.4% of the heifers was older than 20 months at the time of the first positive AGID test. In the case of heifers seroconverting after 6 months of age the average seroconversion period was surprisingly similar: 11.3–12.5 months (extreme values: 7–23 months). The number of animals tested and the positivity rate (%) found at the individual tests between months 19 and 114 of the experiment are shown in Fig. 1. No positive reaction occurred at the last six tests (5,328 sera since 1991). At the previous four tests 8 out of 3,574 sera were positive (all were derived from 7- to 12-month-old animals). It can be seen that by repeating the tests at 2- to 3-month intervals, the prevalence of infection can be kept on a very low level (some tenths of one per cent). As all cows were kept in breeding, the number of pregnant heifers did not decrease during the long period of eradication.

The heifers were regularly tested on the heifer-rearing farm and transferred to the dairy units in the 7th month of pregnancy. On the leukosis-free farms these heifers remained negative also after calving. In contrast, the pregnant heifers that were initially co-mingled with the "seronegative" cows of the farm showing a high prevalence of leukosis sooner or later became positive. (At that time the positive and negative cows yet calved in the same calving barn!). The young cows usually became positive at 26–30 months of age, a few months after calving.

A resounding success was achieved by housing the pregnant heifers brought to the farm in the separated "white" part and making them calve in a separate calving barn. From that time on there were hardly any positive reactors among the young cows. A comparison of this favourable change with the previous situation is contained in Table 3. This shows that while the pregnant heifers were assigned to the "seronegative" group and they shared a calving barn with the infected animals, the more time they spent on the farm, the higher proportion of them gave a posi-

tive reaction. In contrast, there were hardly any positive reactors among the cows assigned to the "white" side and calving in a separate calving barn.

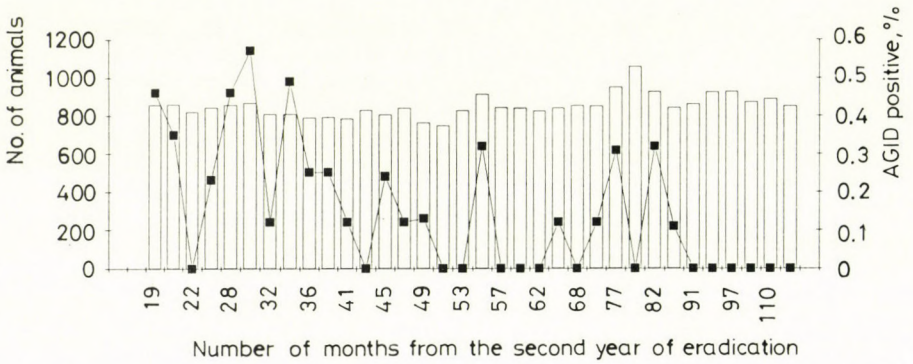

Fig. 1. Changes in the population size and AGID positivity rate of the heifer-rearing unit between months 19 and 114 of the trial

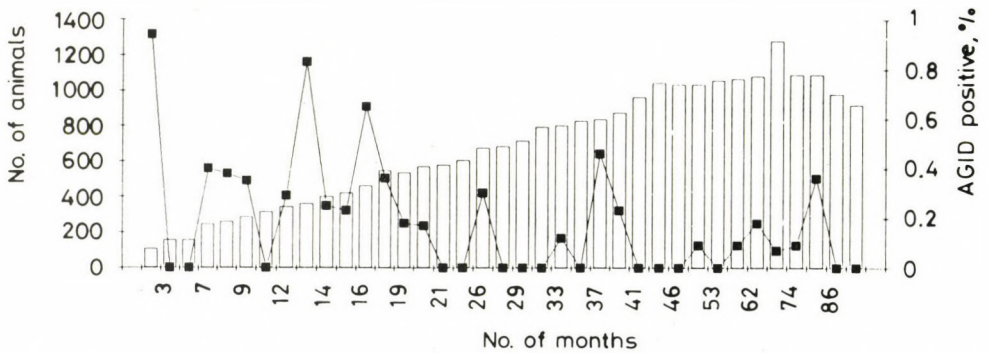

Fig. 2. Changes in the number of animals and AGID positivity of the "white" side

Subsequently the eradication gathered speed: as a result of culling, the number of cows on the "black" side steadily decreased, while the "white" side had to accommodate an increasing number of animals. This was made possible by repeatedly changing the place of the partition wall. The continuous increase in the number of cows kept on the "white" side, with their very low rate of AGID positivity is shown in Fig. 2. The few cows remaining on the "black" side were removed from the farm. Between 1990 and 1993, nine AGID tests were performed on the farm: only 10 out of 9,611 cow sera (0.1%) were positive. The number and results of the annual tests are shown in Fig. 3. The positive sera were derived from 2- to 7-year-old cows that had been individually negative by the previous 4–28 (!) tests. Seven out of the 8,902 test sera of heifers were positive in 1990 while only

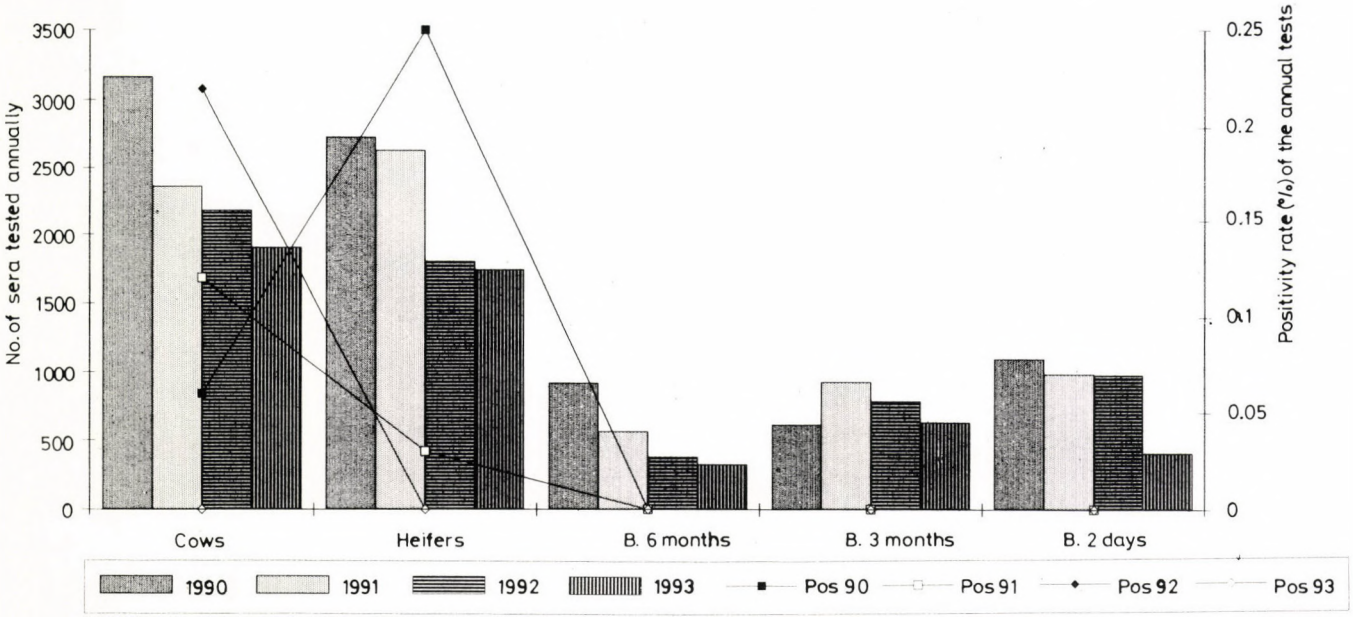

Fig. 3. Annual AGID tests, 1990–1993

one was positive in 1991; since that time, no positive reactor has been found among them. Since 1991 the calves have received their own mother's colostrum. Despite this fact, all the sera from 3,464 two-day-old, 2,950 three-month-old and 2,184 six-month-old calves tested in the past four years were negative.

Table 3

Appearance of AGID positivity in pregnant heifers and cows assigned at quarter-year intervals to the seronegative or "white-side" group of the infected herd after the 7th month of pregnancy, as a function of the time spent in the group

Time spent in the group (months)	No. of heifers assigned to the group at quarter-year intervals and AGID positivity %									
	"Seronegative group" (with joint calving house)					"White side" (with separate calving house)				
	I	II	III	IV	Total	I	II	III	IV	Total
	93	96	99	53	341	115	113	89	128	445
0–3	9.6	3.2	3.1	18.8	–	0.9	0.0	1.1	0	–
3–6	18.2	13.6	23.8	–	–	0.0	0.0	0.0	–	–
6–9	19.2	12.3	–	–	–	2.4	0.0	–	–	–
>–10	25.0	–	–	–	–	1.3	–	–	–	–
Animals becoming positive (in % of animals transferred in to the group)										
	41.9	22.9	18.2	18.8		3.5	0.0	1.1	0.0	

Discussion

It is a remarkable finding that in the three years before the start of eradication the prevalence of BLV infection in the herd increased from 20 to 62%, despite the fact that the general control measures were strictly observed and the calves were isolated from their dams after birth. However, the seropositive and seronegative cows remained in the same air-space and calved in the same calving house. This proved to be a major source of infection, as compared with the probability of intrauterine infection.

According to data of the literature, a few per cent of serum samples taken from calves of BLV-infected cows before the ingestion of colostrum give a positive reaction in the AGID test but first of all in the more sensitive ELISA (Kaaden et al., 1978; Jacobsen et al., 1982; Mewes and Weege, 1985; Gareiss et al., 1985; Lassauzet et al., 1991). This points to intrauterine infection which Thurmond and Burridge (1982) estimate at around 6%. In our study it was quite certain that the calves had no access to colostrum, as colostrum-free calf serum had been collected

on the farm for cell culture purposes for several years. Therefore, every serum was checked chemically to determine whether it had been derived from a colostrum-deprived calf. Thus, we must accept it as an established fact that before the first test the more than one thousand calves did not receive colostrum. The sera of these calves contained no antibodies detectable by the AGID test after birth (Antal et al., 1988*a*). We did not employ the more sensitive ELISA or RIA tests; thus, it cannot be excluded that some of the AGID-negative samples would have given a positive reaction in tests of higher sensitivity (Perlberg et al., 1984; Gareiss et al., 1985; Müller et al., 1985, 1987). By testing 24-hour-old calves we wished to make sure that they had received a colostrum free of BLV antibodies. Namely, this was a precondition of performing the 3rd test as early as at 2.5 months of age, when the calves were transferred from the individual pens to the unit where they were then reared in groups. In the last three years we tested almost 3,500 24-hour-old calves receiving their own dam's colostrum because the negativity of their serum is a very sensitive indicator of the dam's negativity.

The efficiency of that approach is evidenced by the data presented in Table 1, which show that 6.4% of the calves born to positive dams and receiving negative colostrum gave a positive reaction already at 2.5 months of age. That ratio increased to 9.0% by 6 months of age, suggesting the possibility of intrauterine infection or infection during calving. Namely, at 2.5 months of age all the 398 calves delivered by negative cows and fed as described above were negative. Four of these latter calves became seropositive by 6 months of age, probably as a result of horizontal infection, as the calves of positive and negative dams were kept together after 2.5 months of age. This hazard has been pointed out also by other authors (Gulyukin et al., 1985; Deuter et al., 1988). Five out of the 44 calves born to negative cows but receiving colostrum from positive cows for experimental purposes became positive at 2.5 months old, and 4 out of them were still positive at 6 months of age. As these calves had been kept isolated from their dams and herdmates up to 2.5 months of age, it is very likely that their infection can be traced back to the colostrum derived from positive cows (Antal et al., 1988*a,b*). Therefore, whenever feasible, colostrum and milk from a negative herd should be given during leukosis eradication from the progeny herd. Other authors also recommend that approach, or try to eliminate that source of infection by warming up or acidifying the colostrum or milk (Ferdinand et al., 1979; Ferrer, 1979; Liebermann et al., 1983; Müller et al., 1985; Lassauzet et al., 1989; Detilleux et al., 1991;. Sprecher et al., 1991). We were aware of the fact that by supplying negative colostrum we lost the protective effect of anti-BLV antibodies (Van der Maaten et al., 1981; Simoyan et al., 1984; Deuter et al., 1988; Lassauzet, 1988; Lassauzet et al., 1989). In a highly infected environment this could be fatal. In our case, however, the frequent tests and the immediate removal of positive animals (coupled with the strict hygienic measures) substantially reduced the risk of hori-

zontal infection. We would like to repeatedly underscore the advantages of early testing made possible by the use of negative colostrum.

There was a linear correlation between the serological qualification of the dams and the test results of their offspring not only up to 6 months of age but also later on. This is demonstrated in Table 2. Because of the dominance of perinatal infection and the frequent testing, this points to a reduction in horizontal infection. Such a correlation has been demonstrated also by other authors (Crespeau et al., 1986; Deuter et al., 1988).

Both from the epizootiological point of view and as regards the mode of infection, it is interesting to note that during the 8 years of our study the majority of animals became positive at 6–10 and at 11–14 months of age. A substantial proportion of the 6-month-old negative calves became seropositive at the heifer-rearing farm at 11.1–12.5 months of age on the average (Antal et al., 1988*b*). This raises the suspicion that the majority of infections must have occurred at the same age, in our opinion in the perinatal period. Disregarding intrauterine infection assumed to have less than 10% incidence rate (Thurmond and Burridge, 1982), we assumed that the source of infection was in the calving barn. That potential source of infection was suggested also by others, as the bloody secretion arising in the calving house may transfer masses of infected lymphocytes to the calves (Van der Maaten et al., 1981; Pollari et al., 1993). The possibility of infection in the calving barn is supported by the fact that the seronegative heifers transferred to the infected farm became seropositive in the months following parturition (Table 3). In our opinion, this is why eradication gathered speed when the negative heifers were transferred to a separate calving barn to calve (Antal et al., 1988*c*). The simultaneous separation of the "white" side reduced the risk of infection constituted by the nasopharyngeal secretions (Roberts and Bushnell, 1982; Lucas et al., 1993).

One of the lessons to be learned from this extensive experiment is that, when transferred into an infected environment, seronegative pregnant heifers soon develop a high rate of seropositivity. This has been emphasized also by other authors (Ferdinand et al., 1979; Ferrer, 1979; Thurmond and Burridge, 1982; Simoyan et al., 1984; Mewes and Weege, 1985; Lassauzet, 1988). The possibility of infection at calving is supported also by the fact that, as shown in Table 3, the infection rate of the "white" side markedly decreased after isolation from the "black" side and the simultaneous introduction of isolated calving (Antal et al., 1988*c*).

We do not know of any case in which bovine leukosis was eradicated from a herd of more than 1,200 cows showing an AGID positivity higher than 62% by the use of the herd's own offspring. Our objective was to study and eliminate the routes of infection. We also know that ELISA provides results much more quickly, primarily in the final phase of eradication. As we started the eradication with the

AGID test, we kept using that test throughout the study to allow an easy comparison of results.

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PERINATAL OESTROGEN SYNDROME IN SWINE*

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Perinatal oestrogen syndrome (F-2 fusariotoxicosis occurring at the perinatal age) was studied in large pig herds and in animal experiments. The disease markedly lowered the conception rate of sows and gilts, and increased the number of repeat breeders. Litter size decreased and the number of stillbirths rose. Both the newborn piglets and the stillborn fetuses showed swelling of the vulva and teats and oedematous infiltration of the perineal region, ventral part of the abdomen and umbilicus, often accompanied by exudative-crusted inflammation, then necrosis of the teats. The number of piglets with splayleg and trembling increased. Gross and histopathological examination revealed enlargement of the ovary and uterus, with signs of follicle maturation in the ovary, glandular proliferation in the endometrium and epithelial proliferation in the vagina in addition to oedema and hyperaemia. In newborn piglets, the signs of hyperoestrogenism could be induced also experimentally, by feeding an F-2 toxin containing diet to pregnant sows. Intrauterine toxin effect was found to be primarily responsible for inducing the syndrome in newborn piglets. Because of its lower quantity, F-2 toxin excreted in the sow's milk is assumed to have a secondary role.

Key words: Perinatal oestrogen syndrome, swine, F-2 toxin, clinical signs, gross pathology, histopathology, animal experiments

A correlation between vulvar swelling in sows and the feeding of mouldy corn was observed in the United States already in 1927 (McNutt et al., 1928). Soon after that, it was discovered that these signs are induced by metabolic products formed on corn by *Fusarium graminearum* and *F. culmorum* (Stob et al., 1962). Toxin-contaminated feed induces excessive swelling of the vulva and vagina and hypertrophy of the uterus as seen at necropsy (McErlean, 1952; Bristol and Djurickovic, 1971; Ványi et al., 1973; Ványi et al., 1974; Chang et al., 1979). The compounds responsible for inducing these signs are the resorcilic acid-lactone derivatives produced by the above, and possibly by other, *Fusarium* species. The most important of these metabolites is zearalenone (F-2 toxin), which is a com-

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pound possessing oestrogen hormonal activity and hardly any direct toxic effect as evidenced by a barely measurable LD_{50} value (Ványi, 1986).

It was observed long ago that in pig herds where growing animals show signs of hyperoestrogenism due to contamination of the feed with F-2 toxin, similar changes can be found in the newborn piglets and occasionally in the fetuses as well (Domán, 1970; Miller et al., 1973; Ványi et al., 1981). This paper presents our observations and studies on the perinatal oestrogen syndrome of swine.

Materials and methods

Prevalence, clinical studies. The prevalence and manifestation of the disease, as well as the type and extent of the losses caused by it were studied in eight large pig herds.

Gross and histopathological examinations. Gross pathological examinations were carried out in the pig herds on the spot and at the Central Veterinary Institute on 60 newborn piglets and 20 porcine fetuses submitted for diagnostic examination. Since the clinical signs and gross lesions seen in the fetuses and in the newborn piglets were identical, further on no distinction will be made between fetuses and newborn piglets in connection with the individual examinations.

Histopathological examination included samples taken from the liver, kidney, spleen, brain, both ovaries, uterine horns, vagina, vulva and teats of 15 newborn piglets showing the signs of hyperoestrogenism and of 5 healthy (control) piglets. In addition, the same organs from three 4-week-old piglets that had shown the clinical signs of oestrogen syndrome at day-old, and those from three 4-week-old piglets that had been free from such signs, were also examined. Frozen and paraffin-embedded sections were stained with haematoxylin and eosin. Fettrot staining was employed to demonstrate lipids, the periodic acid–Schiff (PAS) reaction for studying the secretory activity of glands, and the Farkas–Mallory procedure for the examination of connective tissue fibres.

Chemical assays. Besides the feeds, occasionally the liver of newborn piglets and fetuses and the sow's milk were also assayed for the presence of the toxin and its metabolites.

Animal experiment. To study the mechanism of action of F-2 toxin in the perinatal period, a toxin-feeding trial was carried out using three pregnant sows. From 2 weeks before term up to farrowing, sow no. 1 continuously received a diet containing 40 ppm zearalenone. Sow no. 2 was fed a diet containing a similar amount of zearalenone for one week before and after farrowing. Sow no. 3 received a toxin-free diet throughout.

Chemical determinations (animal experiment). During the experiment, faecal and urine samples were collected from the sows at 4-day intervals and milk samples were taken on every second day. The samples were assayed for

zearalenone, α-zearalenol and β-zearalenol content after and without glucuronidase treatment.

Results

Prevalence, clinical signs

Farm I had 300 sows. In the months before the investigations, first the number of repeat breeders rose, the conception rate decreased and the number of stillbirths increased (e.g. during the farrowing immediately preceding the investigations there were 48 stillbirths in 11 litters). The newborn piglets showed swelling of the vulva and teats, and the first one or two teat pairs were necrotic. The most conspicuous signs were seen in the litter in which 5 out of 11 piglets had been born dead. The chemical assays demonstrated 15 ppb zearalenone in the milk of one sow and 40 ppb zearalenone in the liver of one piglet.

Farm II had 518 sows. The conception rate had been poor (60–65%) for years, it often dropped below 60% but occasionally results as low as 30% were also recorded. "Purulent" vaginal discharge was found for numerous sows, among them virgin gilts. Fertilized sows either returned to oestrus in the cycle or remained "silent". The farrowing rate gradually decreased and was around 8 at the time of the investigation. Chemical assay of a piglet's liver demonstrated 33.5 ppb zearalenone, 80.0 ppb α-zearalenol and 41.0 ppb β-zearalenol.

Farm III consisted of 2,000 sows. The conception rate had been steadily decreasing for six months. There were many repeat breeders among both the sows and the gilts. The newborn piglets showed mild vulvar and teat swelling. The chemical assays yielded the following results:

		Zearalenone, ppb	α-zearalenol, ppb	β-zearalenol, ppb
Liver	I	27	92	31
	II	24	84	25
	III	22	80	28
	IV	18	68	37
Milk	I	7	62	17
	II	10	45	21
	III	8	59	20

Farm IV had 1,250 sows. The sows had shown a lack of appetite, vomiting, diarrhoea and agalactia for some weeks. The newborn and sucking piglets exhibited vulvar swelling and teat enlargement. Chemical examination of the breeding

sow diet showed T-2 toxin contamination. In addition, the following results were obtained:

	Zearalenone, ppb	α-zearalenol, ppb	β-zearalenol, ppb
Liver (of one piglet)	16	91	23
Milk (of one sow)	12	70	18

Farm V consisted of 950 sows. The farm had a rather long history of poor conception (with a 55% overall conception rate for sows and gilts). The average litter size was 8.7, the stillbirth rate was one piglet per litter, and the rate of abortion was 1–2%. The newborn piglets showed expressed signs of hyperoestrogenism, and each litter contained some piglets with splayleg. Splayleg accounted for approx. 2–3% of the piglet losses. Chemically the sow diet was negative, but in the milk of one sow and in the liver of one piglet the following toxin contamination was demonstrated:

	Zearalenone, ppb	α-zearalenol, ppb	β-zearalenol, ppb
Piglet liver	19	85	27
Sow's milk	23	83	37

Farm VI had 2,000 sows. The weaning to first oestrus interval became longer and the conception rate was around 40–50%. The pigs were reluctant to consume the feed and the sows often had urticaria. There was a striking increase in the number of sows with vaginal and rectal prolapse. Insufficiency of labour was also common. The sucking piglets and sows vomited on several occasions. Death by haemorrhage through the umbilical stump occurred in several instances among the newborn piglets. Chemical examination of the sow diet yielded the following results:

	T-2	F-2
Sow diet I	positive	positive
Sow diet II	positive	positive
Sow diet III	positive	positive

Farm VII consisted of 1,500 sows. The umbilical stump of newborn piglets was regularly ligated, as death by haemorrhage through the umbilical stump was

very common. Even so, numerous piglets died in that way. Gross pathological lesions seen in the newborn piglets included oedema of the subcutaneous connective tissue, primarily on the abdominal wall around the teats and the external genitals. By chemical assay, the liver of a piglet was found to contain 22 ppb zearalenone, 85 ppb α-zearalenol and 28 ppb β-zearalenol.

Farm VIII consisted of 1,400 sows. The sows often showed uterine and rectal prolapse either before or after farrowing. Occasionally vomiting and diarrhoea also occurred. The conception rate had been around 40–50% for years, but sometimes it was as low as 30%. Newborn piglets showed expressed signs of hyperoestrogenism in almost all litters. Many piglets showed a lack of teats. Vulvar swelling subsided around the age of one week, only to become conspicuous again some days after weaning. The chemical assays gave the following results:

	Zearalenone, ppb	α-zearalenol, ppb	β-zearalenol, ppb
Liver (of one piglet)	19	85	38
Milk (of one sow)	23	81	35

Gross and histopathological examinations

By gross pathological examination, sucking piglets showing the signs of hyperoestrogenism had oedematous infiltration of the subcutaneous connective tissue and muscles in the perineal region and on the ventral part of the abdomen, hyperaemia and oedema of the vulva and tissues of the mammary gland, as well as enlargement of the ovaries and uterus, as compared to healthy control piglets. In some cases, first exudative then crusting inflammation of the skin of the ventral abdomen (mainly that covering the mammary glands and the teats) occurred. In advanced cases, the severely affected teats became necrotic and their skin healed by scarring. No pathological lesions were seen in other organs.

As compared to the healthy control piglets' primary follicles being at a state of rest (Fig. 1), in the ovary of the newborn piglets some of the follicles showed histological signs of growth and maturation (proliferation of the granulosa cells and the emergence of a follicular cavity), and several secondary and tertiary follicles could be observed among the primary follicles (Fig. 2). No regressive lesions were seen in the follicles. As compared to the underdeveloped glandular substance of the endometrium seen in healthy control animals (Fig. 3), in the affected piglets the endometrial glands started to grow and became higher and more ramifying (Fig. 4). No signs of secretion were observed in the glandular epithelial cells. In the healthy control animals the multilayered epithelium of the vagina was of uniform thickness and consisted of 2–4 cell rows (Fig. 5). At the same time, in piglets

showing the signs of hyperoestrogenism it had thickened and in some segments consisted of 8–12 cell rows (Fig. 6). Here and there, neutrophilic granulocytes were seen among the epithelial cells. The connective tissue of the vulva and mammary gland showed oedematous infiltration (Fig. 7) as compared to the control. Here and there, the epithelium of the latter organ showed superficial necrosis and concurrent exudative inflammation (Fig. 8). No pathological changes were detectable in the other organs examined (the liver, the kidneys, the spleen and the brain).

In those 4-week-old piglets which had shown the clinical signs of hyperoestrogenism after birth, pathological changes were seen in the ovaries, in the uterus and in the tissues of some mammary gland. As opposed to the resting primary follicles seen in the control animals, the ovaries of affected animals showed atretic remnants of several secondary or tertiary follicles. Ova could no longer be observed in the atretic follicles, the majority of the granulosa cells had become detached and showed the signs of necrosis. In the cavity of the follicle, signs of fibroblast proliferation were seen among these cell residues. The developmental status and ramification of the endometrial glands was similar to that seen in the control animals. In piglets that had shown the signs of hyperoestrogenism the number of so-called "nail-like cells" (dark-staining, degenerated or pycnotic cells of indistinct structure) increased among the superficial and glandular cells of the endometrium. In the connective tissue of the affected mammary gland and around the teat canal the normal tissue structure seen in control animals could no longer be recognized, as it had been replaced by angiofibroblast tissue proliferation, here and there the proliferation of mature split-up connective tissue and focal lymphohistiocytic infiltration.

Animal experiment

The two sows fed a zearalenone-containing diet (sows no. I and II) and the control sow (no. III) farrowed at term without any complications. The litter size was 11 for sows no. I and II and 10 for sow no. III. In contrast to the controls, the female piglets delivered by the two sows fed a zearalenone-containing diet showed swelling and mild hyperaemia of the vulva and teats after birth (piglets of sow no. II exhibited more pronounced lesions). These signs subsided after 24 h and disappeared after 48 h.

Chemical assays (sow experiment)

The excretion of zearalenone and its metabolites with the milk, faeces and urine is shown in Figs 8, 9 and 10.

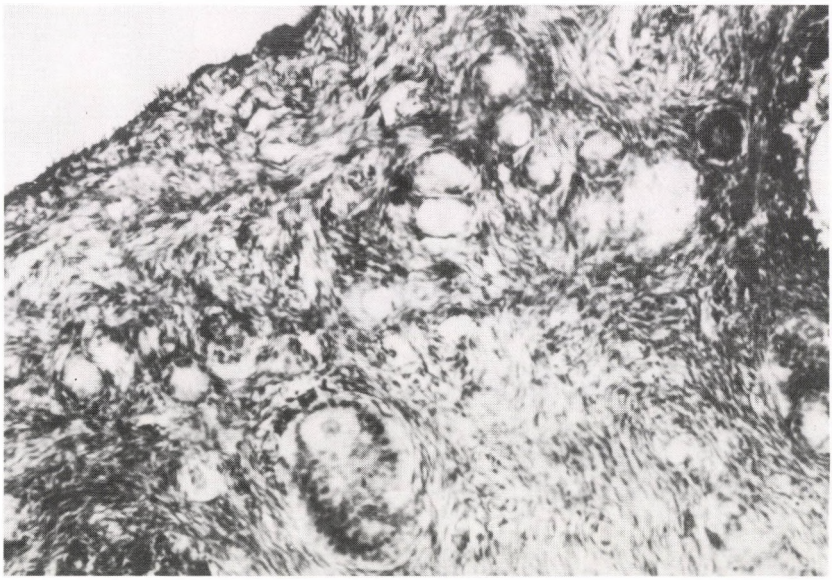


Fig. 1. Ovary from a healthy (control) newborn piglet. Primary (resting) follicles. Haematoxylin and eosin (H.–E.), × 63

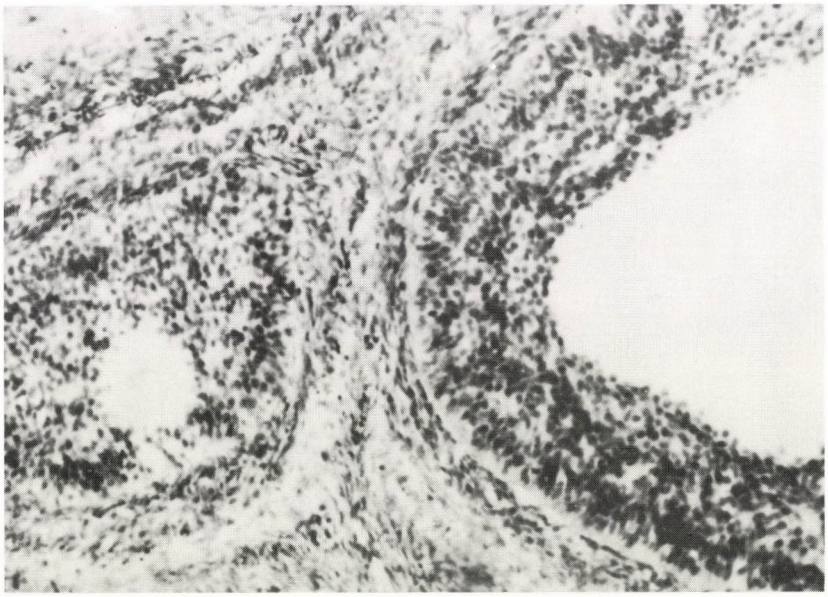
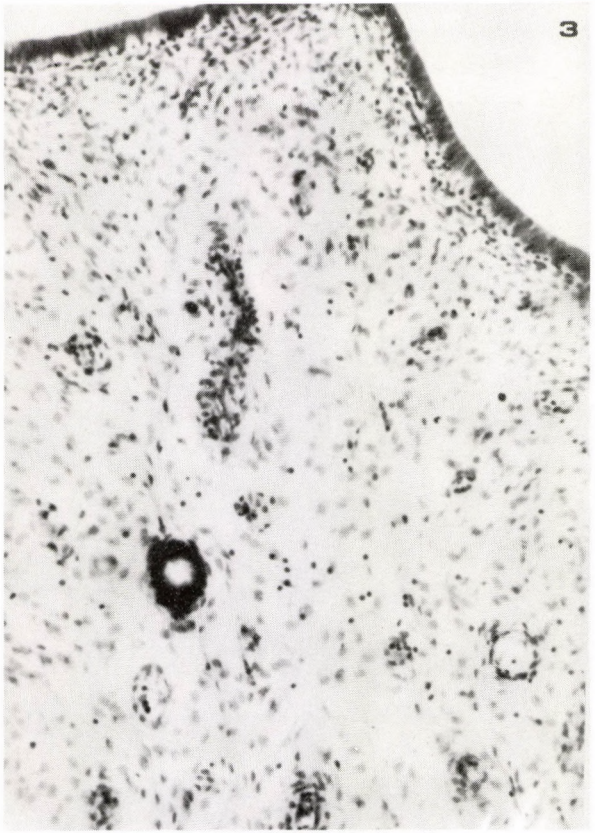


Fig. 2. Ovary from a piglet showing the signs of perinatal oestrogen syndrome. Maturing tertiary follicles. H.–E., × 63



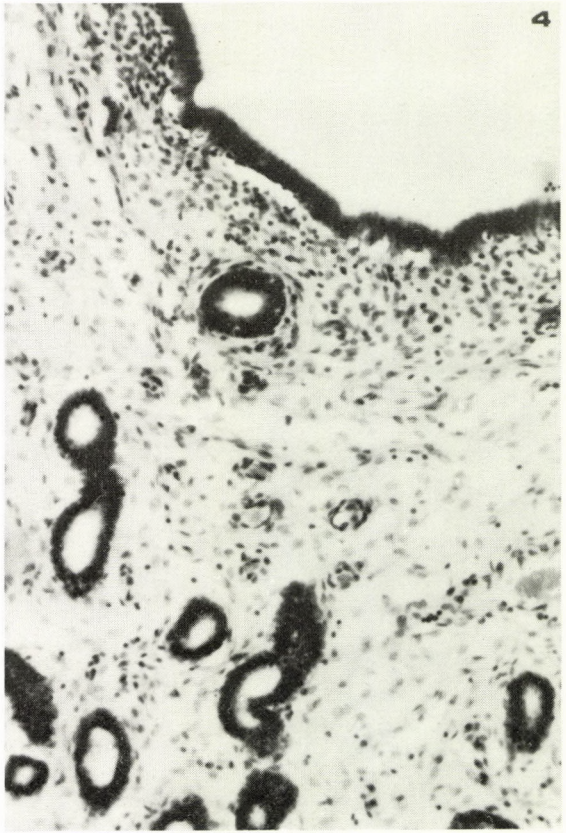
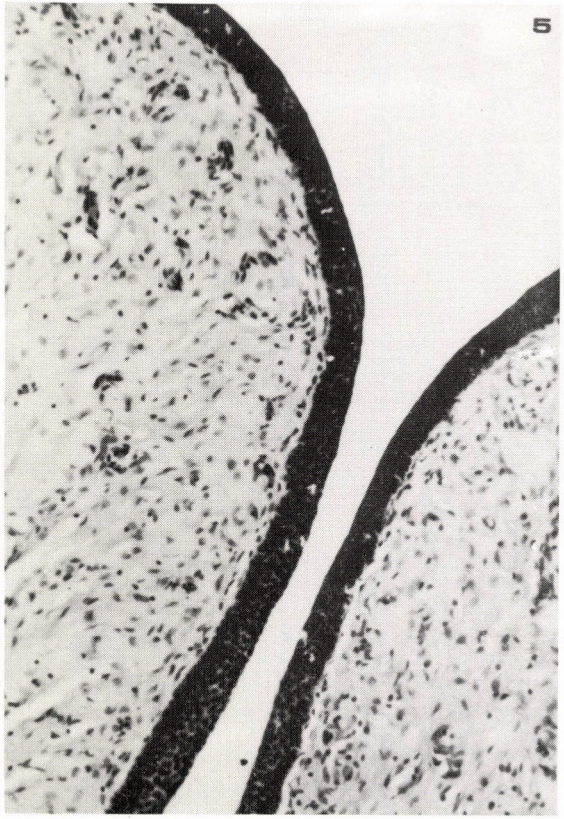


Fig. 3. Part of the uterus from a healthy (control) newborn piglet. The glandular substance of the endometrium is insufficiently developed. H.–E., × 63

Fig. 4. Part of the uterus from a newborn piglet showing the signs of perinatal oestrogen syndrome. The glandular substance of the endometrium has started to develop (note an increase in the number of gland cross-sections). H.–E., × 63



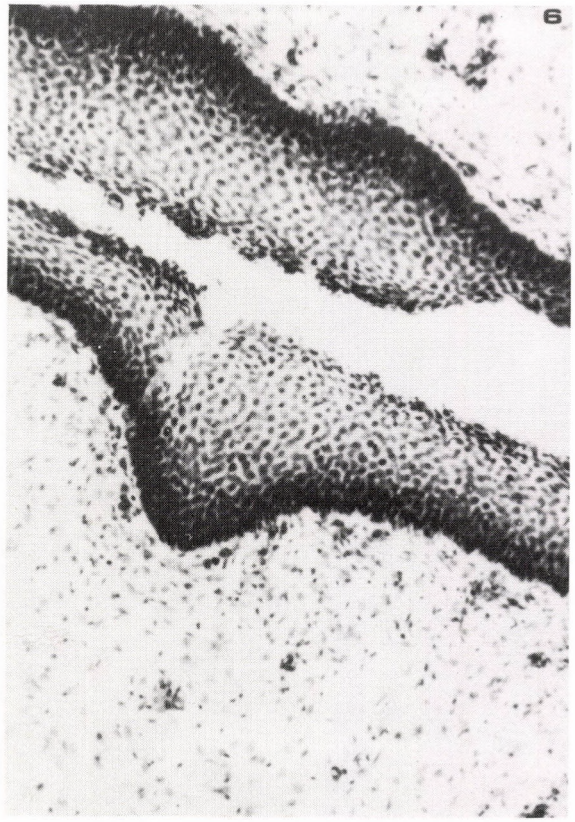


Fig. 5. Part of the vagina from a healthy (control) newborn piglet. The epithelial layer of the mucous membrane is uniformly thin. H.–E., × 63

Fig. 6. Part of the vagina from a newborn piglet showing the signs of perinatal oestrogen syndrome. Note proliferation of the epithelial layer of the mucosa. H.–E., × 63

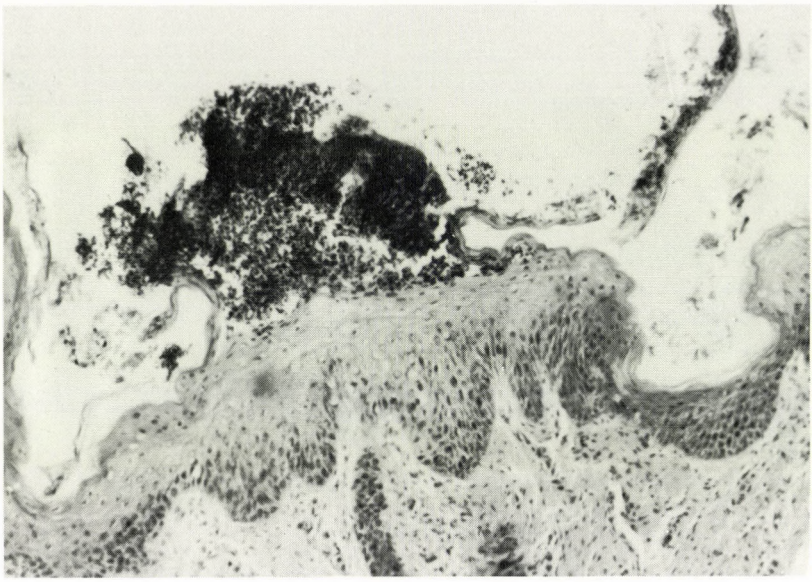


Fig. 7. Part of a teat from a newborn piglet showing the signs of perinatal oestrogen syndrome. Note exudative inflammation accompanied by necrosis of the superficial layers of the epithelium. H.–E., × 63

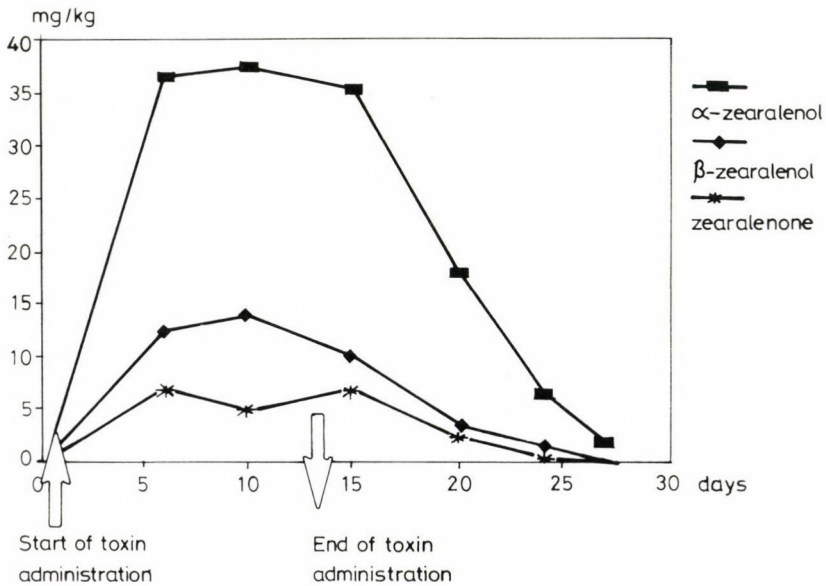


Fig. 8. Excretion of zearalenone in the faeces

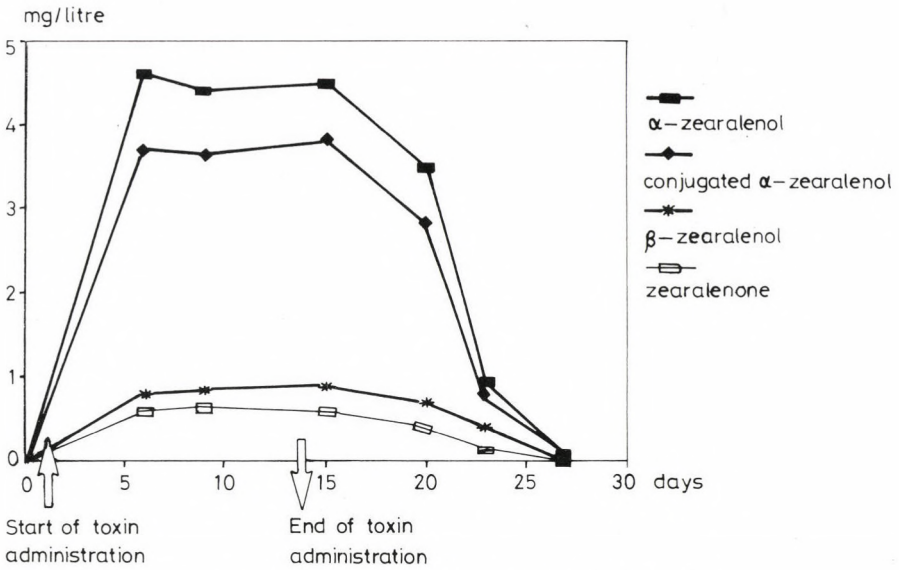


Fig. 9. Excretion of zearalenone in the urine

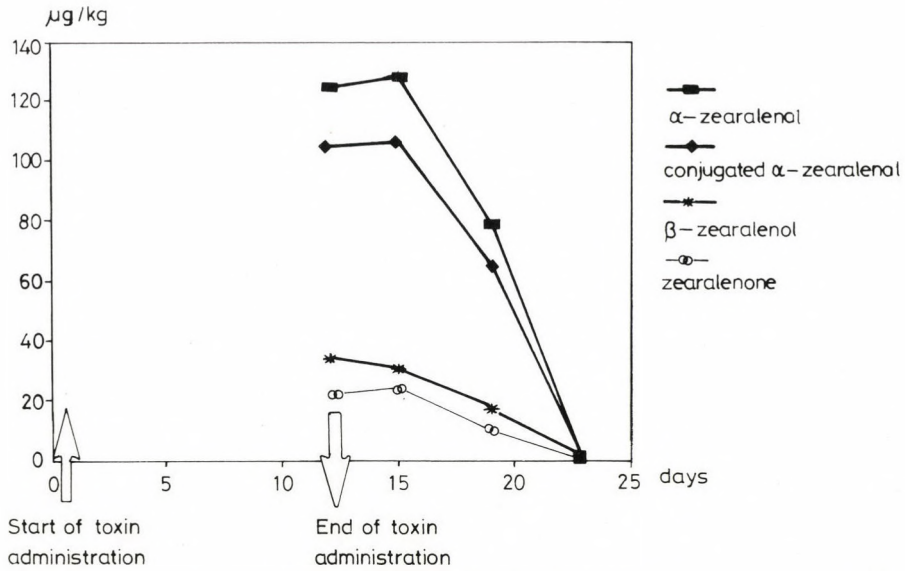


Fig. 10. Excretion of zearalenone in the milk

Discussion

In harmony with the findings of other authors (Domán, 1973; Miller et al., 1973), the present observations made in large pig herds indicate that F-2 toxin contamination of the diet fed to sows may give rise to signs of hyperoestrogenism in newborn piglets and occasionally already in fetuses. These signs include oedematous infiltration of the vulva, the perineal region, the mammary gland and the ventral part of the abdomen, hyperaemia of the vulva, and often the exudative inflammation of the skin of the teats.

In herds showing the signs of perinatal hyperoestrogenism, the conception rate of sows and gilts decreased, the number of repeat breeders and the culling rate of sows increased, the average litter size decreased, the stillbirth rate rose, and vaginal discharge was a common finding in the case of sows and even for gilts. The viability of newborn piglets was reduced, especially in litters of primiparous sows, presumably due to the fact that the gestation period was shortened by a few (3–5) days. Splayleg and trembling were very common findings.

Oedematous and swollen teats are prone to injuries and often become necrotic and detached as a result of secondary bacterial infection. In such cases also the glandular substance becomes atrophic. Animals with such lesions will become unfit for breeding.

The gross lesions were confirmed by the results of histological examinations. Even the internal genital organs showed changes that have an unfavourable prognosis from the point of view of future sexual activity. In contrast to the primary follicles being at a state of rest, which is typical of newborn piglets, some follicles of the affected piglets exhibited signs of growth and maturation. Several secondary and tertiary follicles could be observed among the primary follicles. The endometrial glands started to grow and became ramified. The multilayered epithelium of the vagina thickened, it was sometimes composed of 8–12 cell rows, with neutrophilic granulocytes being present among the epithelial cells. Similar changes have been reported also by other authors (Kurtz et al., 1969; Mirocha and Christensen, 1974).

In the ovaries of 4-week-old piglets that had shown the signs of hyperoestrogenism at day-old the atretic remnants of several secondary and tertiary follicles could be recognized. These follicles no longer contained ova, the majority of granulosa cells had become detached and showed the signs of necrosis. Fibroblast proliferation was observed in the cavity of the follicle among the remnants of cells. The number of the so-called "nail-like cells" (dark staining degenerated or already pycnotic cells of indistinct structure) increased among the superficial and glandular epithelial cells of the endometrium

The results of the sow experiment indicated that zearalenone administered orally at a dose of 40 ppm/day is excreted mainly via the faeces. On day 4 after the start of toxin administration its faecal concentration was 36 mg/kg. That con-

centration did not change considerably during toxin administration; however, after the cessation of administration it rapidly declined and dropped below 1 mg/kg in 14 days.

Elimination via the urine showed a similar trend: here the maximum concentration was 5–6 mg/kg. Subsequently the toxin concentration of the urine abruptly decreased and after 2 weeks no toxin could be detected in the urine.

Only a minor fraction of the administered toxin was excreted in the milk, mainly in the form of metabolites. In none of the cases did the concentration of these metabolites reach 150 μg/kg. Corresponding to the lower concentration, the toxin content of the milk dropped below the detection limit in one week.

It was also established that zearalenone, α-zearalenol and β-zearalenol occurred in the faeces, urine and milk as well. In the urine, the majority of metabolites was present in the form of a glucuronic acid adduct and only a small fraction (10–20%) was present in free form. It was remarkable that α-zearalenol was the dominant metabolite also in the faeces, which contained much less zearalenone (roughly the same amount as β-zearalenol).

Further studies are needed to determine whether this transformation takes place in the intestine, or a substantial part of the already absorbed toxin becomes again released into the intestinal tract.

The female piglets born during the period of toxin effect exhibited the signs of hyperoestrogenism, suggesting that these had developed already during intrauterine life. These signs subsided and disappeared in 3 days after birth. The piglets nursed by sows excreting toxin in their milk did not develop signs of hyperoestrogenism.

This finding indicates that the amount of toxin contained in the sow's milk is not sufficient to elicit or maintain the signs of hyperoestrogenism in sucking piglets.

Finally, it should be mentioned that though in recent years the *Fusarium* and fusariotoxin contamination of feeds has decreased, it still has great importance. The currently used routine methods are unsuitable for detecting F-2 toxin in the feed; however, breeding animals permanently exposed even to small doses of the toxin may experience severe reproductive disorders, impaired reproductive performance, or losses.

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PATHOMORPHOLOGICAL CHANGES CAUSED BY T-2 TRICHOTHECENE FUSARIOTOXIN IN GEESE*

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T-2 trichothecene fusariotoxin was administered to 110 laying geese and 33 ganders in the active egg production period through a tube at 2-day intervals on a total of 10 occasions. After the treatment, the geese were subjected to detailed pathomorphological examination. In the ovaries of layers, a cessation of follicle maturation and follicle degeneration dependent on the toxin dose were observed, accompanied by ovulation and consequent peritonitis in the birds that died and in some of those killed by bleeding. Additional findings included involution of the oviduct, lymphocyte depletion, necrosis and amyloidosis in the spleen, catarrhal enteritis, signs of colloid stasis in the thyroid and large numbers of secretory granules in the cytoplasm of the adrenaline-producing cells of the adrenal gland. In the ganders, toxin administration did not reduce the intensity of spermatogenesis but in the spleen, intestine and endocrine glands it caused changes similar to those seen in the layers.

Key words: T-2 mycotoxicosis, pathology, goose, *Fusarium*

Mycotoxin contamination of the feed exerts an adverse effect on the egg production of poultry species. According to field observations, T-2 fusariotoxin occurring in the feed reduces the egg production of breeding geese (Ványi et al., 1989; Ványi et al., 1992; Ványi et al., 1994; Tóbiás et al., 1992). In the majority of such eggs, embryonic development either does not even commence or the embryo dies some days after the start of development, giving rise to a so-called "bloody" egg as seen at candling. Even for eggs with seemingly undisturbed embryonic development, failure to hatch is rather common. In addition, some of the hatched goslings are non-viable and die at day-old (Ványi, 1990). In view of the fact that T-2 fusariotoxin reduces both the egg yield and the biological value of eggs, in this experiment breeding geese in active egg production were treated orally with T-2 toxin on every second day for 20 days in order to study the result-

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ing pathomorphological changes. To the best of our knowledge, no similar study has been reported in the special literature as yet.

Materials and methods

The breeding geese used in the experiment were selected from a one-year-old flock that had reached a 40% egg production level. The grouping of the experimental geese, the dose of the toxin administered and the resulting deaths are shown in Table 1.

Table 1

The grouping of geese used in the experiment and the number of deaths

	Group										
	I	II	III	IV	V	VI	VII	VIII	IX	X	C*
T-2 toxin through an oesophageal tube, on every second day, 10 times (mg/kg b.m.)	0.1	0.2	0.3	0.4	0.5	0.6	0.8	0.9	1.0	3.0	C
No. of geese per group	10 f 3 m	10 f 3 m	10 f 3 m	10 f 3 m	10 f 3 m	10 f 3 m	10 f 3 m	10 f 3 m	10 f 3 m	10 f 3 m	10 f 3 m
Deaths	–	–	–	–	–	1 f	–	–	3 f 1m	6 f 1 m	–

C* = control; f = female; m = male

Geese that died between day 10 and 20 of toxin administration and those killed on day 20 after the end of toxin treatment were necropsied, then samples of appropriate size were taken from their crop, glandular stomach, small intestine, spleen, liver, kidney, adrenal gland, thyroid, ovaries, infundibulum, magnum and uterus of the oviduct and from the testicle, fixed in 5 (v/v)% formalin (pH 7.2) buffered with NaH_2PO_4 and NaOH, and processed for *histologic examination* by freezing and paraffin-embedding techniques. For general information, the sections were stained with P. Mayer's acid haematoxylin-eosin. The periodic acid–Schiff (PAS) reaction was used for studying the secretory activity of the oviductal glands, Fat Red staining for demonstrating the lipid content of the ovaries, liver and kidneys, while Congo red staining and polarization microscopy for the determination of amyloid content.

Samples from the spleen and adrenal gland of two affected and subsequently sacrificed geese (from the 3 mg/kg b.w. group) were subjected to *electron microscopy*. The organ pieces were postfixed in 2.5 (v/v)% glutaraldehyde buffered with sodium cacodylate, then in 1 (v/v)% osmium tetroxide. After embedding in Durcupan resin, sections were cut with an ultramicrotome, counter-stained with uranyl acetate and lead citrate, then examined by electron microscopy.

Results and conclusions

The results of gross and histopathological examination are summarized in Table 2 and illustrated by Figs 1–10.

Table 2

Morphological changes observed in the geese that died or were sacrificed

Organ and lesion		T-2 toxin, mg/kg b.m.										
		0.1	0.2	0.3	0.4	0.5	0.6	0.8	0.9	1.0	3.0	C
Ovary:	cessation of follicle maturation	+–	+	+	+	++	++	++	++	++	++	
	follicle degeneration	+–	+	+	+	++	++	++	++	++	++	
	abdominal ovulation and peritonitis						+	+	+	++	++	
Oviduct:	involution	+–	+	+	+	++	++	++	++	++	++	
	atrophy of the glandular substance	+–	+	+	+	++	++	++	++	++	++	
Spleen:	amyloidosis						+	+	+	++	++	
	lymphocyte depletion						+	+	+	++	++	
	lymphocyte necrosis						+	+	+	++	++	
Intestine:	catarrhal inflammation						+	+	+	+	+	

+ = mild; ++ = severe

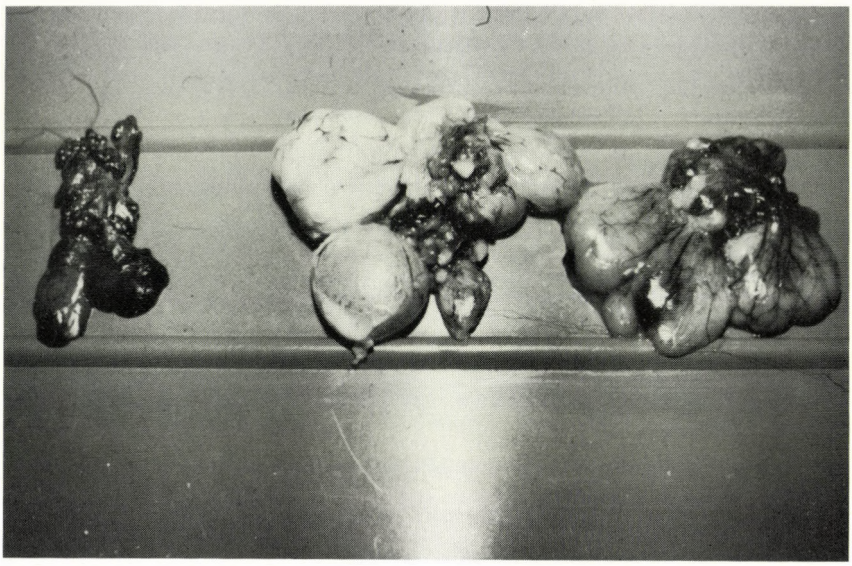


Fig. 1. T-2 toxin causes cessation of follicle maturation and follicle degeneration in the ovary

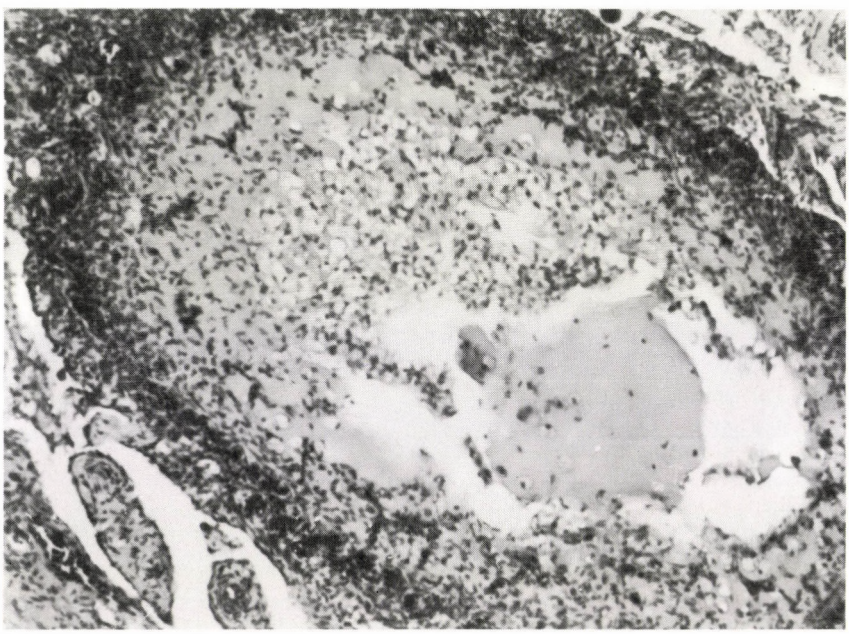
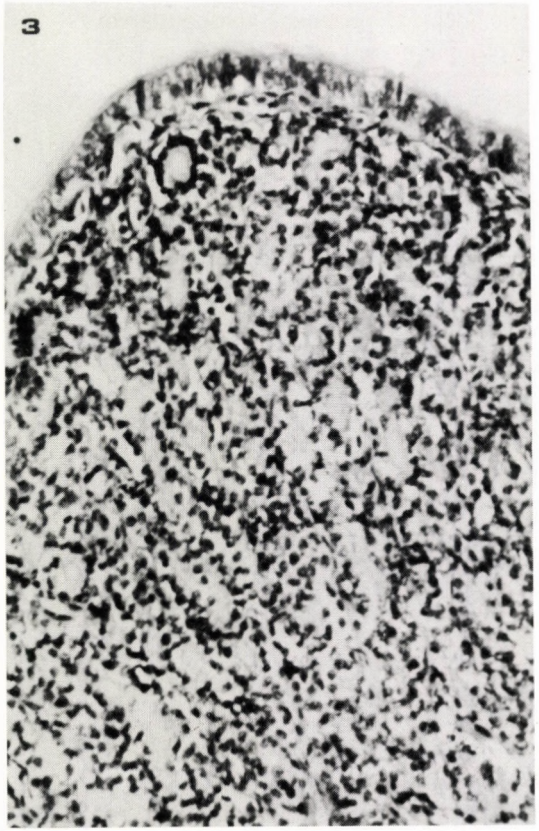


Fig. 2. Degenerated ovarian follicle. Fat Red staining, × 100



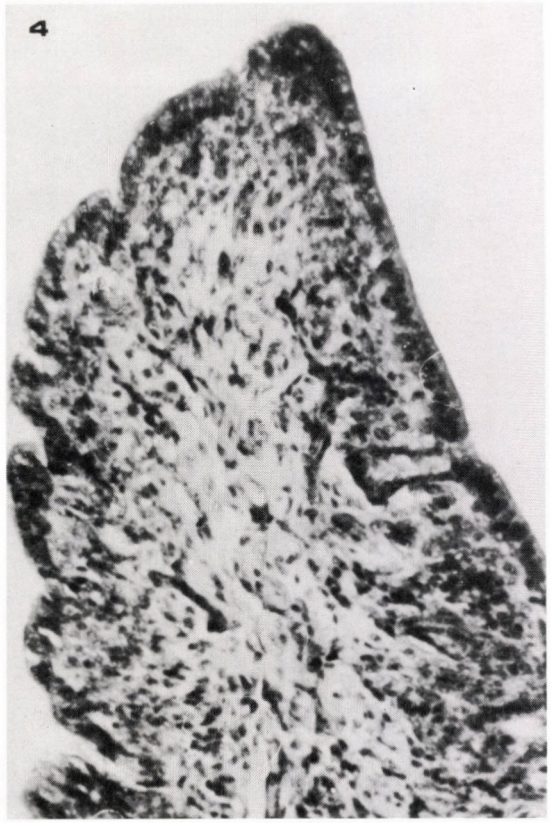
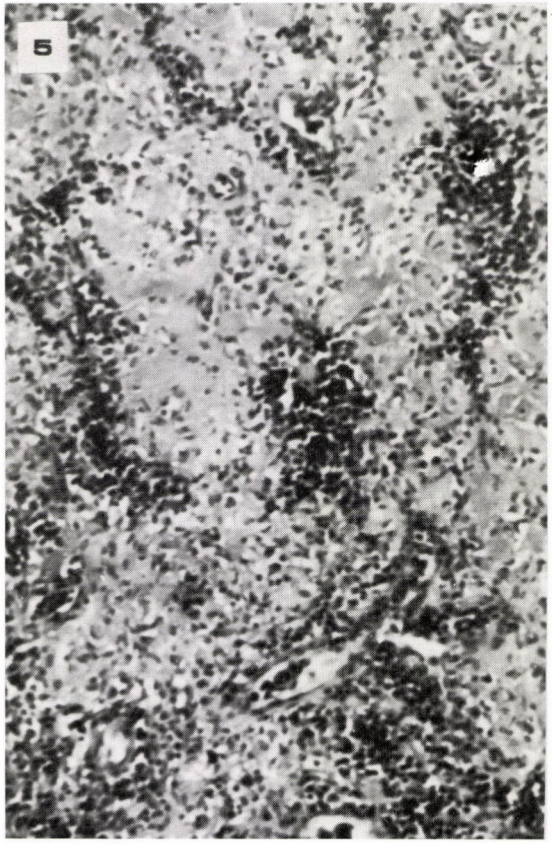


Fig. 3. Intensive secretory activity of the protein-secreting glandular substance in the oviduct of a healthy (control) laying goose. Haemalum–eosin (H.–E.), × 100

Fig. 4. Cessation of secretion and atrophy of the protein-secreting glandular substance as a result of T-2 toxin administration. H.–E., × 100



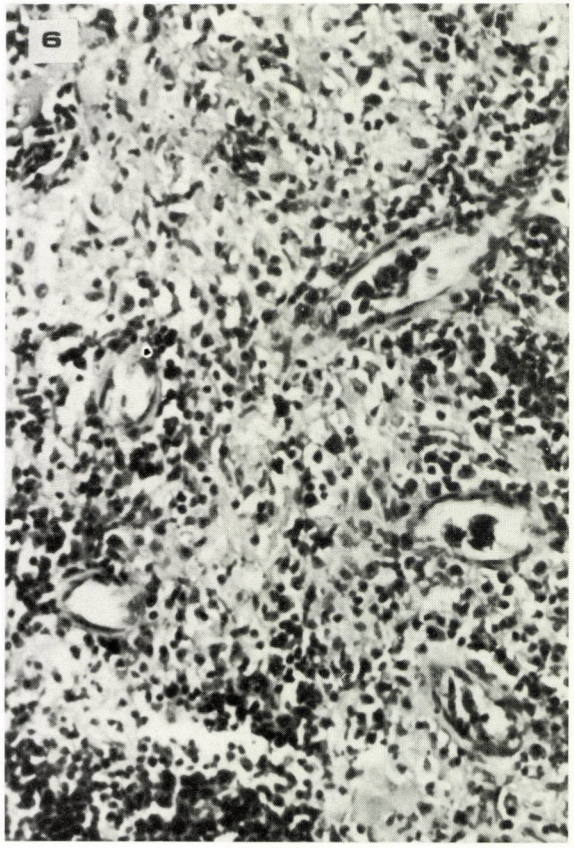


Fig. 5. T-2 toxin induced amyloidosis in the spleen. H.–E., × 100
Fig. 6. T-2 toxin induced lymphocyte depletion and necrosis in the Malpighian bodies of the spleen. H.–E., × 240

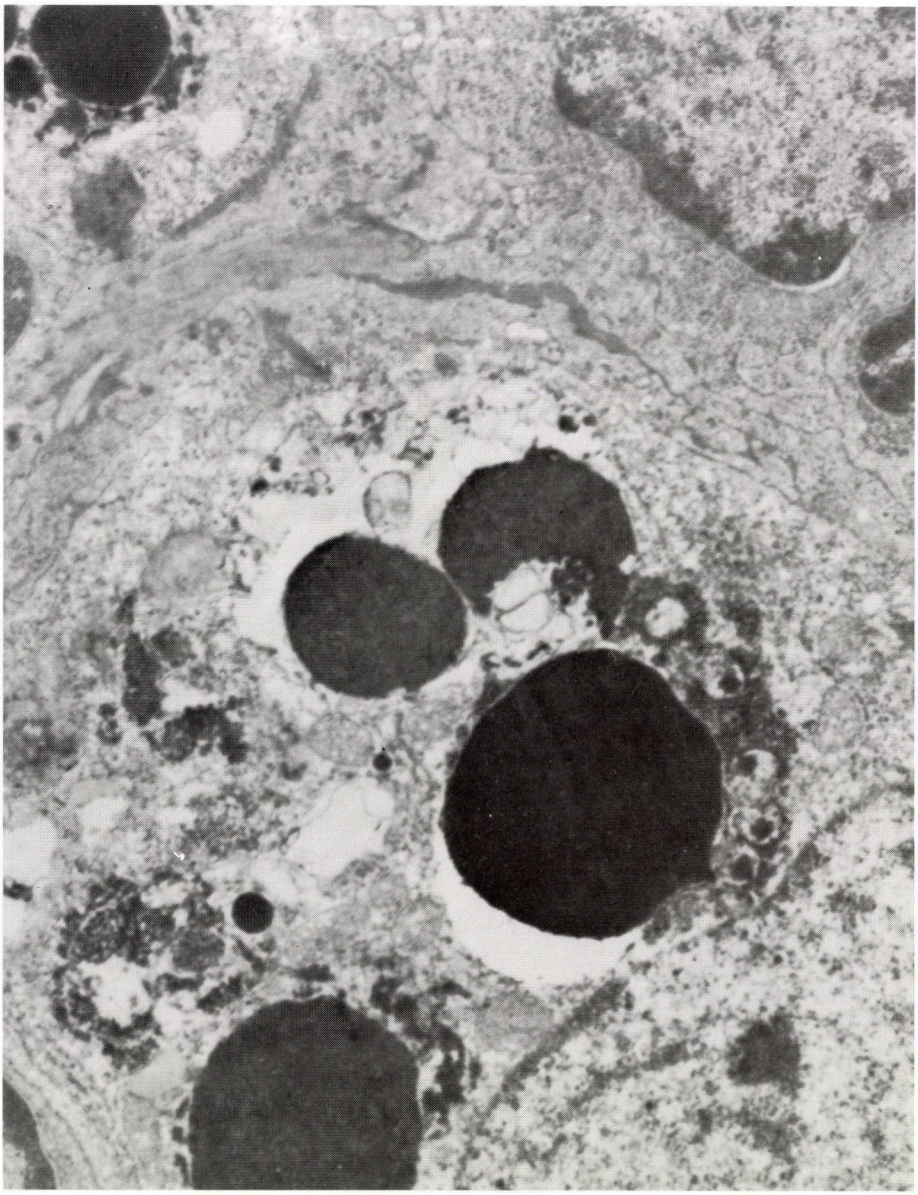


Fig. 7. T-2 toxin induced lymphocyte depletion and necrosis in the Malpighian bodies of the spleen. Electron micrograph, × 9,600

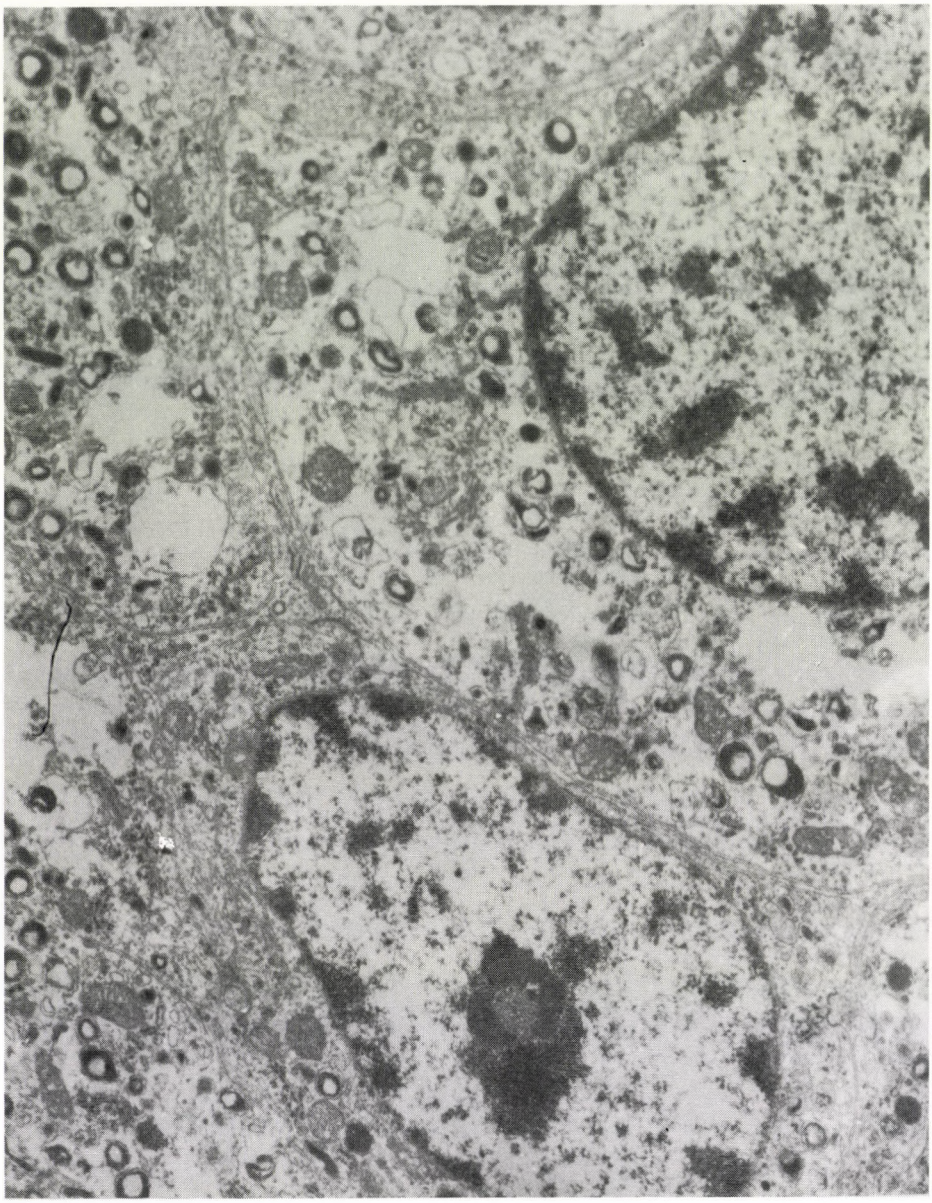
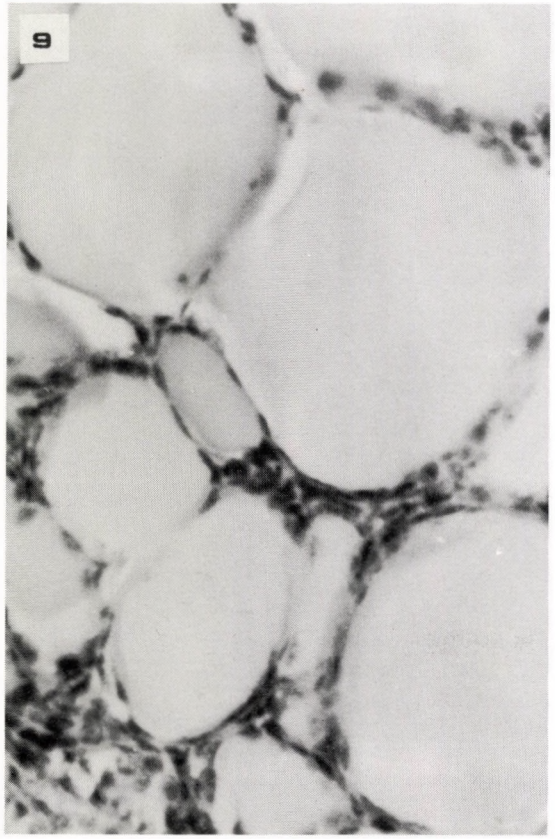


Fig. 8. Presence of large numbers of secretion droplets in the cytoplasm of adrenaline-secreting cells of the adrenal gland as a result of T-2 toxin administration. Electron micrograph, × 9,600



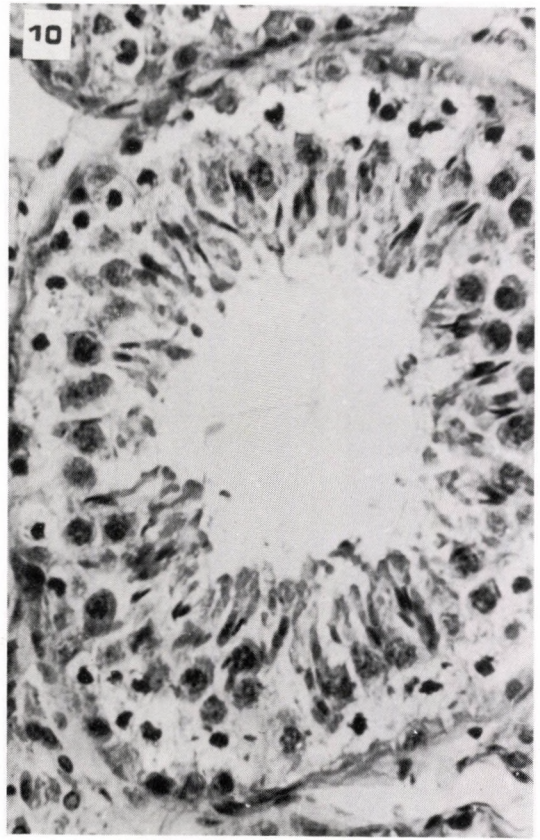


Fig. 9. Stagnant colloid in the acini of the thyroid and atrophy (arrow) of the epithelium lining them. H.–E., × 100
Fig. 10. T-2 toxin does not impair spermatogenic activity. H.–E., × 240

The administration of T-2 toxin through a tube on every second day over a period of 20 days did not cause clinically apparent disease in the adult geese. Even the abrupt deaths were preceded by faintness lasting for a few minutes and slight trembling.

Pathomorphological examination revealed cessation of follicle maturation or reduction of its intensity, degeneration and flaccidity of the maturing follicles, haemorrhages in their wall, and — in the geese that died — abdominal ovulation and peritonitis. Involution of the oviduct and atrophy of the glandular substance in some of its segments could also be observed.

The adverse effect exerted by the trichothecene fusariotoxin T-2 on the reproductive functions of female animals has already been reported by us for swine (Glávits et al., 1983) and for poultry (Tóbiás et al., 1992), and it has been described by other authors (Fazekas et al., 1993) as well.

In contrast to the oestrogenic toxin zearalenone produced by *Fusarium graminearum,* which was reported to impair spermatogenesis (Ványi et al., 1973), in this study T-2 toxin caused no abnormality in the spermatogenic activity and germinal epithelial cells of the ganders' testes.

In this experiment, T-2 toxin was found to cause only mild injury to the mucous membrane of the digestive tract both in the layers and in the ganders, and no clinically apparent diarrhoea was observed. In the spleen, lymphocyte depletion and degeneration as well as necrosis of a certain part of them were seen, which observation is consistent with the finding, supported also by our earlier studies (Glávits and Ványi, 1988), that T-2 toxin and other trichothecene mycotoxins have an immunosuppressive effect.

The amyloidosis seen in the spleen is indicative of a cell damage of toxic origin. In the thyroid, the majority (60–70%) of the acini showed dilatation of the lumen, thinning of the epithelium lining it and, here and there, the absence of acinar epithelial cells. In the adrenal gland, numerous secretion droplets appeared in the cytoplasm of the adrenaline-secreting cells. These lesions are indicative of a dysfunction of the hormonal system presumably associated with toxic and stressor effect. In the sacrificed geese, the above gross and histopathological lesions occurred in a dose-dependent manner. The most severe lesions were seen in those birds which had died.

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EFFECT OF ACUTE SELENIUM TOXICOSIS ON THE LIPID PEROXIDE STATUS AND THE GLUTATHIONE SYSTEM OF BROILER CHICKENS

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The effect of acute oral selenium toxicosis on the rate of lipid peroxidation, on the amount of reduced glutathione as well as on glutathione-peroxidase activity of the blood (plasma and red blood cells) and liver was studied in broiler chicken. Cockerels (28-day-old) were treated with selenium (4.85 mg/kg b.w.) administered intraoesophageally in the form of sodium selenite. Samples were at the onset of clinical signs (3 h after treatment) and 2 and 4 h thereafter. The malondialdehyde content of the blood plasma rose significantly ($P < 0.05$) at the onset of clinical symptoms but decreased later. Malondialdehyde content of the liver was higher than the control value at the first sampling ($P < 0.01$) and steadily increased later. Reduced glutathione content did not change significantly in the blood plasma and liver. Glutathione peroxidase activity of the RBC was significantly elevated ($P < 0.01$) only at the first sampling (3 h after treatment) and decreased to the control level thereafter. Acute oral selenium toxicosis increases the rate of lipid peroxidation in a short period of time (7 h) without exerting a significant effect on the glutathione system.

Key words: Selenium, toxicity, broiler chicken, lipid peroxidation, glutathione, glutathione peroxidase

The role of selenium in biological systems has been associated with its antioxidant activity (Schwarz and Foltz, 1957). Selenium is required for the activity of glutathione peroxidase (Rotruck et al., 1973). Recently the use of selenium preparations for the treatment or prevention of various diseases of livestock has become a common practice. At the same time, high environmental concentrations of selenium have an adverse effect on the reproduction, survival and development of birds (Hoffmann et al., 1988, 1989).

The toxic effects of excess selenium have been suggested to be related to the glutathione redox system. Dickson and Tappel (1969) showed that excess selenium interacts with reduced glutathione. It was also demonstrated that selenium, in oxidised state as selenite, metabolised through a reductive pathway using reduced

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glutathione and NADPH serving as reducing molecules (Ganther, 1986). Other studies have shown that excess selenite administration stimulates the rate of lipid peroxidation (Dougherty and Hoekstra, 1982; Hoffman et al., 1989). Most of the previous studies dealt with the effects of chronic selenium toxicosis. Acute toxicosis is a real problem in the practice of animal nutrition, and the mode of action and short-term effect of excess selenium remain unclear and are probably different from the chronic effects.

The aim of the present study was to study the early effect of a single sublethal dose of selenium on the rate of lipid peroxidation and on some parameters of the glutathione system in broiler chickens.

Materials and methods

Acute selenium toxicity was induced in a group (n=15) of 28-day-old cockerels by oral administration of selenium (4.85 mg/kg b.w.) in the form of sodium selenite dissolved in 0.5 ml of saline. The selenium dose was calculated as half of the LD_{50} according to the previous findings of Sályi et al. (1993). A second group (n=15) of birds served as control and was treated simultaneously with the same volume of sterile saline. At the onset of clinical signs (3 h after treatment) and 2 and 4 h thereafter 5 treated and 5 control cockerels were killed by cervical dislocation. Heparinized blood was separated to plasma and RBC by centrifugation and the RBC were haemolyzed with a 9-fold volume of redistilled water. Liver samples were taken immediately after bleeding and stored deep-frozen until the analyses. Before the analyses the liver samples were homogenised in cold isotonic saline. Malondialdehyde (TBA-reactive substances) was determined in blood plasma and in intact liver homogenate by the thiobarbituric acid reaction (Matkovics et al., 1988). The reduced glutathione content of the blood plasma and liver homogenate was measured using the method of Sedlak and Lindsay (1968). Glutathione peroxidase activity of the blood plasma, RBC haemolysate and 10,000 *g* supernatant fraction of liver homogenate was measured according to Matkovics et al. (1988). The protein content of the plasma was measured by the biuret method (Gornall et al., 1949) and using Folin-phenol reagent in the case of liver homogenate (Lowry et al., 1951). The haemoglobin content of RBC haemolysate was determined by the cyanomethaemoglobin method (Drabkin, 1946).

Statistical analysis of the results was done by the paired *t* test.

Results

Clinical observations. Clinical signs appeared very soon (about 2 h) after selenium treatment. All treated chickens were sitting, and diarrhoea and "vomiting" were general within the group. The clinical signs disappeared 4 to 5 h after treatment. No mortality was observed during the experiment.

Biochemical analyses. The rate of lipid peroxidation in the blood plasma, as measured by the amount of malondialdehyde, increased significantly ($P < 0.05$) 3 h after treatment but decreased to the control level thereafter. The malondialdehyde content of the liver homogenate increased steadily, but a significant ($P < 0.01$) difference was found only at 7 h after treatment as compared to the controls (Table 1). The amount of reduced glutathione did not change significantly either in the blood plasma or in the liver homogenate (Table 2). A significant difference ($P < 0.01$) in glutathione peroxidase activity was found only in the RBC haemolysate 3 h after treatment (Table 3).

Table 1

Changes in malondialdehyde content of the blood plasma and liver homogenate during acute selenium toxicosis (x ± SD)

Group/time of sampling	Malondialdehyde content	
	Blood plasma (mmol/L)	Liver homogenate (μmol/g wet wt.)
Treatment + 3 h		
Treated	15.19 ± 6.84*	2.30 ± 0.72
Control	6.28 ± 3.08	1.66 ± 0.65
Treatment + 5 h		
Treated	7.38 ± 3.28	2.32 ± 0.71
Control	6.21 ± 3.69	1.68 ± 0.20
Treatment + 7 h		
Treated	8.09 ± 1.72	2.75 ± 1.29**
Control	5.69 ± 1.93	1.29 ± 0.14

Levels of significance: * = $P < 0.005$; ** = $P < 0.01$

Table 2

Changes in reduced glutathione content of the blood plasma and liver homogenate during acute selenium toxicosis (x ± SD)

Group/time of sampling	Reduced glutathione	
	Blood plasma (mmol/L)	Liver homogenate (mmol/g wet wt.)
Treatment + 3 h		
Treated	104.20 ± 3.61	3.11 ± 0.63
Control	105.11 ± 2.00	2.78 ± 0.16
Treatment + 5 h		
Treated	101.67 ± 4.10	3.09 ± 0.50
Control	97.48 ± 4.59	3.22 ± 0.60
Treatment + 7 h		
Treated	98.43 ± 3.11	3.20 ± 0.62
Control	95.19 ± 2.52	3.16 ± 0.28

Table 3

Changes in glutathione peroxidase activity of the blood plasma, RBC haemolysate and liver homogenate during acute selenium toxicosis (x ± SD)

Group/time of sampling	Glutathione peroxidase activity		
	Blood plasma (U/g protein)	RBC haemolysate (U/g haemoglobin)	Liver homogenate (U/g protein)
Treatment + 3 h			
Treated	6.20 ± 1.56	61.15 ± 7.87**	15.38 ± 4.08
Control	5.32 ± 0.95	42.82 ± 2.97	13.72 ± 2.87
Treatment + 5 h			
Treated	8.16 ± 4.20	50.64 ± 24.68	14.55 ± 1.22
Control	4.82 ± 0.30	46.67 ± 6.40	15.29 ± 3.18
Treatment + 7 h			
Treated	3.82 ± 1.16	65.10 ± 22.65	14.98 ± 1.43
Control	4.40 ± 2.26	53.33 ± 6.59	17.86 ± 2.65

Levels of significance: ** = $P < 0.01$

Discussion

Excess amount of selenium administered in inorganic form as sodium selenite caused moderate and non-significant alterations in glutathione metabolism but stimulated the rate of lipid peroxidation in broiler chickens. The non-significant changes in glutathione metabolism are contrary to findings described earlier (Dickson and Tappel, 1969; Ganther, 1986). That result may be explained in two possible ways. First, the inorganic form of selenium (sodium selenite in this experiment) had a less expressed effect than did organic selenium compounds in ducks (Hoffman et al., 1989). The second explanation is the acute (short-term) form of toxicosis. The aim of this investigation was to detect only the early effects of selenium toxicity. For this reason, the study was finished 7 h after treatment. The previous findings about the depletion of reduced glutathione were obtained in chronic or sub-chronic experiments (Dickson and Tappel, 1969; Ganther, 1986; Hoffman et al., 1989). It seems that the first step of changes is the increase of the lipid peroxidation rate, which is later followed by the reduction of thiol content.

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ELIMINATION AND METABOLISM OF TRIIODOTHYRONINE DEPEND ON THE THYROID STATUS IN THE BRAIN OF YOUNG CHICKENS+

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The hypothyroid chicken brain has been found to preserve more triiodothyronine (T_3) than expected from plasma T_3 levels. A possible explanation is that the elimination of T_3 from the hypothyroid brain is decreased. In the present experiments, the elimination rate of T_3 was compared in surgically thyroidectomized animals and sham-operated controls. It was found that both T_3 coming from the plasma and T_3 derived locally in the cells from thyroxine have a significantly lower elimination rate in thyroidectomized chickens than in sham-operated ones. Therefore it is concluded that the adaptation of the brain to hypothyroid conditions is partly regulated by reducing the loss of the active thyroid hormone (i.e. T_3) via metabolic and tissue-to-plasma exchange.

Key words: Brain, chicken, thyroid hormone metabolism

It was shown in the rat (Obregon et al., 1981) and in the chicken (Rudas and Pethes, 1986) that even if the levels of thyroid hormones reached close to undetectable levels in the plasma after thyroidectomy (TX), the level of tissue triiodothyronine (T_3) in the brain was kept almost at the euthyroid level. The reasons for such an autoregulative phenomenon might be increased uptake, increased intracellular production or decreased elimination of T_3 from the brain. The main goal of this paper was to show whether or not the elimination rate of brain T_3 is influenced by thyroid status and thus serves as a site of the formerly observed autoregulation. It has been shown that two sources of intracellular T_3 may have importance in the brain (Silva and Matthews, 1984*a*): first the T_3 that comes from the plasma [$T_3(T_3)$] and secondly that coming from the intracellular conversion of thyroxine to T_3 [$T_3(T_4)$]. Therefore we also wanted to gain information on the handling of the two intracellular sources of T_3 in the brain of young chickens.

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Materials and methods

Animals

One-week-old Hunniahibrid chickens were used throughout, purchased from a commercial source ("Vörös Október" MgTsz, Ócsa). Birds arrived at the laboratory just after hatching. Room temperature, water and food supply were regulated according to the supplier's guidelines. On the day of arrival, birds were randomly allotted into smaller groups (15-25) of similar weight. All experiments were carried out with individually labelled chickens.

Thyroidectomy

Surgical thyroidectomy was carried out under Nembutal (25 mg/kg body mass) anaesthesia at 3 days of age. Both the right and the left glands were removed without damaging the airsacs. Thyroidectomy was considered to be complete when both the right and the left organs could be removed in their unhurt capsule (Rudas and Pethes, 1989; Veresegyházy et al., 1986). Fifteen chickens were sham-operated (SH) at the same time when thyroidectomies were performed and these birds served as euthyroid controls.

Determination of the elimination rate of T_3 from the brain

To determine the T_3 elimination rate from the brain tissue, we applied a formerly substantiated method (Silva and Matthews, 1984*a*; J. E. Silva, Harvard Medical School, personal communication). Radiolabelled iodothyronines were injected intravenously and the time point when the tissue concentration of the label was at equilibrium with the plasma concentration was determined. At this point the net flow of the label from the plasma to the tissue equals the net disappearance of this particular label from the tissue. That is,

$$\frac{d[T_3]t}{dt} = [T_3]s \times Cl - [T_3]t \times k$$

where $[T_3]t$ = actual tissue concentration of T_3, $[T_3]s$ = plasma concentration of T_3, Cl = unidirectional clearance from the plasma to the tissue, k = removal (elimination) rate from the tissue. At equilibrium:

$$[T_3]s \times Cl = [T_3]t \times k$$

$$\frac{Cl}{k} = \frac{[T_3]t}{[T_3]s}$$

The right side of the equation was determined experimentally (this equalled the tissue to plasma ratio of the radioactively labelled thyronine at equilibrium). The removal rate (k) on the left side of the equation could be determined by measuring the rate of decrease of T_3 concentration in the tissue after stopping further introduction of T_3 into the compartment (brain). In the case of T_3, sufficient amount of anti-T_3 antibody was given intravenously at the equilibrium time point to stop the inflow of any further label into the cells from the blood stream. The concentration of the label within the tissue was determined several time points after the introduction of such an antiserum. Since no more inflow occurs, the tissue concentration of the label will drop by a speed determined by its removal (elimination) rate (k). For determining the removal rate (k) in the case of T_3 that is converted from T_4 in the brain [T_3 (T_4)] a similar possibility prevails. Labelled thyroxine was injected first, and then its availability was stopped by a subsequent injection of anti-T_4 antiserum at the equilibrium time point. The decreasing concentration of labelled T_3 in the brain thereafter allows to calculate the elimination rate of T_3(T_4) from the brain. The regression of the natural logarithm of the tissue concentrations was calculated over time. The slope of this curve gave the elimination rate. The coefficient of correlation was also calculated to substantiate the supposition of the single exponential characteristic of the elimination.

Experiments

Labelled hormones were injected intravenously (into the v. brachialis, 100 µl/chicken). Purified antibodies were injected into the antelateral brachial vein (250 µl/chicken). At predetermined intervals (see later) birds were bled and blood was collected into plastic tubes. In different experiments, the brain was taken out and the hyperstriate (HS) parts (without brain stem and cerebellum) were dissected out and were snap-frozen on dry ice aceton. In all cases blood vessels and connective tissue in the vicinity of the brain surface were carefully severed and then discarded under magnification. Tissues were then kept at –20 °C until processed as described under the section "tissue extraction".

Experiment 1: Determination of blood volume in the whole animal and in the brain. Blood volume was determined by a standard clinical procedure with Evans blue as marker. The plasma content of the tissues was determined by means of 131-I-albumin prepared by the chloramine T method and purified by standard gel filtration methods. The tissue to plasma ratio of 131-I-albumin was determined after injecting 37 kBq labelled protein (100 µl bolus) into the blood stream of 15 sham-operated (SH) and 15 thyroidectomized (TX) chickens. Twenty min and 40 min after the injection 5 birds were bled from each group and the 131-I-albumin concentrations in the plasma, in the hyperstriate and in the cerebellum were determined. The tissue to plasma ratio obtained in this way was used later on for corrections for plasma content of the organs in the experiments.

Experiment 2: Substantiation of the ability of the antibody to stop plasma to tissue flow of iodothyronines. One MBq/100 g bwt [^{125}I]T_4 was given to two groups of chickens (3 × 5 birds in each group) i.m. + i.p. (50% + 50%, respectively) immediately preceded by an i.v. injection of anti-T_4 antibody. 3 × 5 chickens served as controls which did not get antibody, only saline. One, 2 and 5 h later chickens were bled and the plasma and tissue concentrations of iodothyronines determined. The anti-T_3 antibody was checked similarly: [^{125}I]T_3 was injected i.m. + i.p. preceded by an i.v. bolus of anti-T_3 antibody. The tissue concentration and the amount of [^{125}I]T_3 kept in the circulation was determined.

Experiment 3: Determination of the equilibrium time point of iodothyronines in the brain. One MBq/100 g bwt [^{125}I]T_4 or 1 MBq [^{125}I]T_3 were injected i.v. at time 0 to TX and SH chickens. In the case of T_4, groups of chickens were sacrificed 1, 2, 3, 4 , 5, 8, 12, 16 and 24 h later to determine the specific radioactivities in the plasma and in the brain tissue. Sampling times for labelled T_3 were 0.5, 1, 2, 3 and 5 h after injection.

Experiment 4: Determination of the elimination rates of $T_3(T_3)$ and $T_3(T_4)$. One MBq/100 g bwt [^{125}I]T_4 was injected i.v. at time 0 to sham-operated (SH) or hypothyroid (TX) chickens. Ten h later 200 kBq/100 g bwt [131I]T_3 was injected intravenously. This injection of labelled T_3 preceded the time of equilibrium for $T_3(T_4)$ by 2 h, as obtained from experiment 3. Two hours thereafter [at the equilibrium time point for both $T_3(T_3)$ and $T_3(T_4)$] anti-T_4+anti-T_3 antibody was injected intravenously. One, 2, 3, 4 and 5 h after antibody administration groups of animals were bled to determine the concentration of different labelled compounds in the brain tissue.

Labelled iodothyronines

Radioactively (^{125}I and ^{131}I) labelled hormones were purchased from the Isotope Institute of the Hungarian Academy of Science (Izinta, Budapest). The purity of the commercial label was checked (Weeke and Örskov, 1973) and purified if necessary by paper chromatography (Bellabarba et al., 1968). The major contamination was iodine (usually around 2-5% of the total activity). The label was used within two days after preparation. The label that was stored in ethanol : water (7:3), was dried under nitrogen and redissolved in 0.9% sodium chloride containing 10% normal chicken plasma and 1 mg sodium iodide per ml.

Measurement of the tissue and plasma concentration of labelled iodothyronines

The method decribed by Silva and Matthews (1984*a,b*) was used. Preweighted tubes containing the tissues were taken out from the freezer and their weight and total radioactivity were measured so that tissues did not thaw up during this procedure. This was achieved by the relatively high radioactivity applied (low

counting times). The activity was determined by a gamma counter with automatic background compensation and manual spillover correction for both ^{131}I and ^{125}I activities with less than 2% counting error (NZ 332, Gamma Művek, Budapest). The frozen, individually treated tissues were transferred into the homogenization vessel and 2 vol/weight glycine-acetate buffer (pH 8.6, 0.15 M, containing 10 mM PTU to prevent *in vitro* degradation of iodothyronines) was added to them. An Elvehjem-Potter type homogenizer was used with teflon pestle that was run for 15 sec at constant speed setting in each case. To the mixture 3 vol/weight acidic butanol (butanol saturated with 2 N HCl) was added. The tubes were then vortexed for 20 sec two times. Extraction efficiency was 80% with one set of butanol extraction. A second similar extraction with butanol increased efficiency to 90%, however later on only a one step procedure was used, followed by placing the tubes into the refrigerator for 5 h. After this the efficiency was constantly above 90% per cent either as a per cent of total radioactivity or when checked with non-radioactive tissue samples that were homogenized with known amounts of label. Samples were then centrifuged (3,000 *g*, 10 min, 4 °C). In cases when the supernatant's radioactivity was high enough (activity > 5,000 cpm/100 µl), paper chromatography (Whatmann 3 MM paper, Whatmann Inc., Clifton, NJ) was carried out directly from the supernatant. If not, a concentration step followed using nitrogen stream. Fifty µl (or several times 50 µl aliquots with complete drying between applications) of sample was then applied onto the paper strips. The marker solution (50 µl) contained T_4, T_3, I (NaI) 1 mg/ml each, in ethanol: 2N NH_3 (9:1). The activity of the different iodothyronines was then calculated after measuring the radioactivity of the corresponding spots of the chromatogram.

The plasma samples were usually directly applied onto the chromatographic paper. When the radioactive concentration of the plasma was less then 5,000 cpm in 100 µl, an extraction procedure was used. Fifty µl of the above marker solution and 100 µl of alkaline butanol (butanol saturated with 2N NH_3) was added and mixed with the plasma. After centrifugation (3,000 *g* for 10 min, 4 °C) the supernatant was applied in aliquots so that each aliquot was dried under nitrogen before the application of the next one.

Preparation of antibodies

Highly specific anti-T_3 and anti-T_4 antibodies were formerly obtained from Prof. P. R. Larsen (Harvard Medical School, Boston). These rabbit sera were purified by standard ammonium precipitation and gel filtration methods to obtain the IgG fraction. The binding characteristics of the IgG's were determined by Scatchard analysis. The amount to be injected was adjusted so that the antibody bound at least 10 ng T_3 and 200 ng T_4 in one ml of plasma. Total plasma volume was estimated by using the body weight and the figure obtained from experiment 1 (8.3% blood of bwt). The maximum binding capacity of the antiserum was taken

into account. The *in vivo* ability of the antibodies to keep iodothyronines in the circulation were justified in experiment 2.

Calculation of results

Iodothyronine concentrations in the plasma and in the tissues were expressed as per cent of the injected dose per milliliter of plasma or per gram of tissue, respectively. To avoid the effect of body weight differences all data were standardized for 100 g body weight. The following corrections were made besides taking into account the original purity of the injected label:

Correction for the iodothyronine concentration of the plasma trapped in the tissue:

[corrected tissue label] {% of label dose/g tissue/100 g bwt}

= [tissue label noncorrected] – ([plasma label] * T/S of 131-albumin),

where [] stands for concentration; {} is for dimension; label stands for the iodothyronine in question (T_3, T_4, labelled with ^{125}I or with ^{131}I); T/S is tissue to plasma ratio.

The elimination rate was calculated by linear regression analysis of the natural logarithm of the corrected tissue label concentrations over time. The slope of the line gave the elimination rate (k, $hour^{-1}$) from which the half life was obtained ($t_{1/2} = 0.693/k$, hour).

Standard statistical methods (Student's *t* test and analysis of variance) were used according to Snedecor and Cochran (1967).

Results

Average blood volume was 8.3 ± 0.96% of the body weight. The average plasma volume calculated was 12 ± 0.1 µl/g tissue for the hyperstriate and 16 ± 0.09 µl/g tissue for the cerebellum. TX and SH groups did not differ significantly regarding the above parameters.

The ability of the anti-iodothyronine antibodies to prevent the hormones from entering the organs was justified in experiment 2, where $[^{125}I]T_4$ or T_3 was injected i.p. + i.m. just after the antibody injection. After anti-T_4 injection no drop in plasma $[^{125}I]T_4$ occurred, however with no antibody the half-life of $[^{125}I]T_4$ was 5.78 ± 0.34(5) [average ± SEM(N)] h, that is an acceptable half-life for plasma T_4 in young chickens (Rudas and Pethes, 1986). The $[^{125}I]T_4$ that was injected after the antibody administration did not leave the circulation, since after about 5 h its plasma concentration stabilized and reached 24.7 ± 1.8% of dose/ml plasma/100 g body weight. This means that calculating with 8.3% blood volume (see earlier), the whole $[^{125}I]T_4$ was kept in the circulation. The hyperstriate contained almost no $[^{125}I]T_4$ (less than 0.001% of dose/g tissue/100 g bwt) in the

presence of the antibody, and 0,6 ± 0.04% of dose/g/100 g bwt in the absence of it at the fifth hour. Similarly, no leakage from the surface of T_3 antibody was seen in the *in vivo* experiment.

Table 1

Concentration of labelled iodothyronines in the brain of sham-operated control (SH) and thyroidectomized (TX) chickens*

Groups		Time (hours) after injection of the tracer								
		1	2	3	4	5	8	12	16	24
$[^{125}I]T_4$										
SH	avg	0.51	0.73	0.81	0.76	0.75	0.69	0.64	0.53	0.46
	± SEM	0.032	0.046	0.065	0.053	0.068	0.051	0.045	0.052	0.057
TX	avg	0.72	1.61	1.52	1.42	1.31	1.21	1.15	1.01	0.82
	± SEM	0.061	0.12	0.13	0.15	0.11	0.093	0.13	0.078	0.081
$[^{125}I]T_4$										
SH	avg	0.5	0.8	1.2	1.3	1.7	2.11	3.9	3.6	3.2
	± SEM	0.04	0.06	0.11	0.09	0.19	0.17	0.27	0.32	0.29
TX	avg	1.1	1.3	1.8	2.1	3.5	4.6	5.8	5.2	5.0
	± SEM	0.13	0.14	0.24	0.23	0.28	0.49	0.63	0.47	0.38

Groups		Time (hours) after injection of the tracer				
		0.5	1	2	3	5
$[^{125}I]T_4$						
SH	avg	0.39	0.45	1.16	0.92	0.86
	± SEM	0.017	0.031	0.078	0.061	0.057
TX	avg	0.098	0.33	1.41	1.22	1.12
	± SEM	0.011	0.021	0.105	0.087	0.092

* (% of dose/g tissue/100 g bwt) (average, avg ± standard error of mean, SEM, n = 5)

The accumulation of different iodothyronines in the tissues from experiment 3 are shown in Table 1. Thyroxine reached its maximum concentration in the brain at 3 and 2 h postinjection for sham-operated and thyroidectomized groups, respectively. The concentration of $[^{125}I]T_3(T_4)$ coming from $[^{125}I]T_4$ in the brain of control and thyroidectomized groups reached the maximum 12 h after $[^{125}I]T_4$ injection and was significantly ($P < 0.05$) higher in TX animals. $[^{125}I]T_3(T_3)$

peaked at 2 h after [^{125}I]T_3 administration in both groups with no significant differences in peak values by Student's *t* test. The concentrations of all iodothyronines were significantly ($P < 0.01$) influenced by thyroidectomy over time as shown by two-way analysis of variance (TWAOV).

Table 2

Elimination of [^{125}I]T_3 from the brain of sham-operated control (SH) and thyroidectomized (TX) chickens in experiment 4

Group	Organ		Half-life $t_{1/2}$ (h)	Elimination rate (k) (h^{-1})	Coefficient of correlation (r)
SH					
	Plasma	$T_3(T_3)$	6.13 ± 0.57	-0.113 ± 0.01	
	Brain	$T_3(T_3)$	3.31 ± 0.24^A	-0.209 ± 0.016^C	0.952
		$T_3(T_4)$	2.87 ± 0.19^B	-0.241 ± 0.017^D	0.921
TX					
	Plasma	$T_3(T_3)$	5.13 ± 0.45	-0.135 ± 0.012	
	Brain	$T_3(T_3)$	9.12 ± 0.56^A	-0.076 ± 0.0046^C	0.912
		$T_3(T_4)$	8.23 ± 0.64^B	-0.084 ± 0.0065^D	0.897

$t_{1/2}$ = half life in hours; k = elimination rate in hour^{-1}; average ± SEM. n = 5; r = coefficient of correlation; groups labelled with the same superscripts are significantly different ($P < 0.001$)

The elimination rates of $T_3(T_3)$ and of $T_3(T_4)$ are given in Table 2 as obtained from experiment 4. The elimination rate from the brain of both triiodothyronines was about threefold less in thyroidectomized birds than in the controls ($P < 0.001$). The rate of disappearance from the brain fitted very well on a single exponential as shown by the coefficients of correlation (r). The plasma $T_3(T_3)$ elimination rates were higher in TX animals, but this difference was not significant by Student's *t* test.

Discussion

Formerly we have found that the uptake (Rudas, 1989) and the intracellular deiodination rate of iodothyronines (Rudas, 1988*a*) in the brain of young chickens adapt to hypothyroidism. The main goal of the present experiments was to dem-

onstrate the effect of the change in the thyroid status upon another possible site of regulation, namely the elimination of the active thyroid hormone, triiodothyronine (T_3), from the brain tissue of chickens. $T_3(T_3)$ originates from the plasma, while $T_3(T_4)$ from local tissue sources as a result of the conversion of thyroxine that comes from the plasma into triiodothyronine (Larsen et al., 1981; Silva and Matthews, 1984*b*). According to this the elimination rate of both types of tissue triiodothyronines was examined.

To determine the elimination rates we used the equilibrium technique described by Silva and Matthews (1984*a*,*b*). One critical part of this technique is to determine the ability of the anti-T_4 and anti-T_3 antibodies to keep the circulating label from entering the cell. The results clearly show that the leakage from the surface of the antibody was negligible (see Results). It is also important to suppose that the antibody itself, while present in the blood, would not influence significantly the elimination rate of the triiodothyronine from the brain into the blood. This supposition was substantiated by the developers of the method (Silva and Matthews, 1984*a*) and by our earlier works on rats (not published). In favour of this supposition speaks the fact that the elimination fitted with high coefficient of correlation upon a single exponential curve.

Another concern of the technique applied is to establish the time point when the antibody should be administered. In principle one has to find the maximum of the concentration curve of the tracer in the tissue. This was relatively easy in the case of $T_3(T_3)$, the peak concentration of which was well apparent from the T_3 uptake curve. The maximum concentration of the labelled $T_3(T_4)$ in the brain after administering labelled T_4 was not as easy to determine since the concentration curve was much more extended over time. Pilot experiments and our findings in rats (not published) helped us to determine the appropriate time interval to be investigated. Finally the highest concentrations of $[^{125}I]T_3(T_4)$ were between 11 and 13 h after $[^{125}I]T_4$ administration. Therefore 12 h was accepted as a peak concentration of the tracer, when production and elimination are in equilibrium. Several reasons for such a long time for the peak $[^{125}I]T_3(T_4)$ concentration to occur may exist. Thyroxine gets into the brain with relatively high rate and therefore it is not a limiting factor for the production of $T_3(T_4)$. One may suppose that there is a delay of T_4 to T_3 conversion in the brain after thyroxine is taken up. This is unlikely, however, since it was shown by several authors (Silva and Larsen, 1978; Crantz and Larsen, 1980; Obregon et al., 1981; Ruiz de Ona et al., 1988; Rudas, 1988*a*,*b*) that T_4 is rapidly converted into T_3 in the brain or in brain cell cultures. More likely is the supposition that $T_3(T_4)$ will be cleared not from the same compartment as it is produced in. To be transferred into the compartment from which it is cleared may take several hours.

It is clearly shown in these experiments that the hypothyroid brain accumulates more T_4 and T_3 over the same period of time than does the control (SH) one. This can be seen from the tissue concentrations presented in Table 1 and this was

also confirmed by TWAOV of these data. This increased accumulation may be the result of two factors: increased uptake (Pardridge, 1981) of iodothyronines and their reduced release from the brain. The present data unanimously prove the role of the decreased elimination rate of T_3 from the brain in thyroidectomized chickens as opposed to control (SH) ones (Table 2). Since there was no significant difference in half-life between $T_3(T_3)$ and $T_3(T_4)$, one can suppose that the triiodothyronines of different origin would mix uniformly in a common pool before exiting the brain tissue. The high coefficient of correlation for a single exponential further strengthens this view. It was proved (Gullo et al., 1987) that nuclear receptors for triiodothyronine in the brain of the rat are mainly found in neurons, especially in phylogenetically newer regions. Therefore one may suppose that not the glial but the neuronal compartment is the final pool, where triiodothyronines of different origin would mix. This might hold true also for the chicken, since it was demonstrated that in this species the major portion of triiodothyronine receptors is found in neuronal cells of the brain tissue (Haidar et al., 1983).

It was described (Dratman et al., 1983; Silva et al., 1984) in the rat and also in the chicken (Rudas and Pethes, 1989) that a major factor that helps the individual adapt to hypothyroidism is constituted by the compensatory changes in the deiodination system. This is true for the brain tissue as well (Crantz and Larsen, 1980; Obregon et al., 1986; Ruiz de Ona et al., 1988). According to this the disappearance of triiodothyronine from the tissue is not only via its elimination into the plasma, but also by its deiodination into diiodothyronines by type III deiodination (Kaplan and Yaskoski, 1980; Leonard and Visser, 1986). Therefore it is presumed that the elimination rate obtained here expresses both ways of T_3 removal from the brain. Thus the term "tissue to plasma" movement of the hormone actually involves disappearance into the metabolic sink and the real entry into the plasma, the latter being more pronounced in the case of $T_3(T_4)$. Further experiments are needed to throw light on the proportion of these ways.

The brain tissue belongs to the group of organs that are so called "local producers" (Larsen et al., 1981), meaning that the triiodothyronine that binds to its nuclear receptor is mainly coming from the local conversion of thyroxine to triiodothyronine and not from the triiodothyronine coming from the plasma (Silva and Matthwes, 1984*a*,*b*; Ferreiro et al., 1988). It was demonstrated that this characteristic is not only important in normal situations, but also determines the response of this organ to altered thyroxine supply. In hypothyroidism when thyroxine availability is low, tissues like the brain, which are locally producing T_3, are much more influenced by the lack of T_4 in the plasma than other tissues. In this way, knowledge of the behaviour of intracellular hormonal events has basic clinical implications (Legrand, 1986). Even if triiodothyronine is the main active hormone responsible for most of the thyroidal effects, the correct therapy should take into account the need for thyroxine itself when one wants to restore normal intracellular thyroid status in the brain and in several peripheral tissues (Silva and

Matthews, 1984*a*,*b*). At first glance this has important implications only for humans, still it has practical importance in the fowl, too. Several endocrine defects exist in birds like dwarfism (Scanes et al., 1983; Huybrechts et al., 1989) and malabsorption syndrome (Rudas et al., 1985) where the knowledge of the intracellular handling of thyroid hormones by the peripheral tissues of the bird are of great value for devising therapeutic measures.

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EFFECT OF VITAMIN E TREATMENT ON EARLY POSTNATAL CHANGES OF VITAMIN E STATUS OF CHICKEN

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Day-old chickens were treated with 3.25 mg vitamin E/bird/day per os, via the drinking water, for two weeks. The vitamin E content of both the liver and the blood plasma was significantly higher in the treated chickens than in the untreated controls. The positive effect was more pronounced in the liver than in the blood plasma.

Key words: Chicken, postnatal changes, vitamin E

Vitamin E is the only natural lipid-soluble chain-breaking antioxidant. Deficiency of vitamin E in animals causes pathological changes (Scott, 1969). Among them, cerebellar encephalomalacia developed in chicks raised on a vitamin E deficient diet (Pappenheimer and Goettsch, 1931). The extremely high sensitivity of the nervous system to vitamin E deficiency is due to its very high polyunsaturated fatty acid content which means a higher potential for lipid peroxidation (Mizuno, 1984). In chickens, the enzymatic part of the biological antioxidant defence mechanism has also relatively low activity, especially during the early postnatal period (Mézes, 1988*a*). Besides the nervous system, the liver also became markedly depleted in vitamin E during the early period of life (Mézes, 1988*b*; Sososalanova et al., 1990). Some earlier papers reported a marked decline in vitamin E content of chicken liver during the early postnatal period. The aim of the present study was to investigate the possibility of avoiding vitamin E depletion in the liver by administering excessive doses of a water-soluble vitamin E preparation.

Materials and methods

One-day old meat-type female chickens were kept under common technological conditions and fed a starter diet (vitamin E content: 38 ± 5.2 mg/kg). Ten birds from both groups (treated and control) were sacrificed at 1, 3, 7, 10 and 14 days of age, respectively. Vitamin E treatment was done with a water-soluble preparation of alpha-tocopherol (Hoffmann-LaRoche, Basle) administered at a calculated dose of 3.25 mg/bird/day via the drinking water. The daily water intake

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was calculated by the equation of Ross and Hurnik (1983). The vitamin E content of the blood plasma was measured using the method of Bieri (1964). Liver and dietary tocopherol contents were determined by a HPLC method according to Thompson and Hatina (1979). Statistical analysis of the results was done by a paired *t* test.

Results

The results indicate that vitamin E treatment increased the tocopherol level of both the blood plasma (Table 1) and the liver (Table 2). At the same time, tocopherol level decreased constantly in the liver of the control birds throughout the period of study (from to 14 days of age).

Table 1

Changes in vitamin E content of the blood plasma during vitamin E supplementation (mean ± SD)

Age (days)	Vitamin E content (mmol/L)	
	Treated	Control
1	0.12 ± 0.03	0.12 ± 0.03
3	0.31 ± 0.05***	0.17 ± 0.01
7	0.34 ± 0.08*	0.25 ± 0.01
10	0.38 ± 0.08	0.33 ± 0.01
14	0.40 ± 0.04*	0.33 ± 0.01

Levels of significance: * = $P < 0.05$; *** = $P < 0.001$

Table 2

Changes in vitamin E content of the liver during vitamin E supplementation (mean ± SD)

Age (days)	Vitamin E content (μmol/g wet wt)	
	Treated	Control
1	0.71 ± 0.10	0.69 ± 0.25
3	0.99 ± 0.31***	0.22 ± 0.06
7	1.39 ± 0.18***	0.29 ± 0.09
10	1.26 ± 0.28***	0.20 ± 0.08
14	1.40 ± 0.30***	0.19 ± 0.09

Level of significance: *** = $P < 0.001$

Discussion

The results of the present study confirm some earlier reports on the substantial accumulation of vitamin E in the liver at hatching (Noble and Cocchi, 1990; Noble et al., 1993). After that time a strong depletion was found. That depletion may be associated with lipid transport, because during the incubation period the liver accumulates an extremely high lipid content. After hatching the lipids (triglycerides) as energy sources are mobilised from the liver and vitamin E is also transported by that process. This theory supports the earlier results on the transport of vitamin E with the binding of very-low-density lipoproteins in the laying hen during egg shell formation (Mézes, 1988*b*). For that reason the process of depletion is under physiological control. The supplementation successfully stabilised the vitamin E content of the liver, probably because of the permanent supply. Namely, there are several reports suggesting that the tissue tocopherol concentration of the chick liver is in linear relation to the dietary intake (Frigg et al., 1993). This result is important in those cases in which the antioxidant defence mechanism of the organism is suppressed for any reason (e.g. presence of toxic materials, low level of supplementation, etc.). Dietary supplementation of chicks with high doses (250–300 mg/kg) of vitamin E enhanced their protection against *E. coli* infection (Heinzerling et al., 1974) and also facilitated the development of immunity to coccidiosis (Colnago et al., 1984).

In conclusion: the depletion of vitamin E in the chicken liver is a physiological process which could be reversed by the continuous administration of excess doses of a water-soluble tocopherol preparation, especially during the first two weeks of life.

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EVALUATION OF TESTOSTERONE RESPONSE TO INTRAMUSCULAR INJECTION OF GnRH AND ITS CORRELATION WITH SPERM QUALITY PARAMETERS IN COCKERELS

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Eight 40-week-old New Hampshire cockerels (average liveweight: 3.10 ± 0.2 kg) were used for studying the testosterone response to a single intramuscular dose (5 μg/kg b.w.) of exogenous 6D-Phe gonadotropin releasing hormone (GnRH). Serum samples were collected at 15-min intervals during the first hour and at half-hour intervals up to two hours post injection. The semen samples were collected 24 h before GnRH treatment. Testosterone peaks were found 45 to 60 min after the treatment. The coefficient of correlation between the semen quality parameters and the testosterone response to GnRH was evaluated both by Rank correlation and by multiple regression analyses. Rank correlation showed moderate but not significant values while multiple regression analyses showed a close correlation (multiple R = 0.72). The results show that testosterone response to GnRH may be used as a marker of semen quality in cockerels.

Key words: Cockerels, GnRH response, semen quality, testosterone

Testosterone is known as a hormone essential for reproduction in males. However, differences have been reported in the reproductive performance of cockerels with the same actual value of testosterone. This finding is easily accounted for, as — among other factors — reproductive performance depends also on semen quality. Because of the episodic nature of testosterone secretion, no positive results were obtained in studies where only a limited number of samples was used for determining the testosterone status. A more accurate assessment of testosterone status required a series of samples taken at relatively short intervals. However, such methods of assessment are time-consuming and subject to marked inhibition of testosterone secretion by restraint during blood sampling. The finding that GnRH treatment overcame the depressant effect of restraint on secretion and that the testosterone response to GnRH was found to be highly correlated with the 24-h plasma testosterone profile suggested that the GnRH re-

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sponse test might be a simpler and more accurate method (Post et al., 1987).

Many authors suggesting the importance of evaluating the rate and type of morphological abnormalities, found a correlation between the percentage of sperm anomalies and reduced fertility in different poultry species (Bakst and Sexton, 1979; Clarke et al., 1984; Maeda et al., 1986).

The aim of present study was to use the GnRH response test for assessing the testicular function of cockerels and comparing the results with several parameters of semen quality.

Materials and methods

Eight 40-week-old cockerels of the New Hampshire breed (average body weight: 3.10 ± 0.2 kg) were used. GnRH was given in the form of 6D-Phe analogue of mammalian GnRH (OVURELIN inj. ad us. vet., REANAL, Budapest) in a single intramuscular dose (5 μg/kg b.w.) as was proposed for avian species (Péczely, 1989). Blood samples were taken at 15-min intervals during the first hour and at 30-min intervals up to two hours after injection from the wing vein. The serum testosterone level of serum was determined by 125-I radioimmunoassay method (Institute of Isotopes Ltd., Budapest) after diethyl ether extraction. The intraassay variation was 4.3 % CV.

Semen samples were taken by the massage technique. One drop of fresh sample was used for morphological examination by the eosin-nigrosine staining method (Lake and Stewart, 1978). One hundred sperm cells were counted and the abnormalities recorded (Haye et al., 1981). The motility and the concentration were evaluated by a subjective scale score system (1 to 5 points of each).

Mathematical evaluation of the data was carried out using multiple regression and Rank correlation methods (Snedecor and Cochran, 1976).

Results

The serum testosterone level increased gradually after the GnRH injection. The highest level was found between 45 to 60 min after treatment (Fig. 1).

The semen quality parameters of cockerels are shown in Table 1 and their testosterone values in Table 2. All test parameters showed individual differences among the birds tested.

One of the semen quality parameters (per cent of morphologically normal cells) and the testosterone levels (initial, at 60th min after treatment, and increase) were correlated first according to the Rank order of the testosterone levels and

response, and also according to the Rank order of the semen parameter. A moderately close (Rank = –0.58, –0.59, +0.44) but not significant (P>0.05) correlation was found between them. Correlation was calculated also between the testosterone response (increase from the initial level to the 60th min after treatment) as dependent variable and semen quality parameters (volume, motility, concentration and morphologically normal cells) as independent variables using the absolute values of the different parameters of different cockerels. A close (R = 0.72) multiple correlation was found between them.

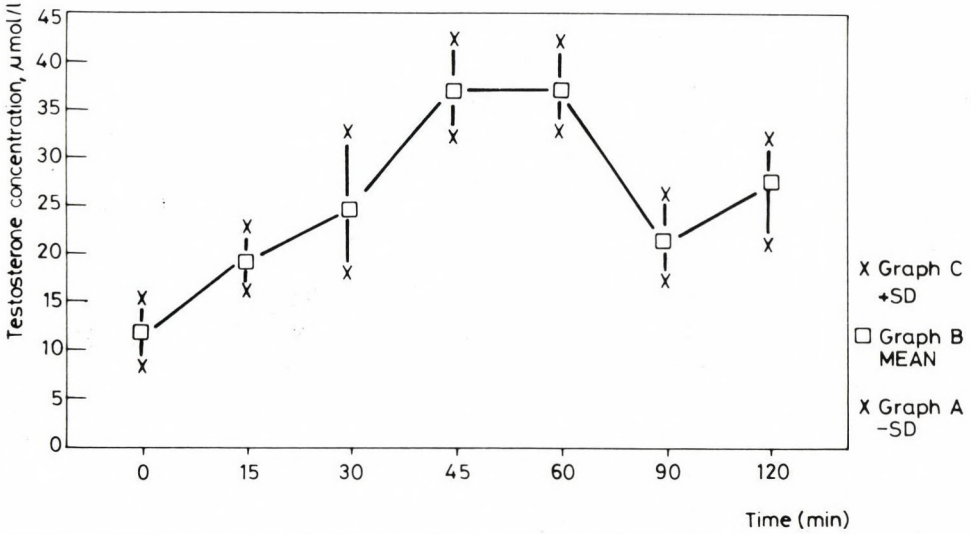

Fig. 1. Changes in serum testosterone concentration after GnRH treatment

Table 1

Semen quality parameters of cockerels

No. of birds	Semen volume (ml)	Motility (score)	Concentration (score)	Morphologically normal cells (%)	[Rank]
1	0.3	3	2	35	[8]
2	0.2	4	3	58	[6]
3	0.4	4	4	53	[7]
4	0.9	5	4	82	[1]
5	0.8	5	3	66	[4]
6	0.4	3	2	60	[5]
7	0.8	4	3	76	[3]
8	0.8	5	5	78	[2]

Table 2

Changes in the serum testosterone level of cockerels after GnRH treatment

No. of bird	Serum testosterone level (nmol/L)		Increase [Rank]
	Before GnRH treatment	60 min after GnRH treatment	
1	10.13 [3]	25.75 [6]	15.62 [6]
2	9.14 [4]	30.51 [4]	21.37 [5]
3	8.28 [5]	21.52 [8]	13.24 [7]
4	5.28 [7]	32.00 [2]	26.72 [2]
5	25.76 [1]	63.70 [1]	37.94 [1]
6	6.98 [6]	29.25 [5]	22.27 [4]
7	21.44 [2]	29.25 [6]	7.81 [8]
8	5.25 [8]	30.67 [3]	25.42 [3]

Discussion

The results of this study showed that the stimulation of testosterone secretion from the testis by exogenous GnRH treatment is also applicable in cockerels as was found in some mammalial species.

The testosterone peaks were found at nearly the same time in all cockerels which enabled the taking of blood samples only two times (prior to and 60 min after treatment) using the above-mentioned dose.

The Rank correlation between testosterone response values and the per cent of viable cells showed a non-significant correlation. All the semen quality traits compared together as independent variables showed a close correlation with the rate of testosterone response as dependent variable. This showed that, besides the well-known other effects (e.g. libido), the Leydig's cell activity of the testis and its ability to produce and release testosterone also affect the quality of semen.

On the basis of the above-mentioned preliminary results it can be concluded that, besides semen qualification, GnRH-induced testosterone production and/or release also seems to be a good marker for the qualification and/or selection of cockerels, especially those to be used for artificial insemination.

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PROTEIN CONTENT AND AMINO ACID COMPOSITION OF THE UTERINE MILK IN SWINE AND CATTLE

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The protein content and amino acid composition of the uterine milk of swine and cattle were analyzed using slaughterhouse-derived samples. For both species, the majority of the dry matter of uterine milk consisted of materials of protein nature (swine: 68.1%, cattle: 73.3%). Free amino acids constituted approximately 0.3% (swine) and 0.5% (cattle) of the dry matter. In the uterine milk of sows, the high ratio of serine, glycine and acidic amino acids (overall ratio: 65%) was remarkable. No such striking values were found for the bovine species; however, the uterine milk of cows was also characterized by the predominance of acidic amino acids. Total amino acid content of the uterine milk was compared with that of freshly milked colostrum. With the exception of glutamic acid in sows and methionine and glutamic acid in cows the uterine milk and the colostrum had identical amino acid content. It is concluded that the embryotroph can be considered a transition between the blood and the colostrum.

Key words: Uterine milk, colostrum, amino acid, immunoglobulin G, swine, cattle

The uterine milk or embryotroph is produced by the uterine glands in the so-called secretory phase of the uterus, when the genital system is under the influence of progesterone hormones. It also contains some tissue debris of maternal origin. In the so-called histotrophic phase the uterine milk serves as exclusive nutriment to the embryo. The histotrophic phase lasts approx. from day 3–4 to day 15–16 in the porcine and from day 6–7 to day 28–30 in the bovine species. Although to a decreasing extent, the embryotroph plays a role in the nutrition of the embryo up to the end of gestation (Michel, 1983). According to data of the literature, embryonic losses of up to 10–20% may occur in both swine and cattle if uterine milk is the sole nutriment available to the embryo (Wekerle and Szőllősi, 1992). The success rate of embryo transfers also depends on the composition of the embryotroph.

Despite the importance of the subject, the special literature available on it is extremely scarce. In the CAB and MEDLINE systems of the international database very few papers can be found on the embryotroph, and even the existing ones

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touch upon the subject rather superficially. No data at all are available on the amino acid and protein composition of the embryotroph, though our studies show that in both swine and cattle the dry matter of uterine milk consists largely of compounds of crude protein nature. These compounds are likely to have an importance commensurate with their relative proportion in the embryotroph.

Materials and methods

Animals. Multiparous sows of miscellaneous breed and of Large White and Landrace type were used. The bovine embryotroph was collected from Holstein-Friesian, Hungarian Fleckvieh and crossbred cows.

Sampling methods. Uteri were removed from the animals at the slaughterhouse on day 13 after the onset of oestrus, and were stored at +5 °C for 24 hours. The uterine horns showing an undulating or arc-like course were straightened out by transecting the ligaments, then suspended by the uterine body and cut through at a site located a few centimetres from the oviductal junction. Subsequently the cut surfaces were cauterized, and the embryotroph was allowed to drip out and was stored deep-frozen until used for the analyses.

Chemical assays. Samples stored in a freezer were placed into water of +20 °C temperature until complete dissolution of the embryotroph. The dry matter content of the samples was carried out as described in Hungarian Standard MSZ-3744-67 by drying to constant weight. Parallel measurements were applied both for the dry matter determination and for all chemical assays. The crude protein content of the embryotroph was determined using a Kjell-Foss 16200 type rapid nitrogen analyzer, in the presence of potassium sulphate as boiling point elevator, mercuric oxide as catalyst and hydrogen peroxide as oxidizing agent. The crude protein content was calculated by multiplying the nitrogen content by 6.25 as conversion factor.

The free and total amino acid content of the embryotroph was determined by an LKB 4101 type amino acid analyzer. For total amino acid determination, approx. 80–120 mg of the thick, viscous embryotroph was hydrolyzed with 6 M hydrochloric acid at 110 °C for 24 hours (Csapó et al., 1986*a*). The cystine content of the embryotroph was determined in the form of cysteic acid (Csapó, 1982) while its methionine content was assayed after mercaptoethanesulfonic acid hydrolysis to prevent oxidative losses (Csapó et al., 1986*b*). For determination of the free amino acid content, the thick and viscous embryotroph was diluted tenfold with distilled water, then its protein content was precipitated with 12% trichloroacetic acid. The supernatant obtained after centrifugation at 8,000 rpm for 5 min was adjusted to pH 2.2 by adding 4 M NaOH, and the clear solution thus obtained was applied onto the ion-exchange column of the amino acid analyzer. The

quantitative evaluation of amino acids was done by the use of cysteic acid as internal standard.

The immunoglobulin G content of the embryotroph was determined by radial immunodiffusion (Mancini et al., 1965).

Results

The free amino acid content of the porcine embryotroph is shown in Table 1, while its total amino acid content in Table 2. The free amino acid content of the bovine embryotroph is presented in Table 3, while its total amino acid content in Table 4. The tables show the mean values and their standard deviation. Table 5 shows the amino acid composition of the porcine (Csapó and Csapó-Kiss, 1993) and the bovine (Csapó et al., 1991) colostrum derived from the first milking, to enable its comparison with proteins of the embryotroph.

Table 1

Free amino acid content of the porcine embryotroph
(mg free amino acid/100 g of embryotroph)

Amino acid	Mean x	Standard deviation ± s
ASP, n=13	3.99	1.03
THR, n=13	10.14	2.77
SER, n=13	96.0	23.8
GLU, n=13	17.06	7.5
PRO, n=13	6.11	2.48
GLY, n=13	64.68	33.03
ALA, n=13	27.86	8.55
CYS, n=13	1.02	0.07
VAL, n=13	5.9	1.82
MET, n=9	2.21	0.63
ILE, n=13	2.12	0.55
ILU, n=13	4.05	1.08
TYR, n=13	5.56	1.43
PHE, n=13	3.92	1.18
LYS, n=13	6.03	2.04
HIS, n=13	4.36	1.24
ARG, n=13	4.98	1.55
NH_3, n=13	28.68	4.68

Table 2

Total amino acid content of the porcine embryotroph
(mg amino acid/100 g of embryotroph)

Amino acid	Mean x	Standard deviation ± s
ASP, n=13	314.0	78.4
THR, n=13	184.0	37.2
SER, n=13	292.0	59.5
GLU, n=13	454.0	74.6
PRO, n=13	235.0	60.0
GLY, n=13	210.3	54.3
ALA, n=13	189.0	32.5
CYS, n=13	54.0	19.8
VAL, n=13	187.1	41.6
MET, n=9	16.02	3.1
ILE, n=13	86.9	17.4
ILU, n=13	301.0	58.5
TYR, n=13	213.8	43.6
PHE, n=13	281.6	102.1
LYS, n=13	293.4	75.3
HIS, n=13	103.1	21.4
ARG, n=13	203.7	56.9
NH_3, n=13	147.5	25.1

All data relate to dry matter on arrival: the low volume of the sample (average volume: 2.5 ml for sows and 1.0 ml for cows) did not allow us to use a given sample also for tests other than dry matter determination. The relatively high standard deviations may be attributed to this fact. The ratio of the different amino acids was almost constant in the case of the free amino acids but primarily for the total amino acid content.

Dry matter and crude protein content of the embryotroph. The dry matter content of the porcine embryotroph ranged between 5.2 and 11.7% (average: 6.9%). The dry matter content of the bovine embryotroph was between 7.2 and 15.4% (average: 11.6%). The average total protein content of the porcine embryotroph was 6.06% (extreme values: 4.42 and 7.51%), and constituted 68.1% of the dry matter content. The average total protein content of the bovine embryotroph was 8.5% (extreme values: 6.92 and 10.11%), and accounted for 73.3% of the dry matter content.

Table 3

Free amino acid content of the bovine embryotroph
(mg free amino acid/100 g of embryotroph)

Amino acid	Mean x	Standard deviation ± s
ASP, n=6	18.12	8.68
THR, n=6	30.87	9.85
SER, n=6	46.9	18.96
GLU, n=6	76.14	26.34
PRO, n=6	44.62	22.35
GLY, n=6	64.04	12.95
ALA, n=6	34.3	17.22
CYS, n=6	4.89	0.79
VAL, n=6	23.35	12.10
MET, n=6	10.31	5.76
ILE, n=6	13.39	7.16
LEU, n=6	30.82	17.51
TYR, n=6	14.83	7.66
PHE, n=6	14.91	8.61
LYS, n=6	28.13	19.81
HIS, n=6	9.87	3.79
ARG, n=6	26.54	17.33
NH_3, n=5	16.76	14.27

Free amino acid content of the embryotroph. Studying the free amino acid content of the porcine embryotroph, the high ratio of serine and glycine is striking. These two amino acids constitute more than 56% of the total free amino acids. Among the other amino acids, the 9.3% ratios of alanine and free ammonia are remarkable, as they stand out among the 1–3% ratios of the other free amino acids. The embryotroph of sows contains strikingly low amounts of sulphur-bearing cystine and methionine. The total free amino acid ratio is 0.31% of the dry matter, and represents 5.1% of the crude protein content.

The bovine embryotroph also consists mostly of glycine and alanine, but it contains considerable amounts of neutral amino acids and 5.2% arginine, an amino acid of alkaline nature. The free amino acids constitute about half per cent of the dry matter and 6.2% of the crude protein.

Table 4

Total amino acid content of the bovine embryotroph
(mg amino acid/100 g of embryotroph)

Amino acid	Mean x	Standard deviation ± s
ASP, n=4	802.2	198.25
THR, n=4	377.1	50.80
SER, n=3	605.27	185.32
GLU, n=4	1142.4	218.65
PRO, n=4	390.9	50.25
GLY, n=4	439.1	65.04
ALA, n=4	414.9	81.44
CYS, n=4	97.5	62.47
VAL, n=4	407.4	103.30
MET, n=4	56.1	13.62
ILE, n=4	256.6	78.99
LEU, n=4	813.1	446.1
TYR, n=5	268.0	167.54
PHE, n=5	354.9	56.70
LYS, n=5	698.2	338.3
HIS, n=5	323.7	112.7
ARG, n=5	577.0	165.76
NH_3, n=5	210.1	38.20

Total amino acid content of the embryotroph. The total amino acid content of the porcine embryotroph accounts for 63% of the crude protein, indicating that the embryotroph contains, besides the true protein and free amino acids, also other substances of non-protein nitrogen content. The total amino acid content of the bovine embryotroph constitutes 95% of the crude protein, suggesting that in cattle the embryotroph contains substantial quantities of non-protein nitrogen materials in addition to the free amino acids.

Immunoglobulin G content of the bovine and porcine embryotroph. By radial immunodiffusion the IgG content of the porcine embryotroph was found to be 1.28 mg/cm^3, which corresponds to the immunoglobulin content of the sow's milk in mid-lactation. By the same method, the average IgG content of the bovine embryotroph was 1.57 g/cm^3, which is consistent with that of bovine milk obtained between day 7 and 9 and in the middle third of the lactation period.

Table 5

Amino acid composition (%) of the porcine and bovine embryotroph and of the porcine and bovine colostrum derived from the first milking

Amino acid	Porcine		Bovine	
	Embryotroph	Colostrum*	Embryotroph	Colostrum**
ASP	8.3	7.8	9.7	8.6
THR	4.9	5.8	4.6	6.2
SER	7.7	6.5	7.4	6.3
GLU	12.0	18.1	13.9	17.4
PRO	6.2	9.1	4.7	7.3
GLY	5.6	3.1	5.3	2.4
ALA	5.0	4.4	5.8	3.7
CYS	1.4	1.8	1.2	2.2
VAL	5.0	5.0	4.9	6.7
MET	0.4	1.7	0.7	1.9
ILE	2.3	2.4	3.1	3.8
LEU	8.0	9.9	9.9	8.3
TYR	5.7	4.0	3.3	4.8
PHE	7.5	4.4	4.3	4.1
LYS	7.8	6.3	8.5	7.3
HIS	2.7	2.1	3.9	2.6
ARG	5.3	6.1	7.0	4.5

*Csapó and Csapó-Kiss (1993); **Csapó et al. (1991)

Discussion

Although free amino acids occur in relatively low amounts, they are very important for the embryo, as they are directly available for absorption. Therefore, the predominance of amino acids of relatively simple structure in both species is remarkable. In our assumption, this may be attributed to the fact that in the period when the uterine milk is the sole nutriment source, the embryo can use these amino acids to build up amino acids of more complex structure and from the latter it can synthesize proteins.

Comparing the composition of total amino acids with that of different body fluids, the highest degree of agreement was found between the amino acid composition of the embryotroph protein and that of the colostrum derived from the first milking. Especially good agreement was found for valine, isoleucine, histidine

and aspartic acid (but not for glutamic acid), but no substantial differences were obtained for the other amino acids either (with the exception of methionine).

In cattle, the amino acid composition of the embryotroph protein shows a higher degree of similarity to that of the first-milked colostral protein than it does in swine. With the exception of the methionine and glutamic acid content, the amino acid composition of the two substances is practically identical.

The similarity of total amino acid content of the uterine milk to that of the dam's own fresh-milked colostrum in both species (Csapó et al., 1991; Csapó and Csapó-Kiss, 1993) is a surprising finding. There are data in the literature to suggest that the colostrum is a transition between the blood and the milk (Starodobtsev et al., 1974), and that there is a transition also between the colostrum and the blood: the embryotroph (Hetzel after Gamgee, 1944). This latter statement is confirmed by the results presented in this paper.

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