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VOLUME 39

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1991

CONTENTS

Pathology

Pathophysiology

Physiology

Virology

CONTENTS

Acta Veterinaria Hungarica 39 (1—2), pp. 3—11 (1991)

EFFECT OF TREATMENT WITH PROLAN OR WITH A GnRH SUPERACTIVE ANALOG ON THE SEXUAL FUNCTION OF SOWS AFTER WEANING

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(Received May 2, 1990)

On a large, closed pig farm using artificial insemination (AI), 29 sows were treated with Prolan S-öl injection (Bayer, FRG) and 31 sows with a GnRH superactive analog (Ovurelin, D-Phe^6-GnRH-EA, Reanal, Hungary) 48 h after weaning. The effect of treatment on the sows' sexual function was monitored by serum progesterone radioimmunoassay (RIA).

The conception rate in the control group (36 sows) was 69.4%. In the groups treated with Prolan and Ovurelin it was 79.3 and 71%, respectively.

The use of Prolan S-öl injection markedly reduced the number of acyclic sows and of those having an irregular oestrous cycle.

Treatment with the GnRH analog inhibited the manifestation of weaning-induced heat; subsequently, however, it induced a regular cycle in 30 out of the 31 sows treated.

Key words: Oestrus induction, sexual function, sow, weaning, Prolan, GnRH superactive analog

Under closed managemental conditions the hormonal function of animals is poorly expressed. This renders it necessary to use preparations which make the oestrous symptoms overt, hereby increasing prolificacy and the profitability of sow keeping. In pig production, well-known means of achieving this aim are PG 600 (400 IU PMGSG + 200 IU HCG, Intervet, The Netherlands) treatment (Polanco, 1976; Schilling and Cerne, 1972; Cerne and Nikolic, 1976) and the use of Prolan S-öl (200 IU chorionic gonadotropin + 1 mg oestradiol benzoate, Bayer, FRG) injections (Maczelka, 1981).

Recently, since the structure of gonadotropin releasing hormone (GnRH) was determined, its synthetic derivatives much more effective than the natural hormone have been developed (so-called superactive analogs, e.g. D-Phe^6-GnRH-EA, Ovurelin, Reanal, Hungary). It seemed, therefore, reasonable to study the effect of these analogs on the conception results of sows.

Materials and methods

The experiments were carried out at a specialized farm of 1100 sows producing hybrid pigs for the market, of the Agricultural Combine of Mezőhegyes. The farm uses artificial insemination (AI) and the piglets are weaned 30 ± 3 days after farrowing.

The experimental sows were housed individually during lactation. From weaning up to insemination the sows were kept in small groups and exercised daily. During and for 10 days after insemination the animals were kept individually and subsequently, up to farrowing, in groups of 8–10 sows again.

Thirty-six farrowed sows were used as control. Forty-eight h after weaning 29 sows received 1.5 ml Prolan S-öl injection (200 IU chorionic gonadotropin + 1 mg oestradiol benzoate suspended in 1 ml oil; Bayer, FRG) and 31 sows were injected with 100 μg GnRH superactive analog (Ovurelin, D-Phe^6-GnRH-EA, Reanal, Hungary), intramuscularly.

Blood samples were taken from the vena cava cranialis of all animals at the time of treatment (from the control sows 48 h after weaning), and then once at weekly intervals. After centrifugation the sera were stored frozen at -15 °C until processed. The progesterone concentration of the sera was determined by radioimmunoassay (RIA) as adapted by us (Faredin et al., 1987; Szabó et al., 1988).

Results

The results obtained for the *control group* (n = 36) are presented in Table 1. Twenty-five out of the 36 sows (69.4%) conceived: out of them, 21 farrowed and 4 aborted. Seventeen sows conceived on first insemination. The serum progesterone profiles of 3 of these sows (no. 5041, 5762 and 4745) are shown in Fig. 1.

Insemination in the second oestrous cycle after weaning was successful in 4 sows (Table 1). The serum progesterone levels of 2 successfully inseminated sows (no. 4719, 44) and 2 out of the 4 aborted sows (no. 358 and 230) are shown in Fig. 2.

Table 1

Reproductive status of sows based upon the serum progesterone profiles

Total number of sows: 36			
Conceived: 25		Not conceived: 11	
Farrowed: 21 on first insemination: 17 on second insemination: 4	Aborted: 4	Acyclic: 7	Irregular cycle: 4

Seven and 4 out of the 11 non-conceived sows (Table 1) were found to have acyclia and irregular cycle, respectively. The serum progesterone profiles of sows no. 231, 4036, 4701 and 4035 are shown in Fig. 3.

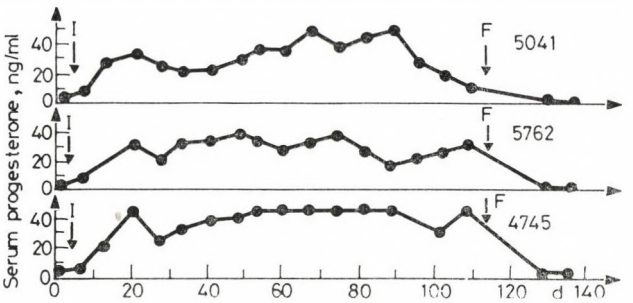

Fig. 1. Serum progesterone profiles in sows. I = insemination; F = farrowing

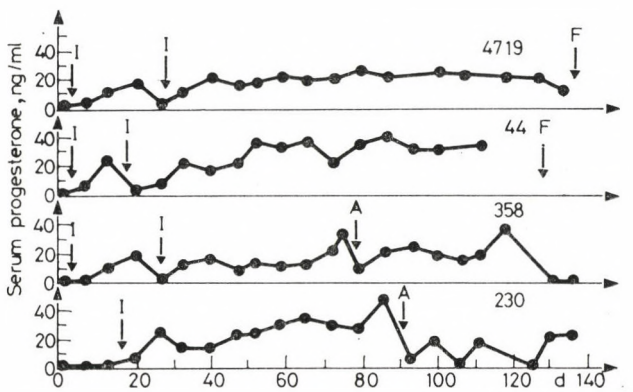

Fig. 2. Serum progesterone profiles in sows. I = insemination; F = farrowing; A = abortion

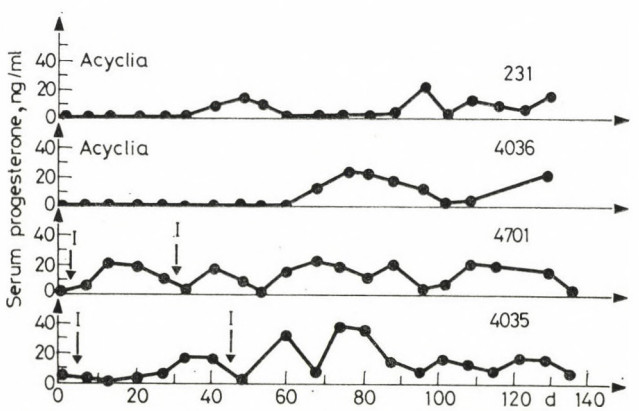

Fig. 3. Serum progesterone profiles in sows. I = insemination

Table 2

Effect of Prolan S-öl treatment on the sexual function of sows after weaning

Total number of sows: 29			
Conceived: 23 (79.3%)		Not conceived: 6 (20.7%)	
Farrowed: 23			
after insemination performed immediately after weaning	after insemination performed after several cycles	"Silent oestrus"	Irregular cycle
22	1	3	3

The effects of *Prolan treatment* are summarized in Table 2. Twenty-three out of the 29 sows (79.3%) treated with Prolan 48 h after weaning conceived and farrowed. Of them, 22 conceived on the first oestrus after weaning, while one sow conceived and farrowed after insemination following several returns to oestrus. The serum progesterone profiles of sows no. 486, 153 and 4985 of this group are presented in Fig. 4.

Six out of the 29 Prolan-treated sows (20.7%) failed to conceive (Table 2). Three of them had a characteristic, regular, so-called "silent" oestrus which was clinically inapparent but could be easily monitored on the basis of serum

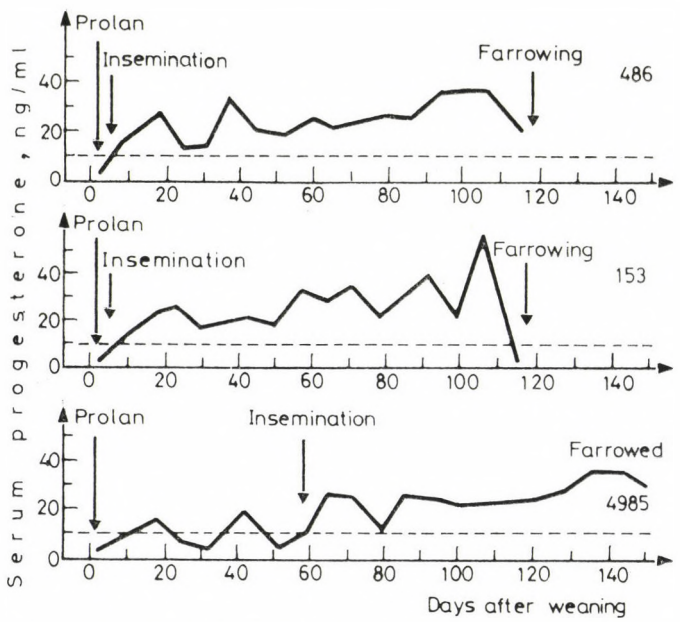

Fig. 4. Effect of Prolan S-öl on the sexual function of sows: Sows that farrowed. The broken line indicates the lower limit of serum progesterone concentration at which sows are considered pregnant

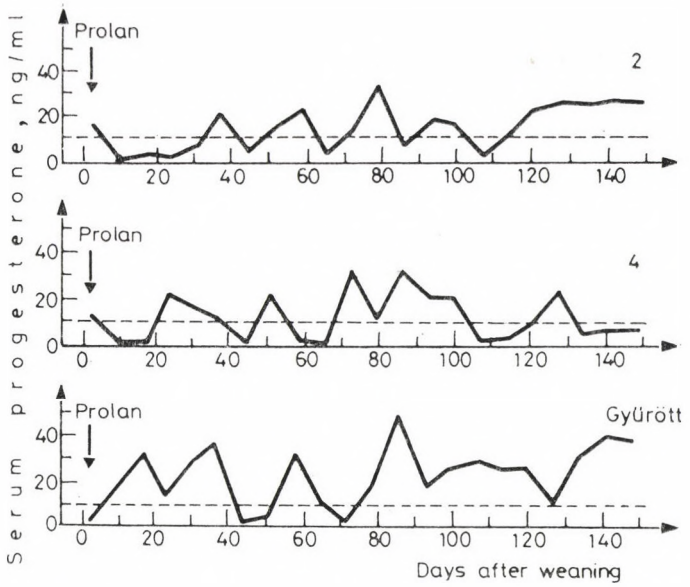

Fig. 5. Effect of Prolan S-öl on the sexual function of sows: Sows with "silent oestrus"

progesterone profiles (Fig. 5). The other 3 sows did not exhibit a regular cycle throughout the entire period of study, and had serum progesterone values corresponding to this (Fig. 6).

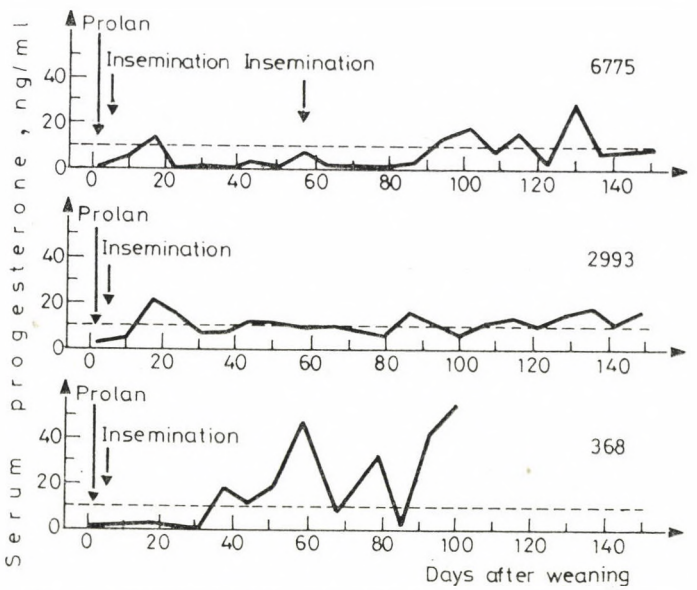

Fig. 6. Effect of Prolan S-öl on the sexual function of sows: Sows with irregular cycle

Twenty-two out of the 31 sows (71%) treated with the *GnRH superactive analog* 48 h after weaning farrowed (Table 3). Of them, 1 sow conceived on insemination done immediately after weaning, 8 sows on oestrus at the end of the first cycle, 2 sows after repeated insemination, and 11 sows on insemination after several "silent" oestruses. The typical serum progesterone profiles

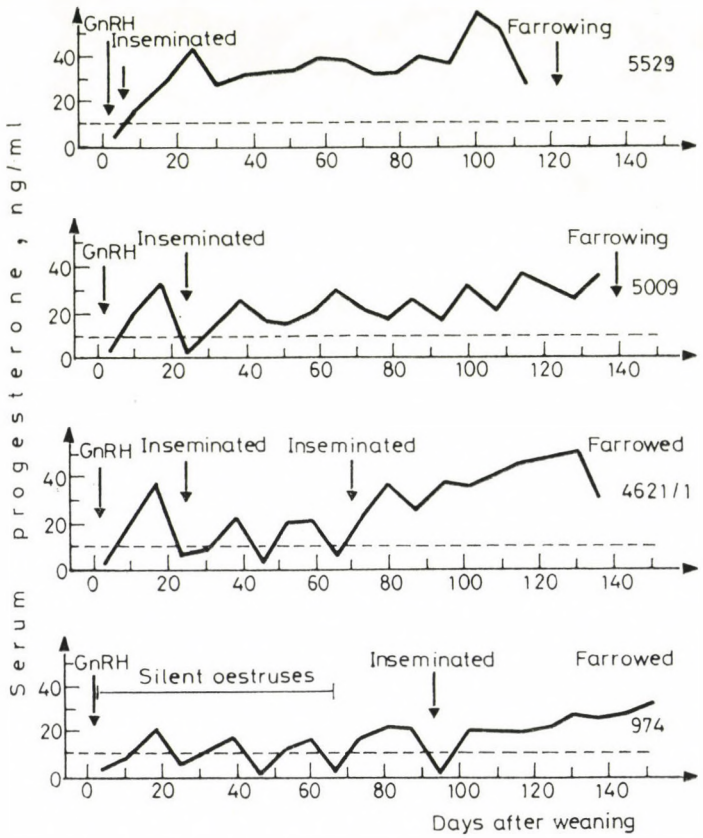

Fig. 7. Effect of GnRH treatment on the sexual function of sows

of one representative of each of these sow groups (sows no. 5529, 5009, 4621/1 and 974) are shown in Fig. 7.

Nine of the sows treated with the GnRH analog failed to conceive (Table 3). Eight of them had regularly occurring so-called "silent" oestrus which was clinically inapparent but could be easily monitored by the serum progesterone profiles. Only 1 out of the 9 sows had an irregular cycle. The serum progesterone profiles of sows no. 266, 973 and 5784 of this group are shown in Fig. 8.

Table 3

Effect of GnRH on the sexual function of sows*

Total number of sows: 31					
Conceived: 22 (71%)				Not conceived: 9 (29%)	
Farrowed: 22					
on insemination immediately after weaning	on insemination in the first cycle after weaning	after repeated insemination	on insemination after several cycles	"Silent oestrus"	Irregular cycle
1	8	2	11	8	1

* Weaning: 30 ± 3 days after farrowing.
Treatment: 2 ml (100 μg) GnRH im. 48 h after weaning.

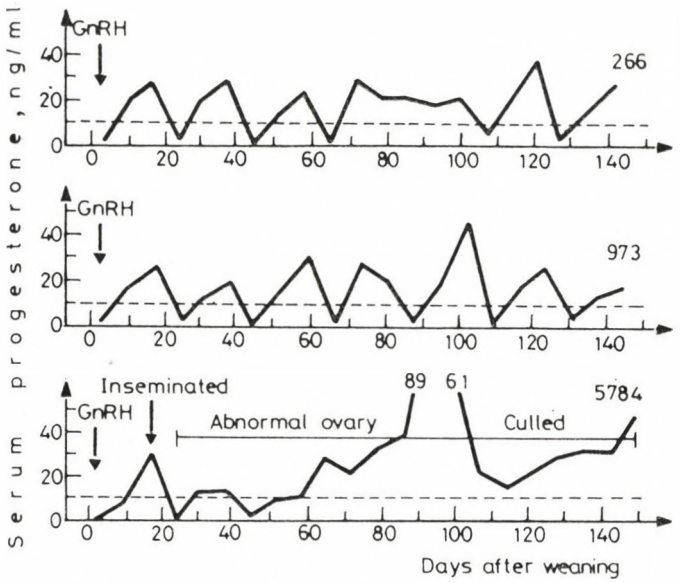

Fig. 8. Effect of GnRH treatment on the sexual function of sows

Discussion

In these experiments, Prolan S-öl treatment markedly increased the conception rate (79.3% vs. the control value, i.e. 69.4%). Furthermore, it reduced the number of acyclic sows which was well reflected in the serum progesterone profiles. Prolan S-öl injection can be used successfully for improving the post-weaning conception rate of sows. This finding is consistent with the conclusions drawn by Maczelka (1981).

The different superactive, synthetic analogs of GnRH have the same biological effect as natural GnRH; however, their longer binding to receptors

of the pituitary makes their efficacy many times higher than that of the latter is. Ovulation induced with GnRH superactive analogs indirectly is followed by a cycle of normal course (Nikolics et al., 1983; Horváth et al., 1986).

In trials involving a total of 1566 sows, Bergfeld et al. (1979) treated the sows with 1250 IU PMSG 24 h after weaning at 5 weeks of age, then on day 3 after weaning they gave 300 μg of GnRH and 400, 300 or 200 IU of HCG. They found no difference in the reproductive indices between the group treated with GnRH and that given only 500 IU HCG.

Using a similar treatment regimen, Schlegel et al. (1984) observed that treatment with HCG alone resulted in larger litter size, while HCG treatment combined with GnRH (300 IU HCG + 300 μg GnRH) led to higher conception rate.

According to Raasch et al. (1982), combined HCG + GnRH treatment improves the reproductive results even on industrial pig farms of good fertility. In trials involving 10 large pig herds, they found that combined treatment improved the conception rate and resulted in a significantly increased farrowing percentage (Raasch et al., 1984). In a more recent study, Bergfeld (1984) also found combined treatment (300 IU HCG + 300 μg GnRH) more effective than treatment with 500 IU HCG alone. He suggested that the use of GnRH superactive analogs might allow reduction of the hormone dose.

When using GnRH superactive analog 48 h after weaning we assumed that it would enhance FSH and LH release, hereby increasing the number of sows coming into oestrus within 3–7 days after weaning and, thus, improving the reproductive indices of the herd.

Contrary to the expectations, the GnRH superactive analog used by us 48 h after weaning failed to facilitate (quite the contrary, it inhibited) clinical manifestation of weaning-induced oestrus. Only 1 out of the 31 sows (sow no. 5529, Fig. 7) came into oestrus within 1 week after weaning and was successfully inseminated at that time. In the other 30 sows, besides inhibition of weaning-induced oestrus, GnRH administration was followed by normal cycles that could easily be monitored by serum progesterone determinations. In 8 sows, a normal cycle was followed by oestrus and successful insemination. Thirteen sows repeatedly returned to oestrus. In 8 sows so-called "silent oestrus" was found which could be observed only by monitoring the serum progesterone profiles.

Based on these results we suggest that treatment of sows with the GnRH superactive analog 48 h after weaning failed to improve the reproductive status of the herd. Therefore, some other mode of application must be looked for, so that weaning-induced oestrus and oestrus induced by the GnRH analog should coincide. (In this work we can utilize the experience that the GnRH analog induces a normal cycle in sows after weaning). Further studies are needed to find a solution for this problem.

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Acta Veterinaria Hungarica 39 (1–2), pp. 13–19 (1991)

ABORTION OF A SOW CAUSED BY *PASTEURELLA AEROGENES*

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Three strains of the *Pasteurella aerogenes* complex were isolated as sole pathogens from aborted fetuses of a sow aborted at the 12th week of gestation on a farm of 600 sows. Gross pathology showed no characteristic lesions. The isolates were biochemically identical and resembled *P. pneumotropica* on the basis of their strong indole and urease positivity but they produced gas, were ornithine decarboxylase negative and fermented mannitol but not trehalose. Only a few differences were apparent in biochemical characteristics between the isolated strains and *P. aerogenes*. They differed from the type strain of *P. aerogenes* in ornithine decarboxylase activity, indole production and lactose and mannitol fermentation; however, such strains do occur within this heterogeneous species.

At the time of abortion the antibody titre of the aborted sow was 1 in 16 when examined with live bacterial suspension and 1 in 128 if boiled antigen was used.

Similar strains could not be isolated from the vaginas of aborted sows or pregnant and newly farrowed sows in the same group.

The bacteriological, serological and histological findings support the opinion of other workers on the occasional pathogenic nature of *P. aerogenes*.

Key words: *Pasteurella aerogenes*, swine, fetus, abortion, Hungary

Several bacteria (leptospirae, brucellae, *Erysipelothrix rhusiopathiae*, chlamydiae, occasionally *Actinobacillus pleuropneumoniae*, streptococci and *Eperythrozoon suis*) may cause abortion in sows (Leman et al., 1986). Members of the *Pasteurella–Actinobacillus–Haemophilus* group are rarely responsible for swine abortions; however, sometimes *Pasteurella* (Carter and Biddy, 1966; Corkish and Naylor, 1982; Hommez and Devriese, 1976; McAllister and Carter, 1974; cit. Sneath and Stevens, 1985) or *Actinobacillus* strains (Ross et al., 1972) can be isolated from aborted swine fetuses.

Pasteurella, *Actinobacillus* and *Haemophilus* species cause different diseases in farm animals including swine. Pneumonia or pleuropneumonia due to *P. multocida* or *A. pleuropneumoniae*, progressive atrophic rhinitis caused by toxin-producing *P. multocida* strains, Glässer's disease induced by *H. parasuis* or septicaemia of piglets produced by *A. suis* are well known (Biberstein, 1981; Kilian and Frederiksen, 1981).

The *Pasteurella* genus comprises six species, namely *P. multocida*, *P. haemolytica*, *P. pneumotropica*, *P. ureae*, *P. aerogenes* and *P. gallinarum* (Carter

1984), however, recent DNA-DNA hybridization studies revealed that *P. haemolytica*, *P. ureae* and *P. pneumotropica* type Jawetz and Heyl are more closely related to the genus *Actinobacillus*. Exclusion of *P. aerogenes* from the genus *Pasteurella* was suggested on the basis of these studies as well (Mutters et al., 1985). The taxonomic position of the above-mentioned genera is not certain yet and needs further examination (Carter, 1984).

Members of the genera *Pasteurella* and *Actinobacillus* are usually regarded as fermentative organisms, which attack carbohydrates both under aerobic and anaerobic conditions and produce acid but not gas from them (Carter, 1984; Mutters et al., 1985; Phillips, 1984). However, besides *P. aerogenes* gas-producing strains do occur among *P. pneumotropica*, *Actinobacillus* sp. "Ross", Group Xb and "SP" group strains (Frederiksen, 1981).

This paper reports on a case of abortion of a sow due to a gas-producing *Pasteurella* species and on the characterization of the cultural and biochemical features of the isolated strains.

Materials and methods

From a farm of 600 sows 3 swine fetuses of a sow aborted at the 12th week of gestation were sent to postmortem examination. One week after the abortion vaginal swabs were taken from aborted, pregnant and newly farrowed sows to study their vaginal bacterial flora.

The postmortem examination was carried out as usual. Samples for histologic examinations were taken from liver, kidney and lungs. They were fixed in neutral 10% formalin, embedded in paraffin and stained with haematoxylin and eosin. Samples from liver and kidney were stained by modified Levaditi's silver-impregnation method to detect leptospirae.

Bacterial strains were isolated from the parenchymal organs and stomach contents of the fetuses by inoculation onto nutrient agar and ox blood agar containing 0.5% yeast extract. Agar plates were incubated at 37 °C for 24 h. Stomach contents were inoculated onto two blood agar plates, one of which was incubated for 24 h in air and the other for 7 days in air with 10% CO_2 content. Smears of stomach contents were stained by Gram's and Stamp's method.

Biochemical tests were carried out according to standard criteria (Biberstein, 1978; Cowan, 1974). *In vitro* antibiotic susceptibility of the isolates was tested by the paper disk method using Resistest (Human Co., Hungary) disks.

Tube agglutination tests were used to measure the antibody titre of the aborted sow against the isolates. MacFarland 4 density bacterial suspensions made from an isolate after 10-min boiling and without heating were used as

antigens. Tube agglutination and complement fixation tests were used to reveal antibodies against brucellae. Antibodies to *Leptospira pomona* and *L. hyos* were tested by the microagglutination test.

Specimens from the parenchymatous organs were inoculated in two passages onto secondary calf testicle cell cultures to isolate Aujeszky's disease virus. Fetal antibodies to parvovirus were detected by haemagglutination inhibition test using peritoneal and thoracic fluid of the fetuses (Kudron and Mocsári, 1979).

Results

Gross pathology

No characteristic lesions appeared in the aborted swine fetuses. Oedema of the subcutaneous tissues and accumulation of some reddish peritoneal and thoracic fluid could be seen. The livers were swollen and there was a fibrin network on their and the bowels' surface, the kidneys were enlarged and became haemorrhagic. The stomachs contained reddish watery fluid, the lungs were congested and atelectatic.

Histology

In the liver the simusoids were dilated and filled with blood, the cells of the mononuclear phagocytic system (MPS) became slightly enlarged and in some parts their proliferation was observed. Several cell colonies of fetal extramedullary haemocytopoiesis could be detected. Lymphocytes and neutrophilic granulocytes infiltrated locally the periportal tissues and the Glisson's capsule.

In the kidney the glomerular capillaries were filled with blood and the endothelial cells were enlarged.

In distinct areas of the lungs inflammatory and necrotic lesions were seen (Fig. 1). The alveolar lumina were infiltrated by desquamated epithelial cells, histiocytes, lymphocytes and neutrophilic granulocytes, together with serum and erythrocytes (Fig. 2).

Bacteriology

Bacteria of similar appearence were isolated in pure culture from the stomach contents of the fetuses. Growth was obtained with incubation both after 24 h in air and 7 days in air containing 10% CO_2. Colonies on blood agar after 24 h were approximately 1–2 mm in diameter, gray, convex, smooth and did not adhere to the medium. They did not show haemolysis but the

agar became greenish under the colonies (Fig. 3). In stained smears the bacteria appeared as Gram negative rods of 1–3 μm length and 0.5 μm width but in fresh culture coccoid forms occurred as well (Fig. 4). Similar bacteria could not be isolated from vaginal swabs taken from aborted, pregnant and newly farrowed sows. Stomach content smears, histological examination of livers and kidneys, bacterial and virus isolation revealed no other pathogens.

The biochemical properties of the isolates are summarized in Table 1.

Table 1

Biochemical properties of *P. aerogenes* strains isolated from swine fetuses

Catalase	+	Adonitol	—
Oxidase	+	Arabinose	+
OF test	fermentative	Dulcitol	—
Gas from glucose	+	Fructose	+
Indole	+	Galactose	—
Urease	+	Glucose	+
NO_3 reduction to NO_2	+	Inulin	—
H_2S (lead acetate strip)	+	Lactose	+
H_2S (TSI)	—	Maltose	+
Gelatine liquefaction	—	Mannitol	+
Beta-galactosidase (ONGP)	+	Mannose	+
Lysine decarboxylase	—	Raffinose	+
Arginine dihydrolase	—	Salicin	—
Ornithine decarboxylase	—	Sucrose	+
		Trehalose	—
		Xylose	+

The isolated strains were susceptible to chloramphenicol, erythromycin, gentamicin, nalidixic acid, nitrofurantoin, oxytetracycline, polymyxin, sulphadimidine and sulphamethoxazole–trimethoprim combination, showed reduced sensitivity to kanamycin, neomycin, oleandomycin and streptomycin. They were resistant to ampicillin, bacitracin, lincomycin, methicillin, novobiocin, oxacillin, penicillin, spiramycin and vancomycin.

At the time of abortion the antibody titre of the aborted sow was 1 in 16 when examined with live bacterial suspension and 1 in 128 if boiled antigen was used. No antibodies could be detected against brucellae and leptospirae. The peritoneal and the thoracic fluids of the aborted fetuses were free of antibodies to parvovirus.

Discussion

Exact identification of strains within the genera *Pasteurella*, *Actinobacillus* and *Haemophilus* is very difficult because of the common features (Carter 1984; Sneath and Stevens, 1985). The situation has become more difficult

since the importance of certain phenotypic features has decreased and nucleic acid relatedness has evolved to be one of the main points in bacterial taxonomy (Mutters et al., 1985). In spite of the reclassification of the genera *Pasteurella*, *Actinobacillus* and *Haemophilus*, the taxonomic position of some species has not become clear partly due to lack of DNA hybridization studies, partly because of the heterogeneous nature of the species. This is especially true in the case of *P. aerogenes* and *P. pneumotropica* (Carter, 1984; Frederiksen, 1981; Mutters et al., 1985).

Based on cultural and biochemical features, the three strains isolated from aborted swine fetuses in Hungary seem to belong to the genus *Pasteurella* (Carter, 1984). They differed from *P. multocida* which is urease, ONPG, lactose and maltose negative, galactose positive and does not produce gas (Sneath and Stevens, 1985). Their strong urease and indole positivity was suggestive of *P. pneumotropica* which generally occurs in rodents (Krüger et al., 1980; Schulz et al., 1977), but with the exception of some type Henriksen strains *P. pneumotropica* strains generally do not produce gas when fermenting carbohydrates (Bisgaard, 1986; Frederiksen, 1981). All the three types of *P. pneumotropica* are mannitol negative and trehalose positive and some other minor differences in biochemical characteristics occurred between our isolates and the different *P. pneumotropica* types (Bisgaard, 1986; Frederiksen, 1981). In comparison to *P. ureae*, which was already isolated from aborted swine fetuses, our strains were less fastidious, indole, arabinose, lactose, mannose and xylose positive, produced gas and H_2S (Corkish and Naylor, 1982; Sneath and Stevens, 1985). Our strains differed from *P. haemolytica* and *P. gallinarum* among others in fermentation pattern, urease, indole and gas production (Biberstein, 1978; Carter, 1984; Sneath and Stevens, 1985).

Our isolates resembled very much *P. aerogenes*, which was originally isolated from swine intestine and seemed to be a member of the normal gut flora of young pigs, occasionally acting as a secondary pathogen (McAllister and Carter, 1974), but later it was isolated from different organs of swine (Hommez and Devriese, 1976). *P. aerogenes* was described as a causative agent of swine abortion and was isolated from vaginal discharge of sows as well (Hommez and Devriese, 1976; McAllister and Carter, 1974). The biochemical characteristics of the strains isolated by McAllister and Carter (1974) differed from that of our isolates in some points. Our strains were indole positive, ornithine decarboxylase negative and fermented lactose and mannitol. Numerical taxonomic studies including the type strain of *P. aerogenes* pointed out that indole, lactose and mannitol positive and ornithine decarboxylase negative strains occur (Sneath and Stevens, 1985). In our view, the strains examined do belong to the *P. aerogenes* complex; however, they share some characteristics with *P. pneumotropica*. Their serological relationship has already been noticed (Chladek and Ellis, 1979). Due to common properties of the above-mentioned

two species misidentification occurs (Sneath and Stevens, 1985). Their exact separation needs further examinations.

We do not know anything about the source of infection. *P. aerogenes* strains could not be isolated from vaginas of aborted sows or sows in the same group on the farm; however, three years ago similar strains were cultured from aborted swine fetuses on this farm but they were not thoroughly characterized (unpublished data). The antibody titre of the blood serum of the aborted sow pointed to bacteriaemia. More data are needed to get conclusive evidence on the pathogenic role of these strains. The bacteriological, serological and histological findings support the opinion of other workers on their occasional pathogenic nature (Hommez and Devriese, 1976; McAllister and Carter, 1974).

Acknowledgement

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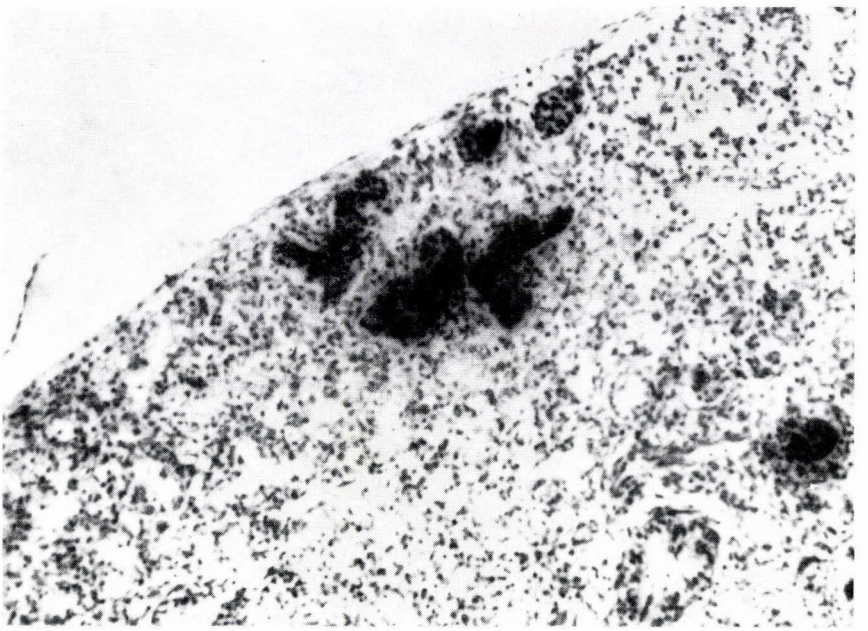

Fig. 1. Inflammatory and necrotic area in the lung. Haematoxylin and eosin (H.-E.) staining, $\times 63$

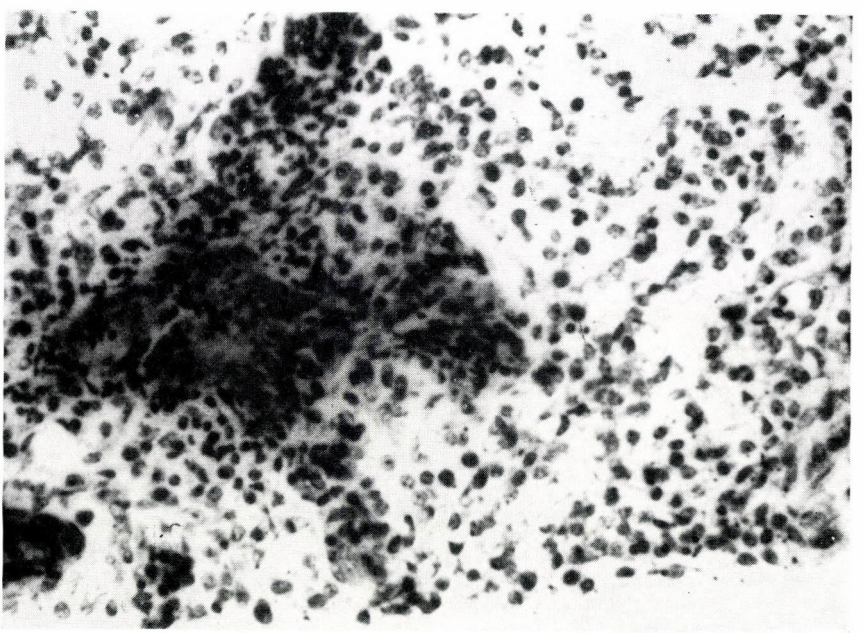

Fig. 2. Enlarged part of Fig. 1 ($\times 160$). The alveolar lumen is infiltrated by desquamated epithelial cells, lymphocytes, histiocytes and neutrophilic granulocytes

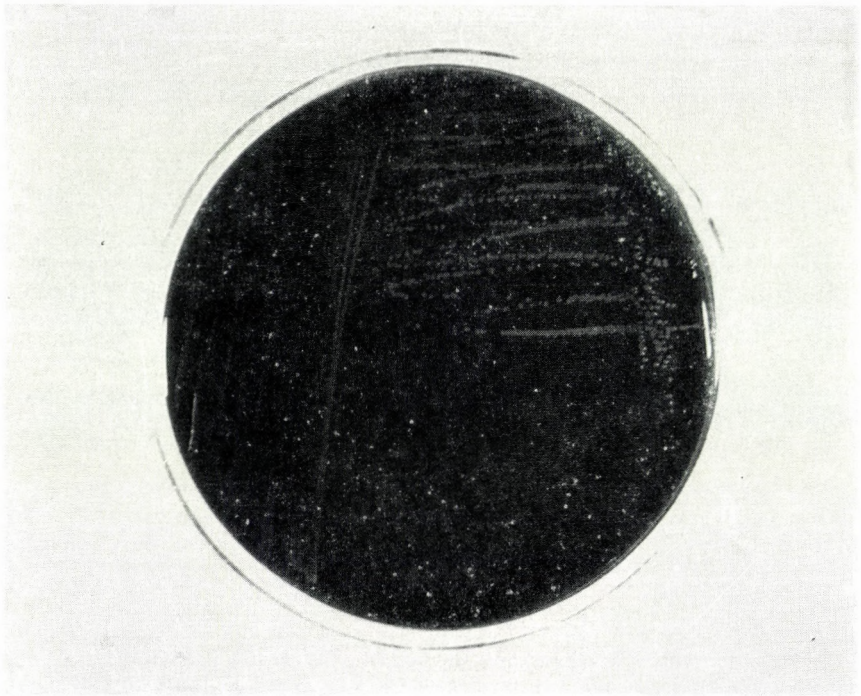

Fig. 3. Colonies of *P. aerogenes* isolated from aborted swine fetuses

Fig. 4. Gram negative 2–3 μm rods in a smear made from blood agar culture of the isolated bacteria

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SUITABILITY OF LACTATE DEHYDROGENASE ACTIVITY AND SOMATIC CELL COUNTS OF MILK FOR DETECTION OF SUBCLINICAL MASTITIS IN MERINO EWES

(SHORT COMMUNICATION)

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The relationship between somatic cell counts (SCC) and LDH activity in milk was examined in Turkey to find out the suitability of these variables for early detection of subclinical mastitis in Merino ewes. A significant positive correlation was found between LDH activity and SCC in ewes' milk. LDH activity in milk samples appeared to be a sensitive and specific indicator of subclinical mastitis in ewes: it was significantly higher in milk from inflamed (mastitic) udders than in normal milk.

Key words: Lactate dehydrogenase activity, somatic cell counts, mastitis, Merino ewes

Subclinical ewe mastitis, being still a severe problem in dairy ewes, is a source of major financial loss. The enzymatic and cytological milk control performed by Hambitzer and Sommer (1987) suggested that there was a high and significant correlation between the lactate dehydrogenase (LDH) activity and the somatic cell count (SCC) of milk. These variables were recommended for use in the diagnosis of subclinical cellular irritation and damage in the udder in the early stage. Apparently, the determination of SCC in ewes' milk can be used as an indirect test for recognition of subclinical mastitis. Milk with a cell count in excess of 10^6 cells/ml, obtained from a gland, was recommended to be examined bacteriologically (Green, 1984; Maisi et al., 1987). It is very useful to determine SCC in the milk to detect damages of the udder tissue. Physiological changes in milk result from its high cell contents (Giesecke and Van den Heever, 1974). Bogin and Ziv (1973) suggested that an elevated LDH activity in milk from chronically or acutely inflamed udders had some clinical diagnostic and prognostic importance.

In the present study, the relationship between SCC and LDH activity in milk was examined in Turkey to find out the suitability of these variables for early detection of subclinical mastitis in Merino ewes.

A total of 24 Merino ewes, 4 to 6 years old, were supplied by the Konya Agricultural Research Centre. Of the ewes, 12 were mastitic and the remaining

12 were healthy. The California Mastitis Test (Schalm et al., 1971) was used for separating mastitic and healthy ewes in the flock. Determination of LDH activity, cell counting and microbiological examination were performed immediately after the samples had been collected. Milk samples were centrifuged at high speed to prepare skim milk in which LDH activity was then measured. A commercially available test combination was used to measure LDH activity in the milk serum (Wako Chemicals GmbH, Nissanstr. 2, 4040 Neuss 1, Germany). Milk samples were cultured on 7% sheep blood agar and MacConkey agar (100 μl milk/plate). The plates were incubated aerobically at 37 °C for 24 to 72 h. Each milk sample was also cultured for fungi, on Sabouraud's

Table 1

LDH activity and cell counts in normal and mastitic milk samples (mean ± SEM)

	Groups	
	Normal	Mastitic
LDH unit/ml	57.08±16.71	875.00±140.77*
SCC/ml milk	699.120±51.143	1,798.804±180.169*

* Significantly different from the normal group ($P<0.01$)

dextrose agar at 25 °C for a week. Individual colonies were picked up for identification by classical procedures (IDF, 1981; Koneman et al., 1983). Somatic cells were counted as described by the IDF (1981).

Results summarized in Table 1 show the significance of variation for LDH activity and cell counts. *Corynebacterium* spp. (5), coagulase-positive staphylococci (5), coagulase-negative staphylococci (2) and *Pasteurella haemolytica* (2) were isolated; however, no microorganism was isolated from milk samples obtained from the healthy group. Like others (Green, 1984; Hambitzer and Sommer, 1987; Maisi et al., 1987; Symons and Wright, 1974), we found that there was a significant positive correlation between LDH activity and cell counts. LDH activity in milk samples appeared to be a sensitive and specific indicator of subclinical mastitis in ewes. Similar results were obtained previously in dairy milk by Sommer et al. (1986), who also showed high positive correlation between LDH activity and cell counts.

In the present study, the LDH activity of milk from mastitic udders was significantly higher than that of normal milk. The results of the present study strongly suggest that LDH activity and SCC are the most effective parameters in separating milk samples with positive bacteriology from samples with negative bacteriological results.

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GOAT SERUM, A SUBSTITUTE OF BOVINE SERUM IN CULTIVATION OF *BABESIA BOVIS*+

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Babesia bovis (a Mexican isolate) was cultivated in MASP culture system using goat serum in various concentrations as substitute of bovine serum. It was observed that 20% goat serum + 20% bovine serum + 60% Parker's medium 199 supported the growth of the parasite, which was maintained in this medium through 8 subcultures. The soluble exoantigen (vaccine) present in the culture supernatant is to be quantified and tested *in vitro*. Goat serum from slaughterhouses may be utilized for *in vitro* cultivation of the parasite and, expectedly, production of vaccine. This study may prove to be useful in reducing the cost of vaccine at least in tropical countries.

Key words: *Babesia bovis*, cultivation, vaccine production, goat serum

Babesia bovis is an intraerythrocytic protozoan parasite of cattle. Difficulties have been experienced in its *in vitro* cultivation, but recently the parasite has been cultivated, following the MicroAerophilus Stationary Phase (MASP) technique. Use of normal bovine serum on commercial scale is as difficult as it is costly. However, in tropical countries, which are rich in goats, goat blood/serum is available as slaughterhouse waste.

A continuous MASP culture technique for cultivation of *B. bovis* (Levy and Ristic, 1980) has been widely accepted. *B. bovis* has been reported to require 40% normal bovine serum (Erp et al., 1978; Levy and Ristic, 1980; James et al., 1987) for *in vitro* cultivation.

The present study was designed to explore the feasibility of using, fully or partly, goat serum instead of bovine serum. It was considered that goat serum, being a slaughterhouse waste, may be utilized for preparing *B. bovis* vaccine and thus reducing the cost of this commercial vaccine.

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Materials and methods

Parasites

B. bovis (a Mexican isolate) has been maintaned in this laboratory for 9 years in liquid nitrogen, bovines and/or *in vitro* cultures. The present *B. bovis* stabilate was obtained from a splenectomized calf, cultivated in MASP culture system and passaged 42 times. The isolate adapted to culture was used in the present study.

Stock medium

This was prepared by dissolving the following ingredients in 1 litre of double-distilled water: (a) Medium 199 (GIBCO) with Hanks' salt, L glutamine, 1 packet powder for 1 litre; (b) sodium bicarbonate 0.35 g; (c) HEPES 5.96 g; (d) penicillin G 0.12 g; and (e) streptomycin 0.29 g.

Working media

Five different working media were prepared from stock medium, bovine serum and/or goat serum. The composition of these working media was as follows.

Ingredient	Working medium, per cent				
	1	2	3	4	5
Stock medium	80	80	60	60	60
Bovine serum	20	—	20	10	—
Goat serum	—	20	20	30	40

The pH of stock as well as working media was adjusted to 6.95–7.00 with 1 N HCl and 5 N sodium hydroxide solution.

Normal culture

Five normal cultures were set by suspending 3.5 ml packed, washed normal bovine red blood cells (RBC) in 30 ml each of the indicated working media. pH was adjusted to 6.96–7.00.

The MASP culture technique (Levy and Ristic, 1980) was followed; 24-well flat-bottomed tissue culture plates were used. The *B. bovis* culture had been adapted to the bovine erythrocyte–serum MASP culture system. The parasitized erythrocyte (PPE) concentration was 9%. This culture was washed with the stock medium to remove bovine serum.

The old culture was diluted 1 : 1, 1 : 2, 1 : 5 and 1 : 12 with normal culture. The supernatants of the cultures were changed every 24 h for the working medium containing the same goat serum concentration. Smears were prepared from each dilution and each set of culture every 24 h, and were stained with Giemsa stain. Morphology, staining character and PPE were recorded.

Results

The Mexican isolate of *B. bovis* was fully adapted to the MASP culture system. Medium no. 1, 2, 4 and 5 containing 0%, 20%, 30% and 40% goat serum and 20%, 0%, 10% and 0% bovine serum, respectively, showed poor growth of *Babesia* and paired organisms could be seen only in 2 consecutive subcultures. Then the organisms became dot- or ring-like, and finally disappeared. Medium no. 3 with 20% bovine and 20% goat serum supported the growth of *B. bovis*. In the same medium cultures were passaged 8 times with good growth, multiplication and morphology (Table 1).

Table 1

Growth of *Babesia bovis* in different media

Medium no.	Bovine/Goat serum (%)	Medium (%)	Number of subcultures	Morphology of parasite	Growth
1	20/—	80	2	Dot and rings	No
2	—/20	80	2	Dot and rings	No
3	20/20	60	8	Normal	Yes
4	10/30	60	2	Dot and rings	No
5	—/40	60	2	Dot and rings	No

Discussion

The present findings indicate that among the 5 media only medium 3 was suitable for this system and supported good growth of *B. bovis*. The cultures contained 9.3, 8.6, 9.6 and 12.0 PPE in 1 : 1, 1 : 2, 1 : 5 and 1 : 12 dilutions, respectively, through 8 subcultures. These findings also indicate that 20% bovine serum or 20% goat serum alone is unsuitable for growth of *B. bovis* in the MASP culture system. Therefore, it is concluded that in the cultivation of *B. bovis* bovine serum can be replaced by goat serum at least to the extent of 50%. Although the cultures changed from bright red to dark coffee colour, indicating the presence of exoantigen in the culture supernatant, this needs to be checked. Goat serum obtained from slaughterhouses as a waste product can be utilized effectively in tropical countries like India.

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CHANGES INDUCED IN NEWBORN PIGLETS BY THE TRICHOTHECENE TOXIN T-2

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Three pregnant sows, being in the last quarter of gestation, were used in an experiment to study the changes induced in newborn piglets by T-2 toxin. One sow was used as control (C). The other two received 24 mg (sow A) and 6 mg (sow B) T-2 toxin, respectively, mixed in the feed, daily, up to the time of farrowing.

The piglets of sow A became ill by 48–72 h after birth, while the litters of sows B and C remained healthy. The clinical symptoms included faintness, diarrhoea, decreased blood glucose level, and collapse followed by death. The milk and urine of sow A and the stomach contents of affected and dead piglets contained T-2 toxin and its metabolites.

Pathological changes seen at necropsy included acute enteritis, degeneration of the liver and kidneys, and oedema of the mesentery. The stomach was filled with clotted milk. Histopathological and electron-microscopic findings consisted of reduced glycogen content and pathological simple fatty infiltration of the liver cells, lymphocyte depletion and necrosis in the lymphoid follicles of the intestinal mucosa, atrophy of the thymic cortex, and hyperfunction of the adrenal and thyroid glands compared to the control.

Key words: T-2 toxin, trichothecene, piglet, sow, clinical signs, pathological changes

In the last three decades, the study of the effects of mycotoxins, the toxic metabolic products of different microscopic fungi, have gradually come into prominence all over the world. In the 1960s, the attention of researchers was focussed on deaths caused by mycotoxins among animals and the resulting economic losses. Recently, high-quality food production and, in relation to this, the food chain and the question of drug residues have come to the centre of interest (Kovács, 1984). This is one of the reasons why it seemed important to investigate further the mass disease of baby piglets which occurred in a similar form on several large pig farms last year. The disease usually occurs after the first sucklings and lasts until the piglets become one week old. Detailed diagnostic investigations into its aetiology ruled out infectious diseases and brought the disease into connection with T-2 fusariotoxin, or its metabolic products, which were detectable both in the sows' feed and colostrum and in the piglets' stomach contents (Palya et al., 1988).

Considering that no similar disease caused by T-2 fusariotoxin has been described in the special literature so far, it seemed reasonable to experimentally reproduce and study the disease in pregnant sows.

T-2 toxin is produced by *Fusarium tricinctum* (*sporotrichioides*) and several other *Fusarium* species. After oral ingestion it is rapidly absorbed from

the small intestine. The toxin is almost completely transformed in a few days and is soon excreted with the faeces and urine. The chief site of its decomposition is the liver. Identified metabolites of the toxin are HT-2 toxin, neosolaniol, diacetyl neosolaniol, T-2 triol, and T-2 tetraol. Its accumulation in the tissues is rather negligible and should be reckoned with in poultry species rather than in swine.

Acute T-2 toxicosis of fatal outcome usually occurs only experimentally. The pathological changes reported for mammalian species dying of acute experimental T-2 toxicosis include dilatation and congestion of blood vessels of the leptomeninges and mesentery, swelling and hyperaemia of the jejunal and ileal mucosa, lymphocyte damage and necrosis in the Peyer's patches, mesenteric lymph nodes and spleen (Smalley and Strong, 1975; Sato et al., 1975). In addition to these morphologic changes, in piglets necrosis of myocardial cells and acini of the pancreas were also observed (Weaver et al., 1978a; Pang et al. 1987).

Further to the alterations described above, in rabbits T-2 toxin was reported to cause reduction of the lipid content and focal haemorrhagic dystrophy of the adrenal cortex, necrosis of the haemopoietic cell colonies of the myeloid and thrombocyte system of the bone marrow as well as of cells of the mononuclear phagocyte system (MPS) of the liver (Glávits et al., 1989).

In broiler chicks T-2 toxin was reported to induce superficial necrosis of the oral, pharyngeal and oesophageal mucosa, acute enteritis, and atrophy as well as lymphocyte depletion in the thymus and bursa of Fabricius (Chi et al., 1977; Bitay et al., 1979).

Chronic T-2 toxicosis of different animal species results in emaciation, reduced growth rate, reproductive disturbances, infertility, decreased white blood cell count of the blood, impaired general resistance, and high incidence of diseases caused by facultative pathogens (Chernov, 1970; Wyatt et al., 1975; Glávits et al., 1983; Corrier and Ziprin, 1986; Ványi et al., 1989).

The effect of T-2 toxin on pregnancy was studied in experiments with sows (Weaver et al., 1978b, c). At the beginning of the last third of gestation two sows each were intravenously inoculated with 0.41 mg and 0.21 mg T-2 toxin per kg body mass, respectively. In the two sows treated with the higher dose vomitus occurred 90 min and abortion took place 48 h after the experimental treatment. The two sows that had received the lower dose aborted 80 h after treatment. The two sows that had aborted after treatment with the higher dose of toxin were successfully inseminated 3 months later and delivered normal litters at term. Another three sows were treated with 12 ppm purified T-2 toxin orally for 38 days in the last third of gestation. The sows brought forth normal litters at term. Subsequently, toxin treatment was continued for 6 weeks up to weaning but the piglets remained free from clinical signs of disease. Within 4 days after weaning 2 sows came into oestrus but could be

inseminated successfully neither at that time nor at their subsequent four oestruses. The third sow failed to come into heat and conceived after weaning.

Robinson et al. (1979) fed a sow a diet containing 12 mg/kg T-2 toxin for 220 days and detected 76 μg/kg toxin in the sow's milk. The same authors treated a cow with 182 mg T-2 toxin per day orally, through a tube, for 15 days and demonstrated 10, 160, 40 and 38 μg/l T-2 toxin in the cow's milk on day 2, 5, 10 and 12, respectively.

The purpose of this study was to monitor pathological changes induced in newborn piglets by T-2 toxin treatment of the sows.

Materials and methods

Design of the experiment. Clinical, gross pathological and bacteriological examinations

Three multiparous (3rd–4th parity) Landrace sows being in the last quarter of gestation were used. The animals were transported away from the large pig farm and housed in individual pens.

The feed of *sow A* contained 8 mg T-2 toxin per kg throughout the experiment, in a daily amount of 3 kg, thus taking up 24 mg T-2 toxin per day on the average.

Sow B consumed 6 mg T-2 toxin per day.

Sow C (control) was fed a toxin-free sow diet.

The sows' behaviour was monitored throughout gestation and at farrowing. The newborn piglets were also kept under clinical observation. One piglet of each litter was killed by bleeding before taking up colostrum and another at 6 days old. These piglets, together with those that died or were killed during the experiment, were necropsied. Samples from their spleen, liver, kidney, small-intestinal and colon contents were taken for bacteriological examination (samples from the colon contents were cultured under both aerobic and anaerobic conditions).

Preparation of T-2 toxin

A *Fusarium tricinctum* (*sporotrichioides*) strain (no. 216), freshly isolated from feed samples in Hungary, was used. It was inoculated onto Sabouraud's slant agar and incubated at 26 °C for 7 days. Subsequently, sterile saline was poured on the culture, and a spore suspension was prepared by gentle mixing. One hundred g of rice was placed into a wide-mouthed Erlenmeyer flask, 100 ml 10% glucose solution was added, the flask was cotton-plugged and subjected to two cycles of fractionated autoclaving at 120 °C for 30 min on two conse-

cutive days. When the contents of the flask had cooled down, it was inoculated with the spore suspension.

The cultures were incubated for 3 weeks, in the first and third week at 20–22 °C and in the second week at 5–8 °C. After the 3 weeks had elapsed, the culture was dried at 60 °C, ground flourfine and assayed for T-2 toxin content.

The toxin concentration of the resulting material (1500 g) was 1.66 mg/g. The material was mixed in the sow diet to give a T-2 toxin concentration of 8.3 mg/kg of diet.

Histological and electron-microscopic examinations

From each piglet that was killed by bleeding or died, spleen, liver, kidney, thymus, thyroid, adrenal gland, brain, sternum, jejunum, ileum, mesenteric lymph node, and colon samples were taken for histological examination. The samples were fixed in 5% (v/v) formalin (pH 7.2) buffered with NaH_2PO_4 and NaOH. The fixed organ pieces were processed both by freezing and by embedding in paraffin. For general orientation the sections were stained with haematoxylin and eosin. Fettrot staining was used to study the lipid content of the liver, kidney and adrenals, and the periodic acid–Schiff (PAS) reaction complemented with diastase digestion was applied to detect glycogen in liver samples. The aldehyde bisulfide–toluidine blue (ABT) reaction and polarization microscopy were used to evaluate the secretory status of goblet cells of the intestinal mucosa.

Liver, adrenal gland and thyroid samples taken from two diseased piglets of sow A and from a healthy piglet of sow C were examined by electron microscopy as described earlier (Glávits et al., 1989).

Mycotoxicological examination

The detection of trichothecene fusariotoxins and their metabolites was attempted from the stomach contents and liver of piglets that had died or were killed, and from the sows' urine and milk samples. Extracts of the above materials were defatted and purified on a silica gel column, then examined on high-performance thin-layer chromatography (HPTLC) plates and, after silylation, by capillary gas chromatography (Ványi et al., 1982). The extracts were assayed for the following toxins: T-2 toxin, HT-2 toxin, neosolaniol, T-2 triol, and T-2 tetraol.

Biochemical examination

Blood samples were taken into heparinized tubes and the plasma was assayed for blood glucose level by photometry.

Results

Clinical, gross pathological and bacteriological examinations

After feeding of the toxin-containing diet was started, sows A and B "tolerated" the toxin dose rising day by day only up to a maximum of 10 mg toxin per day. Approaching this dose range, signs of partial feed refusal were noted, while above it complete feed refusal occurred. On the subsequent day, sow B again refused to consume her entire ration, while sow A consumed it.

The average daily toxin intake of sow A and sow B was around 24 and 6 mg, respectively, up to the day of farrowing (Fig. 1).

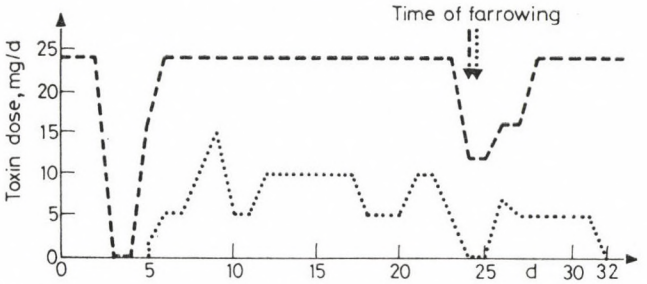

Fig. 1. T-2 toxin ingestion by sows (broken line: sow A; dotted line: sow B)

All three sows farrowed at term, without any complication, and delivered 13 (sow A), 16 (sow B), and 9 (sow C) piglets. Each litter contained one non-viable piglet of substandard body mass (750–850 g); the other piglets were viable and well developed. Sow A and B continued to receive the toxin-containing diet for 1 week after farrowing.

In the first 36 h, piglets of all litters suckled their dams and developed normally. One piglet in each litter was killed before taking up colostrum: these piglets proved to be free from pathological lesions. One day later, however, all piglets of litter A showed a gradually aggravating faintness and diarrhoea. From rectal swabs taken from the piglets a normal mixed intestinal microflora grew out. On the subsequent day the piglets of sow A were strikingly faint and had a shaggy and mat haircoat. The severity of diarrhoea did not decrease with respect to the day before. The piglets huddled together and moved with difficulty. By morning two piglets were "crushed to death" by the sow. Three out of the 9 survivors (piglets no. 5, 8 and 9) were lying in a comatose state.

The results of blood glucose determinations are presented in Table 1. The abnormally low glucose levels indicated that the comatose state of piglets no. 3, 5, 8 and 9 was due to hypoglycaemia. Piglets no. 5, 8 and 9 were killed by bleeding and necropsied. Their stomach was distended and filled with

cheese-curd-like dry, coagulated milk. The liver of all necropsied piglets was light yellowish-brown and its substance was easy to tear. The mesocolon was infiltrated with straw-coloured serum and appeared to be glistening, jelly-like and oedematous.

Table 1

Blood glucose level in piglets of sow A

Piglet no.	Blood glucose level, mmol/l
1	2.65
2	3.34
3	2.30
4	4.25
5	1.59
6	3.36
7	3.01
8	1.23
9	0.70

No signs of inflammation or necrosis were seen in the mucous membrane lining the digestive tract. The intestinal contents was greenish-yellow and liquid. The other organs examined were devoid of pathological changes (Fig. 2).

The bacteriological examination of the parenchymal organs gave negative results and the small-intestinal contents yielded a normal mixed intestinal microflora. During the subsequent 5 days further 5 piglets of sow A died of the clinical signs described above. Their necropsy findings and bacteriological results were similar as above.

The piglets of sows B and C were healthy and developed normally during the same period.

On day 6 after birth only 3 piglets of sow A were alive. Two of them fell ill and died of streptococcosis accompanied by acute septicaemia when 2–3 weeks old. The third piglet came down with streptococcal polyarthritis at 6–7 weeks of age and died, too, in spite of medication.

The litters of sows B and C remained healthy and developed well until weaning at 8 weeks of age and even beyond that time.

Histological and electron microscopic examination

The histochemical as well as light and electron microscopic changes found in the different organs of piglets are shown in Table 2 and Figs 3–9.

Mycotoxicological examinations

The liver and stomach contents of affected and killed piglets and the urine and milk of sows were examined for the presence of T-2 toxin and its metabolites. The results are presented in Tables 3, 4 and 5.

Table 3

T-2 toxin and its metabolites (μg/kg) in the stomach contents and liver of piglets of sow A

Piglet no.	Organ	T-2 toxin	HT-2 toxin	Neoso-laniol	T-2 triol	T-2 tetraol
5	liver	25	30	45	80	0
	stomach	40	40	20	140	0
8	liver	35	70	60	120	0
	stomach	60	50	30	200	0
9	liver	40	60	50	140	0
	stomach	80	40	40	170	0

Table 4

T-2 toxin and its metabolites in the sows' urine (mg/kg)

Sow	T-2 toxin	HT-2 toxin	Neoso-laniol	T-2 triol	T-2 tetraol
A	1.10	0.60	0.40	0.20	0
B	0.55	0.35	0.20	0.10	0
C (control)	0	0	0	0	0

Table 5

T-2 toxin and its metabolites (μg/kg) in the sows' milk 5 days after farrowing

Sow	T-2 toxin	HT-2 toxin	Neoso-laniol	T-2 triol	T-2 tetraol
A	120	80	60	140	0
B	60	55	40	55	0
C (control)	0	0	0	0	0

Discussion

In the experimental design used in this study, piglets of a sow fed 24 mg T-2 toxin per day for 24 days before farrowing became clinically affected by 36–48 h after birth, and the majority of them even died. T-2 toxin and its

metabolites were detectable in the sow's milk and urine and in the stomach contents of the piglets that became affected and died. The piglets of another sow fed 6 mg T-2 toxin per day on the average, and those of an untreated control sow remained clinically healthy. The clinical signs shown by the affected piglets and the gross and histopathological changes correspond to those observed in connection with similar disease cases that occurred on large pig farms earlier. Such a disease has not been described in the special literature. In contrast to the findings of Weaver et al. (1978b and c), who treated pregnant sows with large doses of T-2 toxin intravenously, no abortion took place in our experiment. No clinically recognizable abnormity, attributable to the ingestion of T-2 toxin in the final stage of gestation, was found in the number, body mass and viability of the newborn piglets.

Interestingly enough, feed refusal due to T-2 toxin (McLaughlin et al., 1977) was more expressed in sow B, which received the lower toxin dose, than in sow A. Latter refused feed only once, at the beginning of feeding. No plausible explanation can be offered for this phenomenon: perhaps it may have been associated with individual variation in sensitivity to the toxin.

The clinical signs of disease appeared 36–48 h after birth or the first sucklings. Gradually aggravating faintness was accompanied by diarrhoea, and within 24–36 h several piglets got into a comatose state.

The piglets' stomach was consistently distended by coagulated milk. This indicated a satisfactory milk supply on the one hand and a disturbance in gastric function on the other. Namely, the diseased piglets did not suckle their dam in the last 5–8 h before death or exsanguination. In our view, the T-2 toxin ingested with the sow's milk could have elicited a defensive response manifesting itself in pylorospasm or disturbed gastric motility as a unique sign of feed refusal. As a result, milk could not pass on to the intestine and, in spite of undisturbed milk supply, this may have played a role in inducing hypoglycaemia. In the presence of a normal mixed intestinal microflora, catarrhal enteritis and oedema of the mesocolon can also be accounted for by toxin effect.

The observed reduction in liver glycogen content, the development of comatose state, and fatty degeneration of the liver and kidney may be due to the combined effect of hypoglycaemia and the direct action of T-2 toxin. Therefore, hypoglycaemia seems to play an important role in the pathogenesis of T-2 toxicosis in newborn piglets.

The cessation of fetal haemopoiesis in the liver and spleen, the lymphocyte depletion and necrosis seen in the lymphoid follicles of the Peyer's patches, in Malpighian corpuscles of the spleen, and in the cortex of the thymus (McLaughlin et al., 1977; Weaver et al., 1978a; Pang et al., 1987; Glávits et al., 1989) are suggestive of a cytotoxic, immunotoxic and stressor effect of T-2 toxin (Glávits et al., 1983, 1989). Namely, according to some observations, acute or

Table 2

Histochemical, light and electron microscopic changes seen in different organs of the piglets

Animals		Changes																					
		Liver				Intestine		Kidney		Adrenal gland			Thyroid		Spleen			Lymph node		Thymus	Bone marrow		
		Vacuolation of the cytoplasm of hepatocytes	Decrease of glycogen content	Fatty infiltration of hepatocytes	Number of cell colonies of fetal haemopoiesis (per visual field, magnification: x40)	Acute catarrhal enteritis	Lymphocyte depletion and necrosis in lymphoid follicles of Peyer's patches	Fatty infiltration of the tubular epithelium	Decrease of lipid content in the cortex	Fatty degeneration of the cytoplasm of corticoid-secreting cells	Circumscribed haemorrhages in the medulla	Signs of hyperfunction of epinephrine-secreting cells	% of colloid-containing acini	Signs of thyreocyte hyperfunction	Average number of cell rows in the periarteriolar (T-dependent) lymphocyte zone of the Malpighian corpuscles	Blastogenic transformation of lymphocytes and appearance of germinal centres (B-dependent zones) in the Malpighian corpuscles	Average number of cell colonies of fetal haemopoiesis in the red pulp (per visual field, magn.: x100)	Blastogenic transformation of lymphocytes and appearance of secondary follicles	Infiltration of the medulla with neutrophilic granulocytes	Ratio of cortex and medulla	Ratio of undifferentiated (myeloblast) and differentiated (myelocyte) stages of myeloid haemopoiesis
Colostrum-free, killed by bleeding		+			5–10								30		5–10		10–20			1 : 1	8–10 : 1		
Piglet that fell ill 36–48 h after birth (no. 5)		+	+	+		+	+	+	+	+	+	+	60–80	+	1–4				+	1 : 1	8–10 : 1		
Piglets of sow A fed 24 mg T-2 toxin per day	"—" (piglet no. 8)	+	+	+		+	+	+	+	+	+	+	60–80	+	1–4				+	1 : 1	8–10 : 1		
	"—" (piglet no. 9)	+	+	+		+	+	+	+	+	+	+	60–80	+	1–4				+	1 : 1	8–10 : 1		
	"—" (piglet no. 3)	+	+	+		+	+		+	+	+	+	70–90	+	0				+	0.5 : 1	ND		
	Piglet killed by bleeding at 6 weeks old						+						60–80	+	10–20	+			+	3 : 1	8–10 : 1		
Piglets of sow B fed 6 mg T-2 toxin per day	Colostrum-free, killed by bleeding	+			1–4								40		1–4		10–20			1 : 1	8–10 : 1		
	Killed by bleeding at 6 weeks old												60–80	+	10–20	+	10–20	+		3 : 1	8–10 : 1		
Piglets of sow C (control) toxin-free	Colostrum-free, killed by bl.	+			1–4								40		5–10		5–10			1 : 1	8–10 : 1		
	Killed at 6 weeks old												60–80	+	10–20	+		+		3 : 1	8–10 : 1		

ND: not done

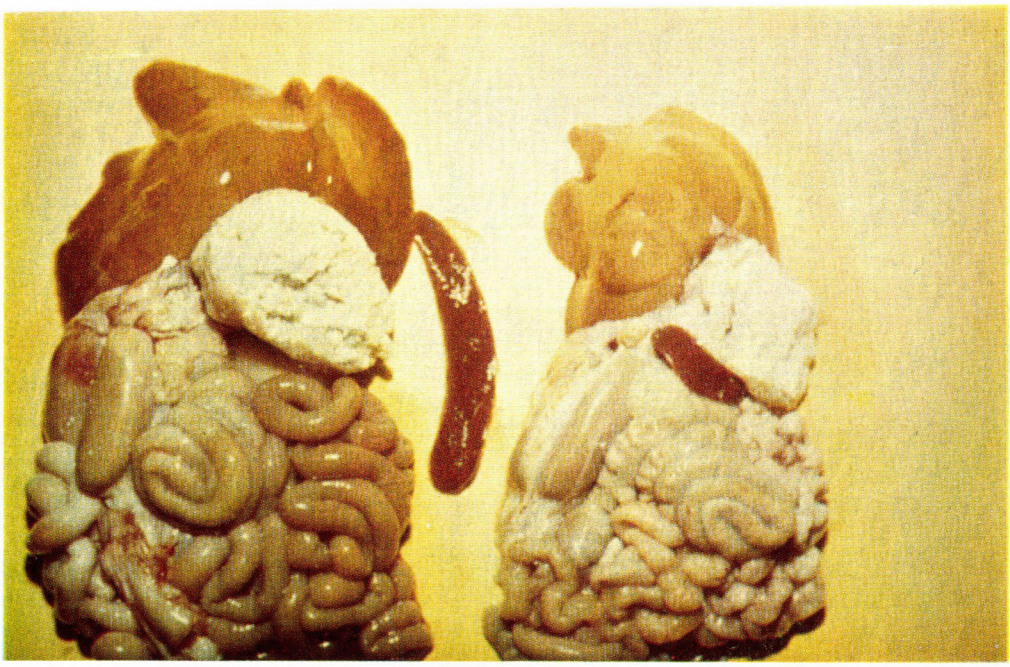

Fig. 2. Organs from a clinically ill piglet of sow A fed 24 mg T-2 toxin per day. The piglet was killed by bleeding at 3 days of age. Curd-like, dry, coagulated milk wells out when cutting open the distended stomach. The liver is pale, yellowish-brown and the mesentery is oedematous

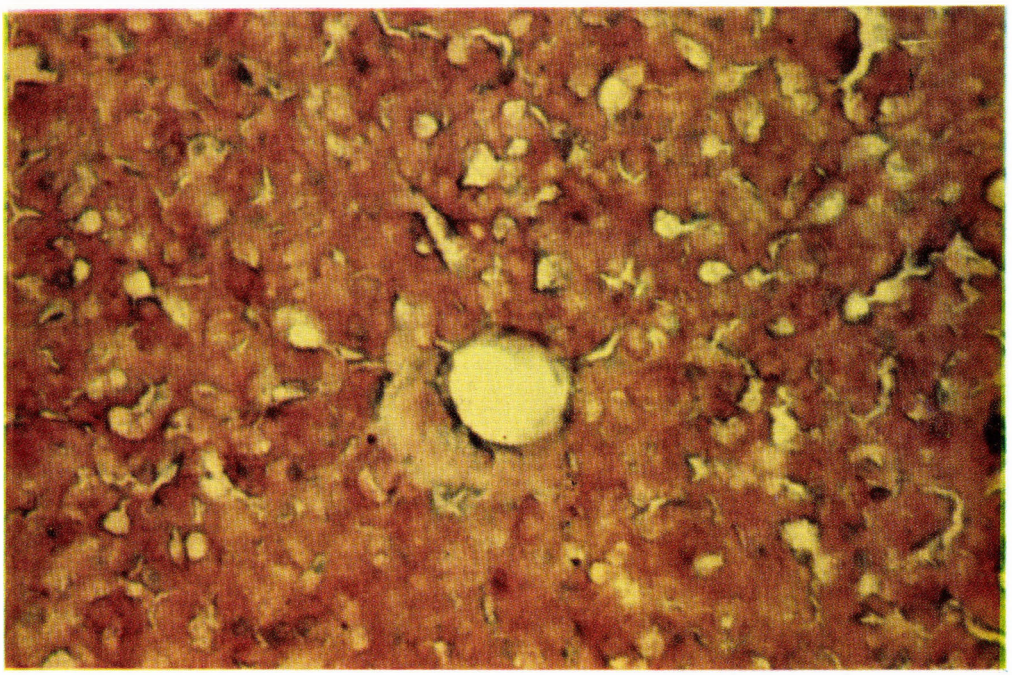

Fig. 3. Detail of liver from a newborn piglet of sow C (untreated control). The cytoplasm of hepatocytes is abundant in red-staining glycogen. Periodic acid–Schiff (PAS) reaction, ×63

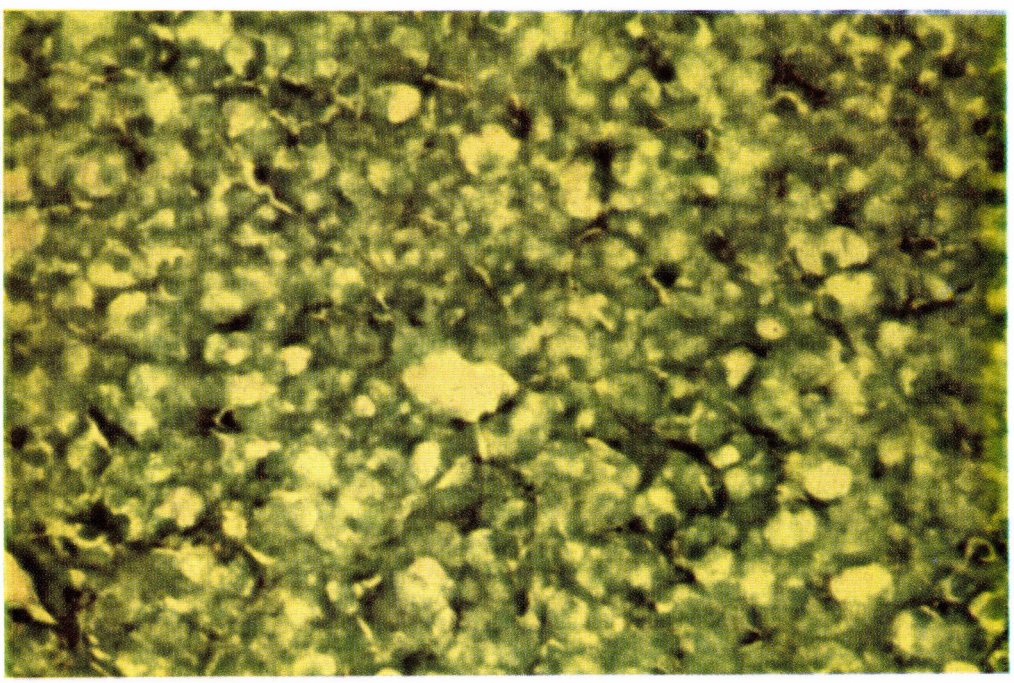

Fig. 4. Detail of liver from a piglet of sow A fed 24 mg T-2 toxin per day. The piglet became ill at 3 days of age. The cytoplasm of hepatocytes does not contain glycogen. PAS reaction, ×63

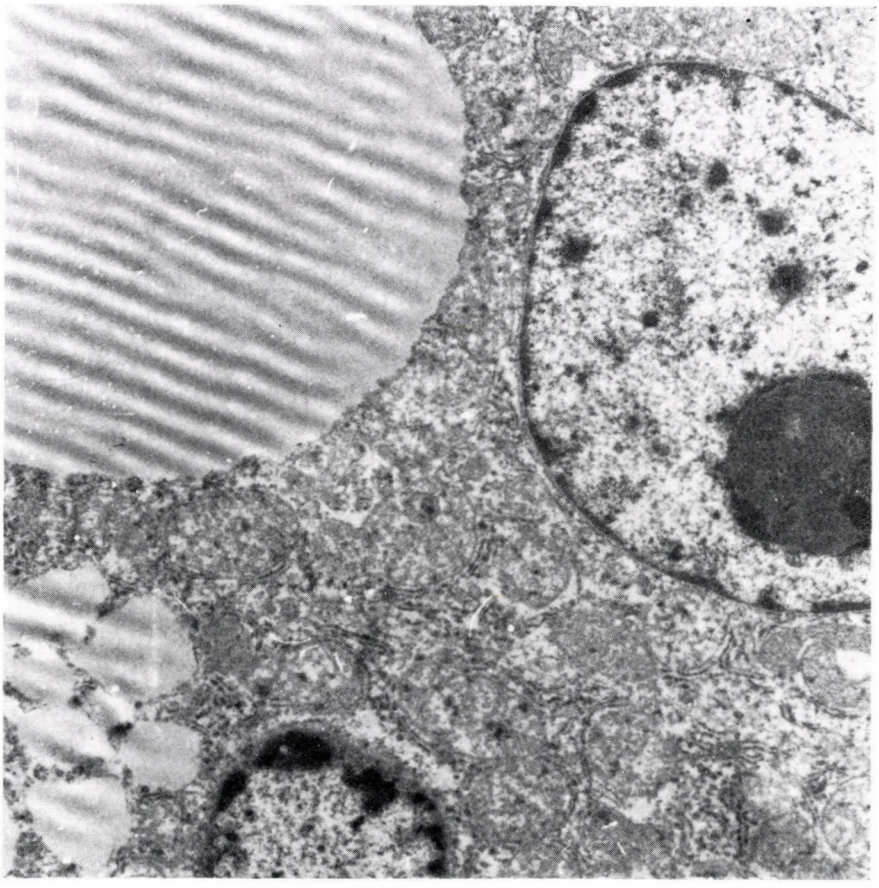

Fig. 5. Detail of liver from a piglet of sow A fed 24 mg T-2 toxin per day. The piglet became ill at 3 days of age. In the cytoplasm of hepatocytes note a lipid-containing vacuole. Electron micrograph, $\times 9{,}670$

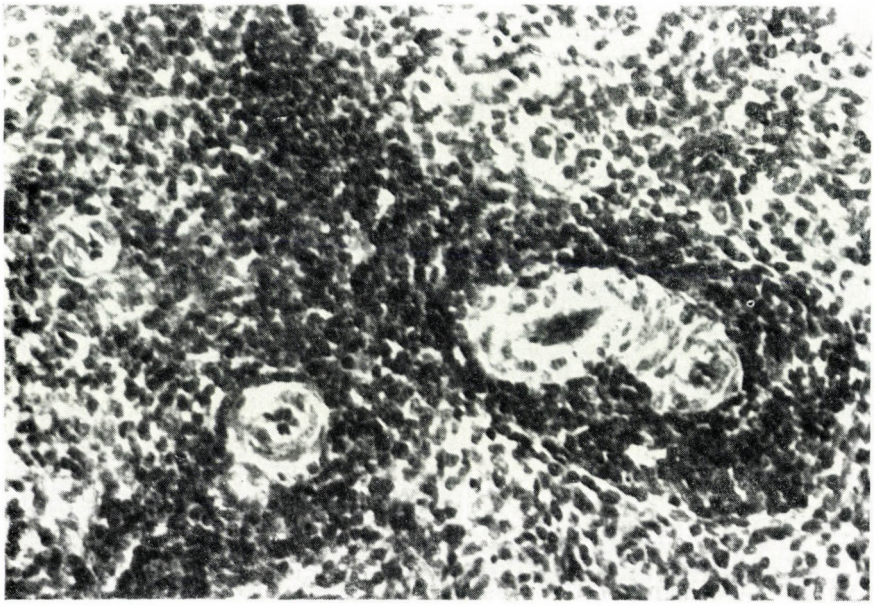

Fig. 6. Detail of spleen from a newborn piglet of sow C (untreated control). Note a lymphocyte zone of 5–10 cell rows around the brush arteries. Haematoxylin–eosin, × 160

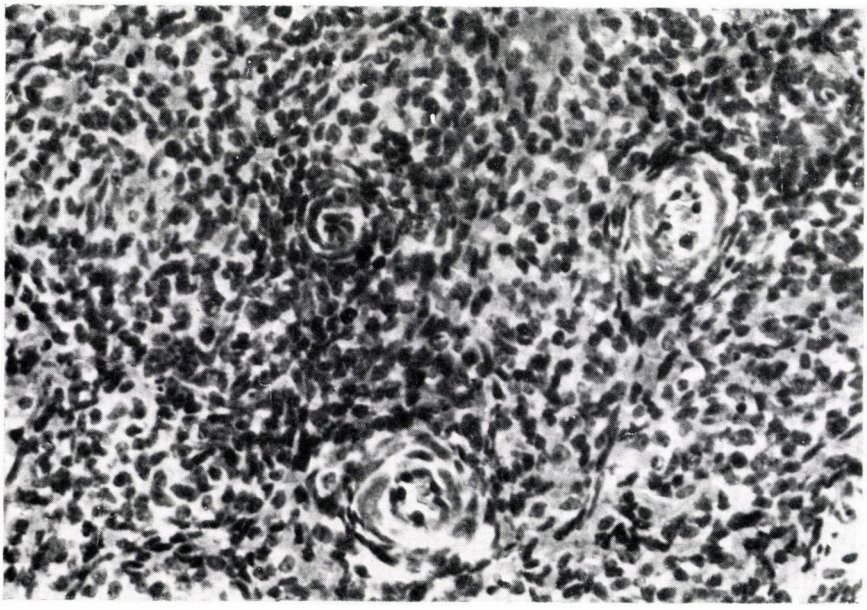

Fig. 7. Detail of spleen from a piglet of sow A fed 24 mg T-2 toxin per day. The piglet became ill at 3 days of age. Note the low number of lymphocytes around the brush arteries as compared to the control. Haematoxylin and eosin, × 160

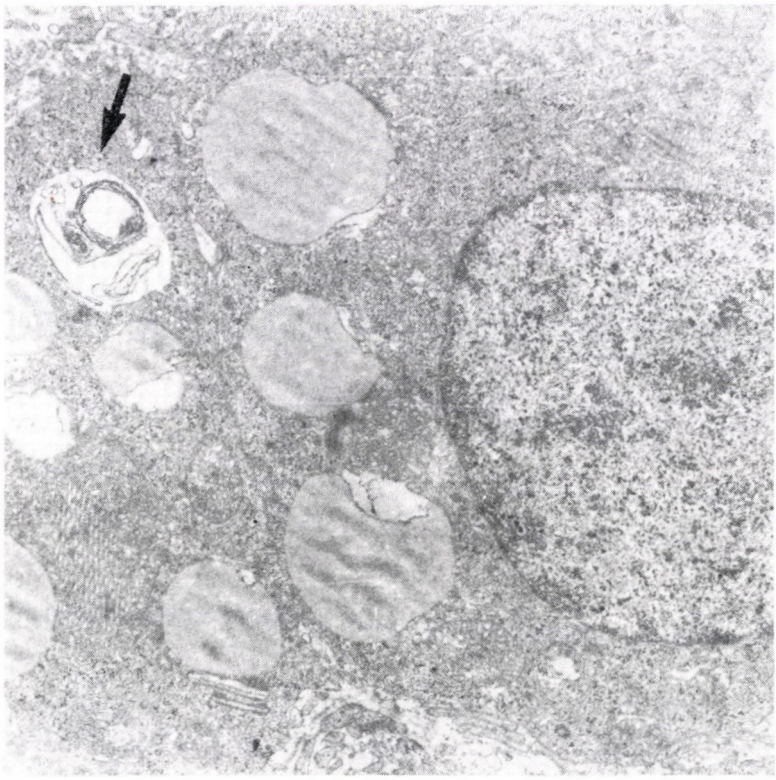

Fig. 8. Detail of adrenal cortex from a piglet of sow A fed 24 mg T-2 toxin per day. The piglet became ill at 3 days of age. Note secretory vacuoles and focal necrosis (arrow) in the cytoplasm of the corticoid-producing cell. Electron micrograph, $\times 9{,}670$

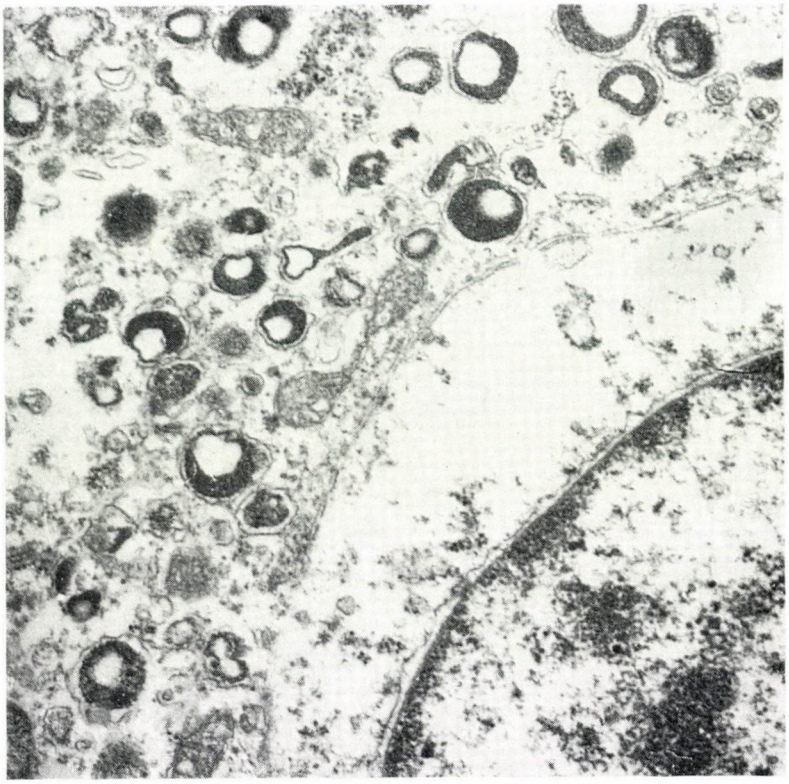

Fig. 9. Detail of adrenal medulla from a piglet of sow A fed 24 mg T-2 toxin per day. The piglet became ill at 3 days of age. The cytoplasm of the epinephrine-secreting cells contains numerous secretory granules. The perinuclear space has widened. Electron micrograph, × 22,300

chronic T-2 toxicosis is accompanied by morphological changes in certain endocrine glands. In our opinion, the lesions seen by us in the piglets' adrenal cortex and medulla and thyroid gland can be regarded as morphologic signs indicative of stressor action.

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PATHOLOGICAL AND IMMUNOLOGICAL STUDY OF GOOSE EMBRYOS AND GOSLINGS INOCULATED WITH AN ATTENUATED STRAIN OF DERZSY'S DISEASE VIRUS

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An attenuated Derzsy's disease virus strain, designated BAV, was studied in goose embryos. A total of 248 embryonated goose eggs, coming from a susceptible laying flock with no yolk-derived immunity (group I) and from a vaccinated laying flock (group II) were used. The eggs were inoculated into the allantoic cavity with $10^{1.9}$, $10^{2.9}$ or $10^{3.9}$ EID_{50}/0.2 ml virus on day 12 or day 20 of incubation. Embryos were killed at 5-day intervals. The dead embryos and the hatched goslings (up to 2 weeks of age) were examined by gross and histopathological methods. Reisolation of the virus from the organs was attempted, serum samples were tested for the presence of antibodies, and lymphocytes separated from the circulating blood were used in the lymphocyte stimulation and immunorosette formation tests.

Embryos of both groups I and II, inoculated at either time of incubation, showed a body mass gain inferior to that of the controls. Sixteen (group I) and 12 (group II) of the embryos inoculated on day 12 of incubation died. Some (group I: 15, group II: 6) of the embryos inoculated on day 20 of incubation failed to hatch. The pathomorphological changes seen in the embryos killed between days 17 and 22 of incubation were of degenerative character. In embryos killed later (between days 23 and 58 of incubation) the degenerative changes were accompanied by infiltration by inflammatory cells. Reisolation of the virus strain was mostly successful between postinoculation (PI) days 5 and 10. Specific virus-neutralizing antibodies and cellular immune response were demonstrable already at hatching. The hatched goslings showed no clinical signs of infection during a 2-week period of observation.

Key words: Derzsy's disease, goose embryo, attenuated virus, pathology, immunology, experimental infection

Investigating the aetiology of the gosling disease later named Derzsy's disease on the basis of an international agreement, Derzsy and his co-worker, Kisary, established that the disease was caused by a parvovirus (Kisary and Derzsy, 1974). Later on this was confirmed by other researchers (Gough et al., 1981; Peter, 1982). By attenuating the pathogen, Derzsy's team obtained a virus strain which failed to kill susceptible goose embryos and day-old goslings. The strain induced primary humoral immune response when used for experimental infection of 40 days old goslings and adult laying geese (Kisary et al., 1977).

Palya and Kisary (1978) studied in detail the pathomorphological changes experimentally induced by the virulent and the attenuated virus in day-old goslings. According to their observations, in acute cases the virulent parvovirus produced serous hepatitis accompanied by parenchymal injury and formation of intranuclear inclusion bodies, and degeneration as well as focal necrosis of myofibrils. Subacute cases were characterized by fibrinous perihepatitis, interstitial hepatitis, and lymphohistiocytic inflammantion of the damaged myocardial areas. The attenuated parvovirus caused neither gross nor histopathological changes in the organs of goslings. By pathomorphological examination of goslings naturally infected by Derzsy's disease virus, Bergman (1987) found similar changes. Intranuclear inclusion bodies were observed only in the myocardium. Electron microscopy revealed parvovirus particles in these inclusion bodies.

Under natural conditions both the virulent parvovirus causing Derzsy's disease and the attenuated strain can be transmitted from laying geese to goose embryos. The virulent virus consistently kills the embryos (Csontos, 1972; Kisary et al., 1977), while the attenuated strain does not (Kisary et al., 1977). Due to vaccinations, the attenuated strain has become fairly widespread in Hungary. Therefore, it seemed reasonable to study in more detail how this virus affects the development and viability of the embryo and whether it causes pathomorphological changes during growth in embryonated goose eggs. Also, it seemed important to study the cellular and humoral immune status of the embryos and the hatched goslings.

The results obtained by experimental inoculation with attenuated Derzsy's disease virus of goose embryos and day-old goslings derived from susceptible laying geese with no yolk-derived immunity to Derzsy's disease (I) and from vaccinated layers (II) are reported in this paper.

Materials and methods

Birds

A total of 248 preincubated goose eggs were used (124 each from laying flocks I and II). According to the virological and serological examinations, flock I was free from parvovirus, herpesvirus, adenovirus and mycoplasma infections, and had not been vaccinated.

Laying flock II was kept on a large farm and comprised layers that had been vaccinated against Derzsy's disease. To rule out possible bacterial or fungal infections, the eggs and the liver and bone marrow of the hatched goslings were sampled and cultured on suitable media.

Experimental infection and sampling

An attenuated Derzsy's disease virus strain designated BAV, developed by Kisary et al. (1977) and contained in a given batch of Deparvac vaccine, was used as inoculum. Inoculation of goose embryos (124 from each flock) was done as shown in Table 1.

Table 1

Experimental infection of goose embryos from flock I and flock II

Group		Number of embryos	Time of inoculation (day)	Treatment	Dilution of inoculum	Virus titre (EID_{50}/0.2 ml)
A	A_1	14	12	Virus suspension into the allantoic sac	1 : 30	$10^{3.9}$
	A_2	14	12		1 : 300	$10^{2.9}$
	A_3	14	12		1 : 3000	$10^{1.9}$
B	B_1	14	20	Virus suspension into the allantoic sac	1 : 30	$10^{3.9}$
	B_2	14	20		1 : 300	$10^{2.9}$
	B_3	14	20		1 : 3000	$10^{1.9}$
PC		20	12	Vehicle into the allantoic sac	—	—
C		20	—	—	—	—

The eggs were candled daily. Five embryos of the groups inoculated at different times of incubation (A and B) and of the control group were killed at 5-day intervals. These embryos, together with those that had died, were weighed, dissected and their organs were examined histologically by light and electron microscopy. The hatched goslings were kept under observation until they reached the age of 14 days. On days 5, 10 and 14 after hatching 5 birds per group were killed and examined in a similar manner.

Entire embryos were used for *light microscopy*. For *electron microscopic examination*, liver and heart samples were taken from 2 embryos each in groups A of flocks I and II, killed between PI day 5 and 10, and examined as described earlier (Glávits et al., 1986).

Virus isolation was attempted in goose embryo fibroblast cell cultures from the liver of each goose embryo and gosling killed at 5-day intervals. Serum samples from embryos killed at hatching and from goslings killed on day 5, 10 or 14 of life were titrated by the virus neutralization (VN) test, against 100 EID_{50} of virus.

Blood samples from embryos killed at hatching and from goslings exsanguinated at different times after hatching were examined by the *lymphocyte stimulation* and *immunorosette formation tests*, as described earlier (Glávits et al., 1986).

Results

After experimental infection, embryos from both flocks I and II showed a *body mass gain* inferior to that of the controls (Figs 8 and 9). In surviving embryos and goslings retardation in growth was demonstrable up to the end of the observation period, i.e. until 2 weeks after hatching.

In the group of embryos inoculated on day 12 of incubation, 16 and 12 embryos derived from susceptible laying geese (flock I) and from those having yolk-derived immunity (flock II), respectively, died. Some of the embryos inoculated on day 20 of incubation failed to hatch out (I: 15; II: 6). The size of the virus inoculum did not affect the mortality rate significantly (probably due to growth of the virus in the embryos).

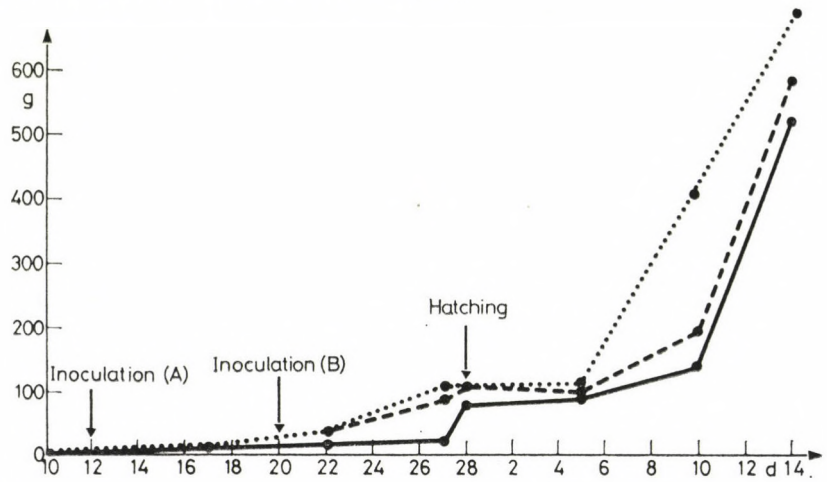

Fig. 8. Changes in the body mass of goose embryos and goslings derived from a susceptible flock not vaccinated against Derzsy's disease, and inoculated with attenuated virus. Solid line: goose embryos inoculated on day 12 of incubation (A); broken line: goose embryos inoculated on day 20 of incubation (B); dotted line: control (C and PC)

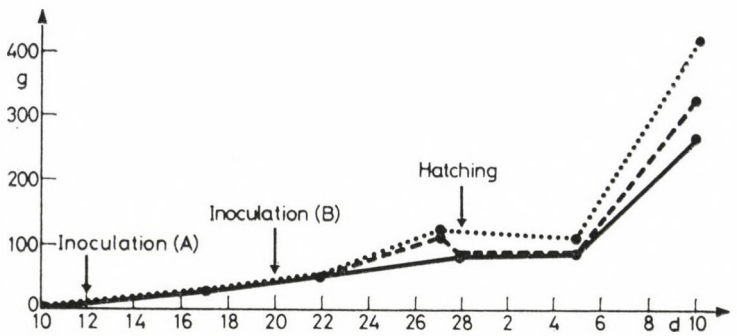

Fig. 9. Changes in the body mass of goose embryos and goslings from a large flock vaccinated against Derzsy's disease, and inoculated with attenuated virus. For explanation see footnote to Fig. 8

Table 2

Pathomorphological changes in goose embryos and goslings after inoculation of the embryos with an attenuated strain of Derzsy's disease virus

Changes	Stage of development		
	Days 17–22 of incubation	Days 23–28 of incubation	From hatching to 2 weeks of age
Retardation in growth	+	+	+
Oedema of the CAM	+		
Haemorrhages under the serosa of certain organs	+		
Hepatic dystrophy (focal)	+		
Icterus	+		
Intranuclear inclusion bodies in hepatocytes and myocardial cells	+	+	
Interstitial hepatitis with infiltration by heterophilic granulocytes		+	
Myocardial degeneration	+	+	
Serous epicarditis	+	+	
Myocarditis with infiltration by heterophilic granulocytes		+	
Poorer colonization by lymphocytes of the follicles of the bursa of Fabricius		+	
Focal lympho-histiocytic peritonitis			+
Focal lympho-histiocytic pancreatitis			+

The results of the pathomorphological examinations are summarized in Table 2 and the findings are shown in Figs 1–7. In both group I and group II embryos inoculated at an early stage of incubation, some organs showed signs of circulatory disturbance (oedema of the chorioallantoic membrane (CAM), haemorrhages in the subcutaneous connective tissue and under the serous membranes) and degenerative changes (myocardial degeneration, intranuclear inclusion bodies in the liver and heart). In some embryos the liver exhibited focal dystrophy and icterus. The lesions were more pronounced in embryos from flock I than in those from flock II. In embryos inoculated in a later phase of incubation, and also in those surviving an earlier inoculation, the degenerative changes were accompanied by infiltrations by inflammatory cells both in the liver and the heart. Colonization by lymphocytes of developing follicles of the bursa of Fabricius was poorer in the experimentally infected embryos than in the controls. Infiltration by inflammatory cells and poorer lyphocyte colonization were observed also in the unhatched embryos and in those killed at hatching.

No viral particles were seen in the liver and heart samples by electron microscopy.

During the 2-week period of observation after hatching the goslings showed no clinical signs, and no deaths occurred. Goslings exsanguinated in the 1st and 2nd week of life were devoid of gross lesions. Histologically, focal

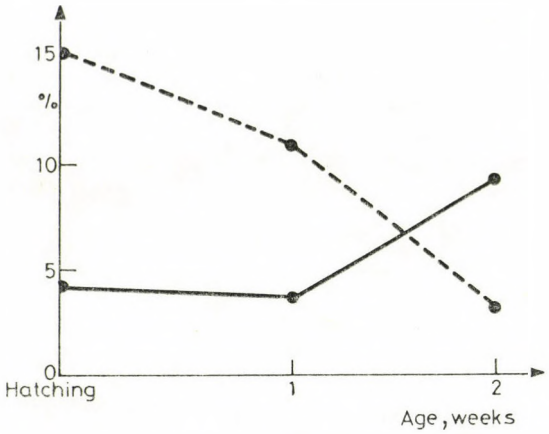

Fig. 10. Results of lymphocyte stimulation tests of embryos and goslings derived from a susceptible flock not vaccinated against Derzsy's disease, and inoculated with attenuated virus. For explanation see footnote to Fig. 8

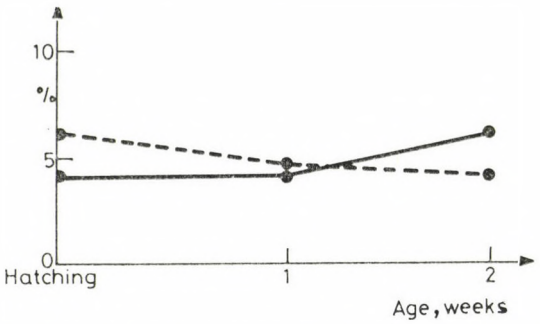

Fig. 11. Results of immunorosette formation tests of embryos and goslings derived from a susceptible flock not vaccinated against Derzsy's disease, and inoculated with attenuated virus. For explanation see footnote to Fig. 8

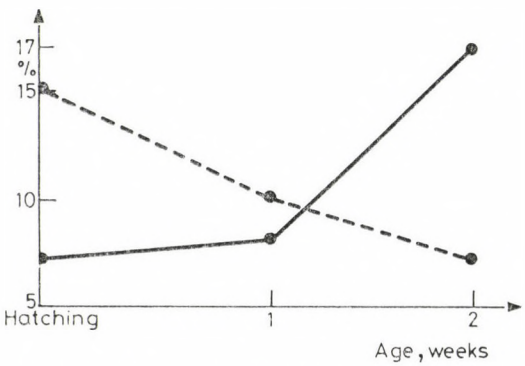

Fig. 12. Results of lymphocyte stimulation tests of embryos and goslings derived from a large flock vaccinated against Derzsy's disease, and inoculated with attenuated virus. For explanation see footnote to Fig. 8

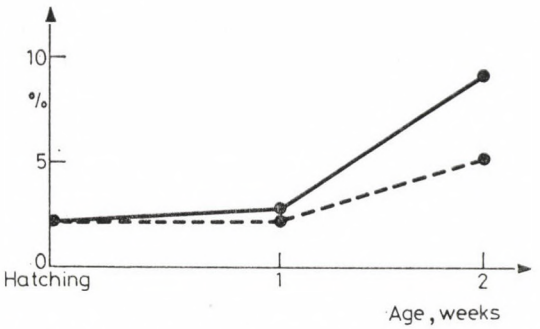

Fig. 13. Results of immunorosette formation tests of embryos and goslings derived from a large flock vaccinated against Derzsy's disease, and inoculated with attenuated virus. For explanation see footnote to Fig. 8

infiltration by inflammatory cells was seen only in the peritoneum and pancreas.

The type of infiltration varied by the age of the goose embryos and goslings: from the third quarter of incubation on, it was dominated by heterophilic granulocytes, while from the last quarter of incubation up to the end of the observation period lympho-histiocytic infiltrations, indicating the appearance of cell-mediated immunity, were typical.

Reisolation of the virus strain

Was successful between PI days 5 and 10 from goose embryos derived from flock I. The success rate of reisolation was higher in embryos inoculated on day 12 of incubation (65%) than in those inoculated on day 20 (18%). From eggs of flock II the virus was reisolated at a lower rate (35%) and only from embryos inoculated on day 12 of incubation. Virus isolation failed from the control groups of both flocks I and II.

Specific VN antibodies were demonstrated in blood samples taken at hatching from embryos of both flocks. In embryos from the susceptible flock, the level of VN antibodies tended to decline, and disappeared at 5 days of age. In blood samples from embryos of the immune flock, on the other hand, VN antibodies were still demonstrated even at the end of the observation period, on day 14 of life.

Lymphocytes separated from blood samples taken at hatching recognized the specific antigen in the *immunorosette formation test* and showed blastogenic transformation in the *lymphocyte stimulation test* (Figs 10, 11, 12 and 13). Hatched embryos from the susceptible flock showed different behaviour in the cellular tests, depending on the time of experimental infection. Namely, in embryos inoculated on day 20 of incubation the positivity rate of

both cellular tests tended to decline, similarly to the level of VN antibodies. Contrarily, in embryos inoculated on day 12 the rate of positive cellular tests began to rise. In embryos from flock II having yolk-derived immunity this was true only for the lymphocyte stimulation test.

Discussion

In the experiment reported here, the attenuated strain BAV of Derzsy's disease virus caused a slight retardation in growth both in goose embryos either susceptible or having yolk-derived immunity. Furthermore, in contrast to observations made by Kisary et al. (1977), a small proportion of the embryos was killed by the strain. The present results do not provide an explanation for this contradiction. It should be noted, however, that, instead of the original attenuated strain, a variant of it obtained from a batch of Deparvac vaccine was used in our experiment. Of the pathomorphological changes seen in the affected embryos, the degenerative lesions (myocardial and hepatic degeneration, Cowdry A type intranuclear inclusion bodies) were similar to those described in goslings by other investigators (Palya and Kisary, 1978; Bergman, 1987). The type of proliferative changes (inflammatory infiltration by heterophilic granulocytes, then by lympho-histiocytes) depended also on the embryo's age. Compared to susceptible embryos, in embryos with yolk-derived immunity the retardation in growth was less pronounced and the mortality rate, the severity of pathomorphological lesions and the rate of virus reisolation were lower. Goslings that hatched out infected remained free from clinical signs throughout the 2-week period of observation.*

Humoral and cell-mediated immune response to the attenuated virus was demonstrable already at the time of hatching. In embryos lacking yolk-derived immunity this was the result of virus infection. As regards the immune status that developed after hatching, further studies are needed for its reliable evaluation.

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* All production batches of Deparvac are tested for safety by intraperitoneal inoculation of day-old goslings susceptible to Derzsy's disease virus. The lack of clinical signs over a 30-day period of observation indicates that the given vaccine batch is safe.

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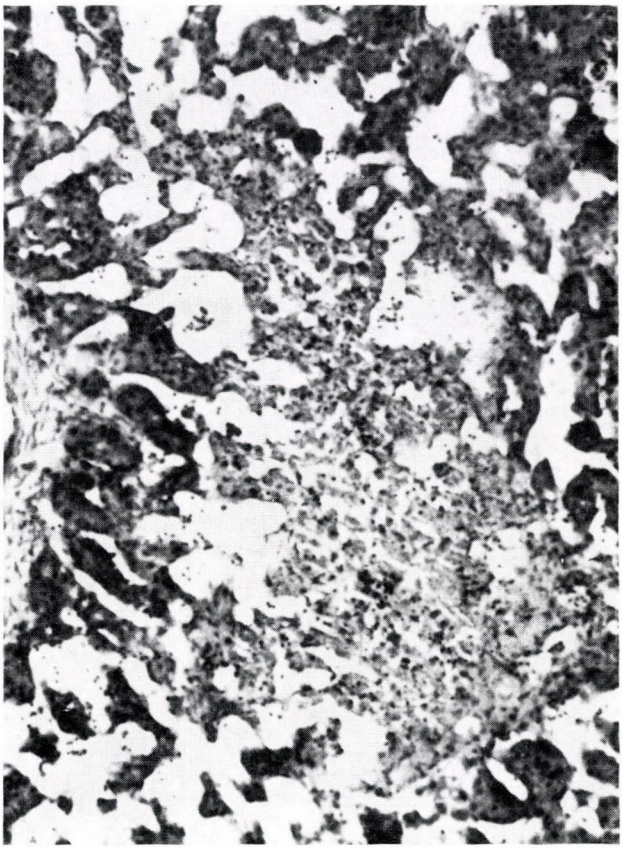

Fig. 1. Detail of liver from a goose embryo without yolk-derived immunity. The embryo was inoculated with $10^{3.9}$ EID_{50}/0.2 ml attenuated virus on day 12, and killed on day 17, of incubation. Focal dystrophy with necrotic hepatocytes. Haematoxylin and eosin (H.–E.), ×63

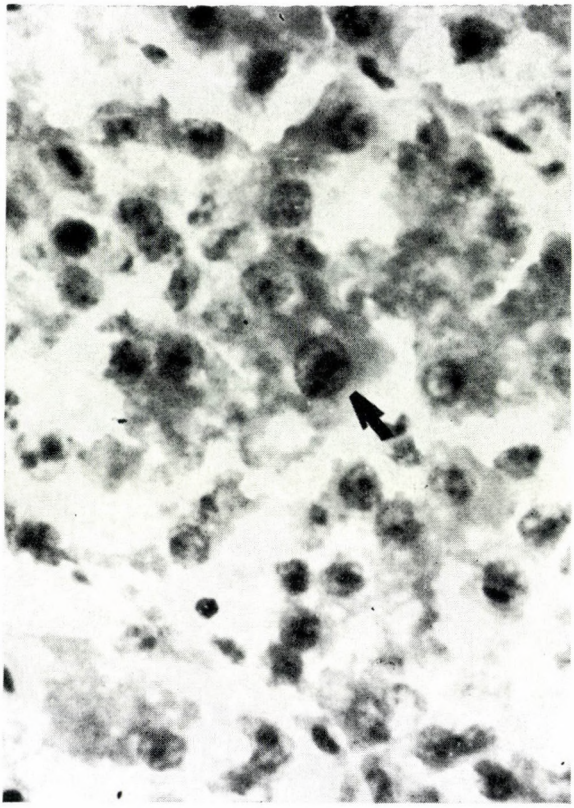

Fig. 2. Detail of Fig. 1. Note the intranuclear inclusion body (arrow) in the swollen nucleus of a hepatocyte. H.–E., ×400

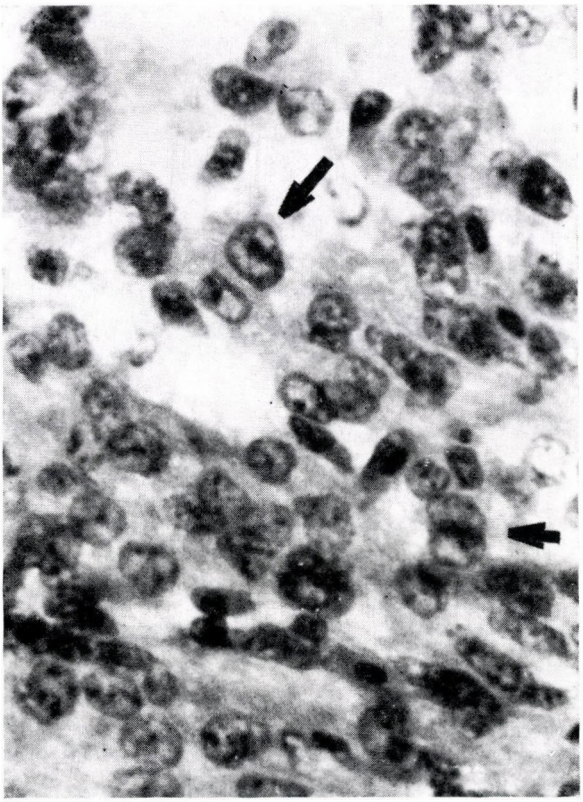

Fig. 3. Detail of heart from a goose embryo without yolk-derived immunity. The embryo was inoculated with $10^{3.9}$ EID_{50}/0.2 ml attenuated virus on day 12, and killed on day 17, of incubation. Note swelling of the nuclei of certain myocardial cells, perichromasia and inclusion body (arrow). H.–E., $\times 400$

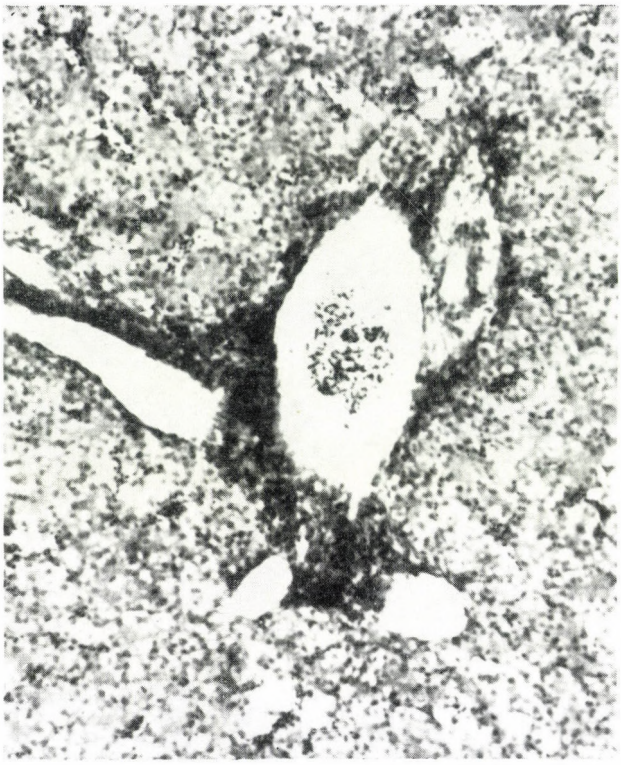

Fig. 4. Detail of liver from a goose embryo having yolk-derived immunity. The embryo was inoculated with $10^{3.9}$ EID_{50}/0.2 ml attenuated virus on day 12, and killed on day 27, of incubation. Note infiltration of the periportal tissue with heterophilic granulocytes. H.–E., ×63

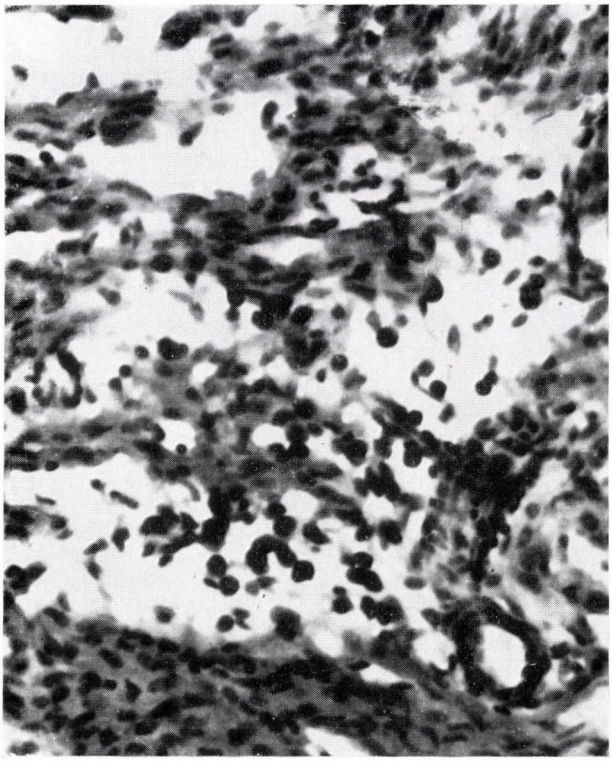

Fig. 5. Detail of heart from a goose embryo having yolk-derived immunity. The embryo was inoculated with $10^{3.5}$ EID_{50}/0.2 ml attenuated virus on day 12, and killed on day 27, of incubation. Myocarditis with infiltration by heterophilic granulocytes. H.–E., $\times 160$

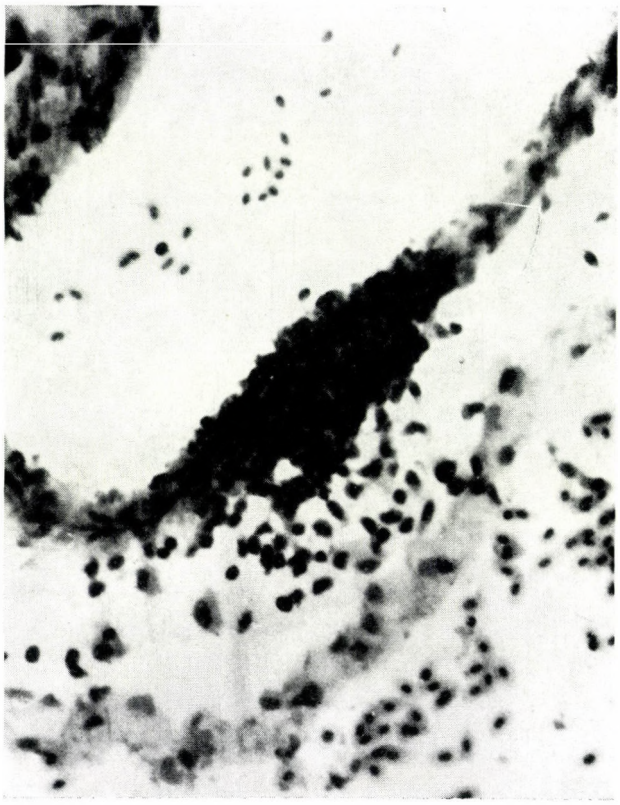

Fig. 6. Circumscribed lympho-histiocytic infiltration of the peritoneum in a one-day-old gosling that had hatched out after inoculation with $10^{2.9}$ EID_{50}/0.2 ml attenuated virus on day 20 of incubation. No yolk-derived immunity to Derzsy's disease. H.–E., $\times 160$

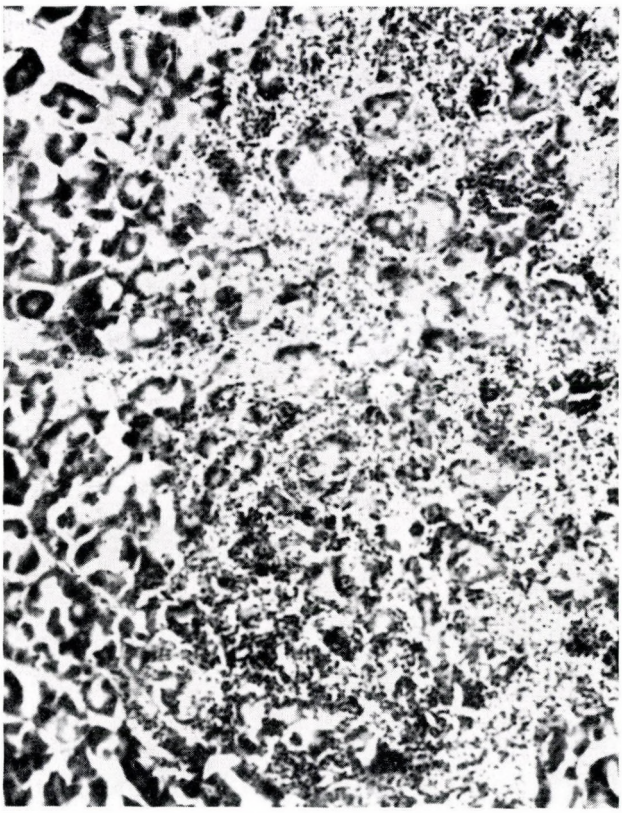

Fig. 7. Detail of pancreas from a day-old gosling that had hatched out after inoculation with $10^{2.9}$ EID_{50}/0.2 ml attenuated virus on day 20 of incubation. No yolk-derived immunity to Derzsy's disease. Note the lympho-histiocytic inflammatory infiltration. H.–E., $\times 63$

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EFFECT OF VOLATILE FATTY ACID INFUSION ON BLOOD PLASMA FREE AMINO ACID PROFILES IN GROWING LAMBS

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Five ram lambs (average body mass: 25 kg) were given, through a catheter inserted into the left ruminal vein, a total of 28.8 mM sodium acetate, 14.4 mM sodium propionate and 4.8 mM sodium butyrate per kg body mass as a 2-hour infusion. During and at 0, 1, 2, 4, 6, 10 and 24 h after the infusion blood samples were taken from the jugular vein and the blood plasma was assayed for free amino acid (FAA) and immunoreactive insulin (IRI) concentrations.

Volatile fatty acid (VFA) infusion significantly decreased the blood plasma concentrations of all FAA but cystine. The lowest FAA concentrations were measured in plasma samples taken at the end of the 2-h infusion. Subsequently the level of all amino acids rose and by 24 h after the infusion the blood plasma concentration of all FAA came close to the preinfusion value. The largest differences were observed in the concentration of glutamate, glycine, leucine and isoleucine.

In contrast to FAA, IRI concentration was increased significantly (almost fivefold) by VFA infusion. By 10 h after the infusion IRI concentration returned to the initial level.

The results reported here indicate that energy supply given in the form of VFA infusion significantly affects blood plasma FAA profiles, supposedly as a result of changes induced in protein synthesis in tissues. Insulin presumably plays a role in the regulation of these changes.

Key words: Volatile fatty acid, infusion, blood plasma, free amino acid profiles, growing lambs

Changes in blood plasma free amino acid (FAA) profiles are closely correlated with protein status. Based upon this correlation, in farm animals blood plasma FAA profiles provide useful data for studies on the nutritional and metabolic aspects of protein status (Bergen, 1979).

The blood plasma FAA pool serves as the primary amino acid (AA) source for protein synthesis in tissues. AA released during proteolysis also appear in the pool. Thus, factors affecting protein synthesis, proteolysis and, depending on the ratio of the two, also protein deposition, probably exert a major influence on blood plasma FAA profiles.

Protein biosynthesis requires much energy. As a result, the energy supply of animals markedly affects the intensity of protein synthesis in the tissues. In experiments with growing pigs, Reeds et al. (1981) found that non-protein energy supplementation of the feed enhanced protein synthesis. As the AA

source of protein synthesis is the FAA pool of the blood plasma, factors affecting protein synthesis in the tissues, including the animals' energy supply status, probably have a significant effect on blood plasma FAA profiles. Slater and Mellor (1977) induced energy deficiency in pregnant ewes by fasting and could not observe important changes in total plasma amino acid (TAA) concnetration. At the same time, the blood plasma concentration of essential amino acids (EAA) considerably increased; this rise, however, was counterbalanced by a drop in nonessential amino acid (NEAA) concentrations. In fasted sheep, Heitmann and Bergman (1980) found an increase primarily in the concentration of branched-chain amino acids (BCAA) including leucine, isoleucine and valine.

Blood plasma FAA levels may also be affected by AA released from skeletal muscles during fast-induced catabolism which become the most important glucose precursors in the organism. The FAA pool of the blood plasma plays an important role in supplying AA that have been used up for gluconeogenetic processes. Thus, during fasting the blood plasma level of primarily those AA declines which are important in glucose synthesis: in ruminants, first of all alanine and glutamate are such AA (Lindsay, 1981).

In contrast to fast-induced energy deficiency, the intravenous infusion of compounds rich in energy in most cases results in decreased blood plasma FAA concetrations through enhancing protein synthesis in the tissues. Following the intravenous infusion of glucose, the blood plasma level of almost all FAA decreased significantly in experiments with ewes (Prior and Christensen, 1978) and beef cattle (Huntington and Prior, 1985).

In ruminants the larger part of the energy required for vital processes is provided by volatile fatty acids (VFA) produced during rumen fermentation. Little is known, however, about the effect of VFA absorbed from the rumen or supplied by exogenous VFA infusion on blood plasma FAA concentrations of ruminants. Therefore, in the experiments reported here the influence exerted by intravenous VFA infusion on the blood plasma FAA profiles of growing lambs was studied. As insulin was shown to play an important role in regulating the metabolism of VFA, amino acids and protein (Manchester et al., 1971; Call et al., 1972; Young, 1980; Ahmed et al., 1983; Debras et al., 1987; etc.), changes in the blood plasma concentrations of insulin were also monitored.

Materials and methods

Five Merino ram lambs (average body mass: 25 kg) were used. One week before the beginning of the experiment, catheters (Clinical Plastic Products SA, 13G) were implanted surgically into the lambs' jugular and left ruminal veins. The animals received a conventional lamb diet *ad libitum* and

200 g alfalfa hay per lamb daily. Licking salt and drinking water were available also *ad libitum*.

After having been deprived of feed overnight (for 12 h), the lambs were placed into individual boxes. Through a cannula inserted into the left ruminal vein, with the help of a peristaltic pump (Pharmacia, P-3) 10 mM VFA mixture per min was infused at a rate of 2 ml/min, for 2 h. The composition of the VFA mixture was as follows: sodium acetate (3 Mol/l), sodium propionate (1.5 Mol/l) and sodium butyrate (0.5 Mol/l). During 2 h a total of 28.8 mMol sodium acetate, 14.4 mMol sodium propionate and 4.8 mMol sodium butyrate

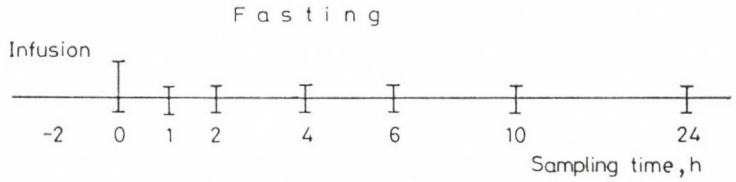

Fig. 1. Design of the experiment

per kg body mass was infused. Before and at the end of the infusion and for 24 h following it, blood samples were taken through the cannula inserted into the jugular vein. The sampling times are shown in Fig. 1.

The plasma samples obtained from heparinized blood samples were deproteinized by adding sulfosalicylic acid. The protein-free plasma sample were assayed for free amino acid (FAA) concentration by ion-exchange chromatography using an LKB-4101 type amino acid analyser (Kedenburg, 1971). Immunoreactive insulin (IRI) concentration was measured using a Boehringer ELISA kit (Cat. 199648).

After determining the FAA concentrations, total essential amino acids (TEAA = Thr + Cys + Val + Met + Leu + Ile + Tyr + Phe + Lys + + His) and total nonessential amino acids (TNEAA = Asp + Ser + Glu + + Glu-NH_2 + Gly + Ala + Arg) were calculated. The sum of TEAA and TNEAA was taken as the total free amino acid (TFAA) content of the blood plasma. The results were evaluated by statistical analysis.

Results

The TFAA, TEAA, TNEAA and IRI concentrations of the blood plasma are shown in Figs 2 and 3. It can be seen that by the end of the 2-h infusion period, infusion of VFA into the ruminal vein caused a significant ($P < 0.001$) decrease in blood plasma FAA concentration. Blood plasma TNEAA and TEAA concentrations measured at the end of VFA administration were less

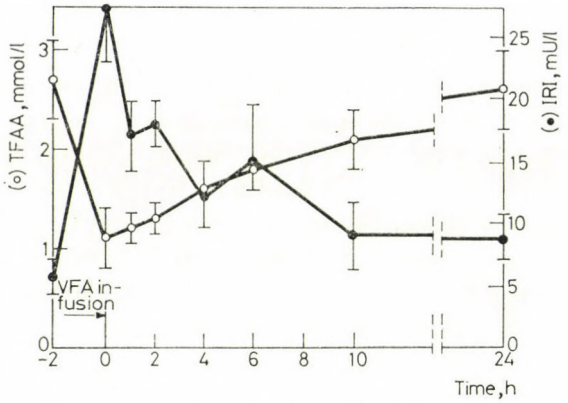

Fig. 2. Changes in the blood plasma concentrations of total free amino acids (TFAA) and immunoreactive insulin (IRI) after intravenous infusion of volatile fatty acids to lambs (n = 5)

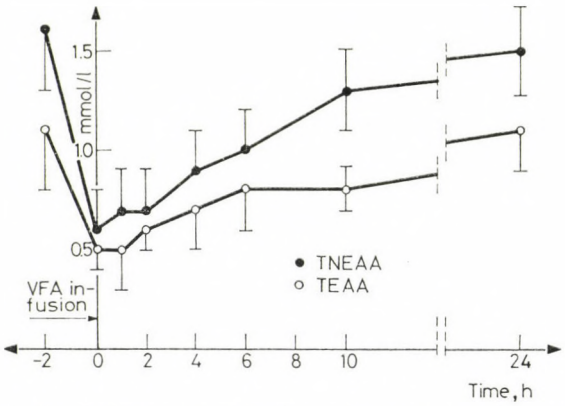

Fig. 3. Changes in the blood plasma concentrations of total essential amino acids (TEAA) and total nonessential amino acids (TNEAA) after intravenous infusion of volatile fatty acids to lambs (n = 5)

than half of the preinfusion values. In the period following VFA infusion, the blood plasma concentration of both FAA groups (TEAA and TNEAA) started to rise: a significant ($P < 0.05$) elevation, however, was demonstrable only 4 h after VFA administration. As a result of the increase in FAA concentrations after the infusion, at 24 h the blood plasma concentration of TEAA and, within it, both TEAA and TNEAA came close to the preinfusion values and did not differ from them significantly ($P > 0.05$).

Changes in the blood plasma concentrations of individual AA are shown in Tables 1 and 2. The plasma concentration of all AA but cystine showed significant ($P < 0.05$, $P < 0.01$, $P < 0.001$) changes. The tendency of the observed changes was the same for all AA. By the end of VFA administration, the concentration of all individual AA had declined considerably. Of the essen-

Table 1

Mean concentrations ± standard error (μmol/l) of nonessential amino acids in the free amino acid pool of the blood plasma in five lambs after intraruminal infusion of 48 mmol VFA per kg body mass

Aminoacid	Time of sampling (h)								P<	L.S.D.*
	−2[a]	0[b]	+1[c]	+2[c]	+4[c]	+6[c]	+10[c]	+21[c]		
Asp	28±6	3±3	2±2	6±3	9±7	11±4	13±5	25±5	0.01	6
Ser + Glu-NH_2	241±29	112±21	215±36	123±19	172±25	173±17	163±27	200±38	0.001	31
Glu	115±27	13±9	12±17	20±15	28±8	29±7	53±11	76±7	0.001	23
Gly	840±101	278±67	260±92	355±28	416±57	490±34	697±71	799±90	0.001	87
Ala	293±31	165±21	174±48	198±17	182±20	188±20	269±18	285±17	0.001	27
Arg	145±28	65±18	61±19	67±17	91±10	90±22	109±37	141±21	0.01	33

[a] before VFA infusion; [b] at the end of 2-h VFA infusion; [c] times after VFA infusion (h); L.S.D.* = least significant difference ($P<0.05$)

Table 2

Mean concentrations ± standard error (μmol/l) of essential amino acids in the free amino acid pool of the blood plasma in five lambs after intraruminal infusion of 48 mmol VFA per kg body mass

Aminoacid	Time of sampling (h)								P<	L.S.D.*
	−2[a]	0[b]	+1[c]	+2[c]	+4[c]	+6[c]	+10[c]	+24[c]		
Thr	146±20	64±15	66±9	70±12	99±18	100±18	94±8	141±26	0.001	24
Cys	30±18	10±9	11±17	31±14	27±15	37±18	30±11	28±9	NS	—
Met	39±17	9±5	10±7	18±9	15±8	19±16	24±6	43±15	0.01	16
Val	156±39	85±22	80±18	102±15	142±28	148±12	140±21	151±41	0.01	37
Ile	164±20	55±16	55±9	63±7	62±7	53±8	82±10	146±15	0.001	21
Leu	125±20	41±21	35±15	39±15	41±15	45±6	56±15	112±30	0.05	45
Tyr	86±11	36±15	32±12	36±11	48±9	56±16	60±16	116±10	0.001	22
Phe	61±15	37±18	40±9	55±12	69±7	79±24	62±8	73±9	0.05	20
Lys	165±35	69±13	81±17	89±24	117±18	174±20	165±22	165±31	0.01	41
His	85±11	52±10	45±9	62±8	98±10	82±11	94±8	85±15	0.01	28

[a] before VFA infusion; [b] at the end of 2-h VFA infusion; [c] time after VFA infusion (h); L.S.D.* = least significant difference ($P<0.05$)

tial AA, branched-chain AA (BCAA), first of all leucine and isoleucine showed the most pronounced decrease. Of the nonessential AA, glutamate and glycine exhibited the greatest concentration drop. In the period following the infusion, the concentration of all AA increased gradually. As a result, in blood plasma samples taken 24 h after the infusion the concentration of all AA came close to the preinfusion values. At that time, significant differences from the preinfusion value were found only for three AA: namely, at 24 h after the infusion the concentration of glutamate and serine + glutamine was lower ($P < 0.05$) while that of tyrosine was higher ($P < 0.05$) than the preinfusion values.

Immunoreactive insulin (IRI) concentration of the blood plasma showed opposite changes: during VFA infusion it underwent a significant ($P < 0.001$), almost fivefold increase as compared to the preinfusion value. After VFA administration blood plasma IRI concentration declined rapidly: by 1 h after VFA infusion it dropped to almost half of its peak concentration, then at 10 h after the infusion it came close to the preinfusion value and did not differ from that significantly ($P > 0.05$).

Discussion

In the experiments reported here, intravenous infusion of VFA produced considerable changes in the blood plasma concentration of all free amino acids. VFA administration rapidly decreased the blood plasma levels of all detected AA. With time after the infusion, the blood plasma concentration of all AA rose. No appreciable differences were found in the degree and type of changes between amino acids of different metabolic features, thus between essential and nonessential AA.

In agreement with our results, Theuer et al. (1966) and Purser et al. (1966) also reported a decrease in blood plasma FAA concentration 2–4 h after feed intake or intraruminal infusion of a glucose–starch mixture. After isocaloric intravenous infusion of different energy sources, glucose was found to produce the most expressed decrease in blood plasma FAA concentration, followed by propionate, acetate and, finally, butyrate (Potter et al., 1968). According to the results of Eskland et al. (1974), the blood plasma FAA concentration diminishing effect of intravenous energy infusion was independent of the quality of the energy source for several AA including lysine, arginine and methionine.

The drop of blood plasma FAA concentration observed after the infusion of compounds rich in energy, e.g. VFA in ruminants, may probably be explained by the fact that rapid energy supply considerably enhances the rate of protein synthesis in the tissues. The amino acid source of cellular protein synthesis is the free amino acid pool of the extracellular fluid and, within it, the blood plasma (Bergen, 1979).

The experiments described here were conducted on fasting lambs being in the postabsorptive phase in which there was probably no considerable amino acid absorption from the alimentary tract. Thus, a fresh supply to the blood plasma of AA used up for protein synthesis was not available. Consequently, enhanced protein synthesis resulted in a decrease of blood plasma FAA concentrations.

The question arises to what degree insulin participates in the regulation of the above processes. According to Garlic and Lobley (1987), accelerated protein synthesis stimulated by feed intake is mediated by insulin. At the same time, the results of Early et al. (1988) indicate that insulin is less important in regulating protein synthesis in the muscles in ruminants than in non-ruminant animals. In the experiments described in this paper, a close negative correlation was found between blood plasma insulin and FAA concentrations. The lowest FAA levels always coincided with the highest insulin concentration in the blood plasma. This relationship allows us to conclude that insulin also has a regulatory role in enhanced protein synthesis stimulated by VFA.

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BLOOD pH, PLASMA CAROTENE, VITAMIN A, ALBUMIN AND GLOBULIN CONCENTRATIONS IN NEWBORN CALVES AND IN DAMS DURING THE FIRST THREE DAYS OF THE POSTNATAL PERIOD

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Blood pH, total plasma carotene, plasma vitamin A fractions and some plasma proteins were determined in newborn calves (n = 38) and their dams. Calves were assigned to one of two groups according to their blood pH immediately after birth: group 1 (normal) with pH above 7.2 (n = 27) and group 2 (slightly acidotic) with pH 7.0 to 7.2 (n = 11). The difference between the neonatal blood pH values of the two groups immediately post partum disappeared 24 h after birth. The blood pH of the dams was normal. The extremely low plasma carotene concentration (group 1: 32.7 μg/l; group 2: 20.5 μg/l) and the low plasma globulin fractions (group 1: 19.6 g/l; group 2: 19.2 g/l) immediately after birth were elevated by the intake of colostrum. There was no significant change in plasma vitamin A fractions (retinol, retinyl ester) during the early postnatal period (3 days post partum) in newborn calves. The acid-base balance of newborn calves does not have any influence on the physiological alterations of plasma vitamin A, carotene and some plasma proteins during the first three days of life.

Key words: Cow, calf, blood pH, plasma vitamin A, plasma carotene, plasma proteins

The early days in the neonatal life of calves are crucial with regard to their subsequent rearing success and performance. The adverse effects of perinatal acidosis beyond the physiological level may produce a considerable economic loss (Eigenmann et al., 1981; Held et al., 1985; Szenci, 1985). The optimal supply of vitamin A and carotene is important for maternal reproductive performance as well as for somatic and immunological development of calves (Ahlswede and Lotthammer, 1978; Fadle, 1966).

The structural arrangements of proteins are mainly affected by the physicochemical characteristics of their surroundings such as ionic strength and pH (Nunnally and Craig, 1980). The fine structural alterations may induce changes even in their function (Aqvist et al., 1986). Since both vitamin A and β-carotene are converted into soluble matters in the blood with the help of transport protein complexes and/or lipoprotein micelles (e.g. retinol-binding protein (RBP), transthyretin (TTR) and chylomicrons; Muto, 1978; Goodman, 1984), the actual blood pH may influence the effects of these protein–lipoid complexes.

This study was designed to detect the variations of blood pH, plasma carotene, plasma vitamin A and some plasma proteins in neonatal calves and their dams during the first three days after birth.

Materials and methods

Holstein-Friesian (HF) and HF × Hungarian Fleckvieh cows (n = 38) and their calves were examined in a dairy herd. Dry cows were confined to stanchion barns and received a mixed corn silage and fodder ration along with grass hay or alfalfa. The delivery of the 38 calves, all in normal anterior presentation, was uncomplicated; one to three attendants assisted with traction, and delivery was completed within 30 to 60 min after the appearance and rupture of the membranes. The newborn calves were naturally suckled during the experiment.

Blood samples were withdrawn anaerobically by needle puncture from the jugular vein of dams and calves into heparinized syringes. The syringes were immediately sealed airtight with a rubber cap and stored on ice until analysed for pH, which was performed within 10 min after sampling. The first blood samples were taken immediately after delivery. Sampling was repeated 24 and 72 h after birth. Blood pH was determined by a Biological Microanalyser (Radelkis, Hungary) at 37 °C.

After birth, the newborn calves were assigned according to their blood pH values to one of two groups: group 1, with blood pH above 7.2 (normal) and group 2, with blood pH between 7.0 and 7.2 (slightly acidotic). A similar grouping had been used by others (Eigenmann, 1981; Szenci and Taverne, 1988).

The plasma was separated by centrifugation (1500 g, +4 °C) and stored at −20 °C until analysed.

Plasma total carotene was extracted from 1 ml of the sample with petroleum ether containing 4% ethyl ether. Quantitation of total carotene level was accomplished by relating the absorbance at 450 nm to a standard curve (Vahlquist, 1974). Due to the extremely low plasma level in newborn calves, a 5-fold plasma volume was extracted.

Plasma total vitamin A was determined photometrically by the trichloroacetic acid (TCA) method. Then one half of the ether phase was evaporated under vacuum. The residue was dissolved in chloroform. Addition of 7.34 mol/l aqueous TCA solution was followed by a colour reaction (Bárdos, 1988). The other half of the ether phase was poured into a test tube containing alumina. When the tube was shaken, alumina containing 8% water selectively adsorbed retinol, leaving the retinyl esters in the supernatant. After settling, the supernatant was subjected to the TCA reaction. The retinol concentration was calculated as the difference between the first and second TCA values.

Plasma total protein was measured using the biuret reaction (Weichselbaum, 1946), and plasma albumin by the bromocresol green colour reaction (Keay and Doxley, 1983).

Plasma total globulin was calculated as the difference between plasma total protein and albumin.

Statistical comparison of the variables between and within groups of calves and dams was done using Fisher's F test, Student's *t* test and the Pearson–Bravis correlation formula (Sokal and Rohlf, 1981).

Results

Changes in the blood pH of newborn calves and their dams during the experiment are shown in Table 1. The difference between the two groups of newborn calves seen immediately after birth disappeared 24 h after birth. The blood pH of the dams was normal throughout.

Values for plasma total carotene level and levels of vitamin A fractions of calves are given in Table 2. Except for neonatal plasma total carotene (24 h), there were no significant differences between groups 1 and 2 (calves and dams). The values for calves increased remarkably ($P < 0.001$) from birth to 24 h in group 1. This change was less marked in the acidotic calves (group 2). At birth, plasma carotene and vitamin A fractions were much lower ($P < 0.001$) for calves than for cows (Table 4).

Plasma proteins showed only non-significant difference between acidotic and normal calves (Table 3). Plasma total protein and globulin were significantly ($P < 0.001$) lower at birth than 24 h after birth. Immediately after birth the plasma protein levels were significantly lower ($P < 0.001$) for the

Table 1

Blood pH values (mean ± SD) of calves and dams

	Calves			Dems		
	Group 1 (n = 27)		Group 2 (n = 11)	Group 1 (n = 27)		Group 2 (n = 11)
pp	7.25±0.04	xx	7.11±0.11	7.40±0.07	NS	7.41±0.06
	xxx		xxx	NS		NS
24 h	7.38±0.04	NS	7.36±0.06	7.43±0.05	NS	7.43±0.06
	NS		NS	NS		NS
72 h	7.39±0.04	NS	7.38±0.04	7.41±0.05	NS	7.42±0.05

Symbols and abbreviations:
pp: immediately post partum
24 h: 24 hours after calving
72 h: 72 hours after calving
NS: not significant; x $P<0.05$; xx $P<0.01$; xxx $P<0.001$

Table 2

Total plasma carotene and plasma vitamin A fractions (mean ± SD)

	Calves			Dams		
	Group 1 (n = 27)		Group 2 (n = 11)	Group 1 (n = 27)		Group 2 (n = 11)
			Total carotene (μg/l)			
pp	32.7±16.3	NS	20.5±16.4	3374.9±1028.2	NS	3040.4±1037.9
	xxx		x	NS		NS
24 h	119.9±97.2	x	58.1±48.0	2988.8±762.8	NS	2812.9±796.6
	NS		x	NS		NS
72 h	105.0±91.2	NS	142.8±94.0	2783.3±802.6	NS	2416.6±796.1
			Total vitamin A (μg/l)			
pp	141.9±37.7	NS	139.8±39.2	283.1±74.9	NS	319.2±64.3
	xxx		xx	NS		xx
24 h	215.0±60.8	NS	213.2±49.2	257.5±50.9	NS	241.7±35.0
	NS		NS	NS		NS
72 h	194.8±50.8	NS	223.6±46.1	264.8±58.8	NS	240.2±38.9
			Retinol (μg/l)			
pp	115.3±42.0	NS	126.4±47.5	233.5±52.9	NS	265.2±58.6
	xxx		NS	NS		x
24 h	187.0±58.9	NS	177.5±37.3	221.1±33.7	NS	215.2±30.7
	x		NS	NS		NS
72 h	154.7±41.5	NS	164.1±46.5	218.0±50.4	NS	198.5±43.7

Symbols and abbreviations:
pp: immediately post partum
24 h: 24 hours after calving
72 h: 72 hours after calving
NS: not significant; x $P<0.05$; xx $P<0.01$; xxx $P<0.001$

calves than for their dams. The differences were still present for albumin 24 h after birth (Table 4).

Table 5 contains the correlations between the investigated parameters. Only $r \geq 0.5$ values are indicated in the matrix. Close correlations were found between the functionally related parameters (total vitamin A/retinol; total protein/globulin) in both groups of calves. Thus, close ($r \geq 0.7$) and very close ($r \geq 0.9$) correlations were noted 24 and 72 h after birth, between total vitamin A and retinol and between total protein and globulin, respectively.

Discussion

The difference in blood pH, seen in the calves immediately after birth, disappeared by 24 h after birth, due to the metabolic compensation of the acid–base balance (Bodenberger, 1979; Schlerka et al., 1979; Szenci et al., 1981).

Table 3

Plasma protein concentrations (mean ± SD)

	Calves			Dams		
	Group 1 (n = 27)		Group 2 (n = 11)	Group 1 (n = 27)		Group 2 (n = 11)
			Total protein (g/l)			
pp	44.30±9.35	NS	45.12±5.45	61.07±7.51	NS	64.93±6.51
	xxx		x	NS		NS
24 h	60.84±13.42	NS	58.51±16.13	59.82±7.53	NS	64.11±3.51
	NS		NS	NS		NS
72 h	60.52±15.58	NS	60.22±13.35	63.25±9.89	NS	59.58±10.13
			Albumin (g/l)			
pp	24.72±3.74	NS	25.86±3.39	31.39±2.92	NS	31.82±1.84
	NS		NS	NS		NS
24 h	24.78±2.63	NS	23.90±2.35	31.23±2.93	NS	31.46±2.17
	NS		NS	NS		NS
72 h	25.53±2.31	NS	25.67±2.64	31.39±2.94	NS	29.30±2.42
			Globulin (g/l)			
pp	19.60±9.93	NS	19.25±8.09	29.20±8.42	NS	33.17±7.51
	xxx		x	NS		NS
24 h	36.05±14.47	NS	34.58±17.70	28.61±9.35	NS	32.65±2.57
	NS		NS	NS		NS
72 h	35.27±16.48	NS	33.90±14.00	31.72±8.17	NS	31.06±8.75

Symbols and abbreviations:
pp: immediately post partum
24 h: 24 hours after calving
72 h: 72 hours after calving
NS: not significant; x $P<0.05$; xx $P<0.01$; xxx $P<0.001$

The blood pH values of dams are in good agreement with those considered physiological (Mülling et al., 1979; Held, 1983; Szenci, 1985).

In newborn calves plasma total carotene increased after birth. This suggests an intensive intestinal absorption of carotene (Tomlinson et al., 1974; Surynek et al., 1976), due to the highly intensive β-carotene secretion of udder in the first 48 h after delivery (Pethes et al., 1987). A similar change was found for vitamin A (total and retinol), as it was observed by others, too (Özpinar et al., 1988). The variations observed in retinol level are fairly stable both in cows and in their calves. This can probably be associated with the function of the retinol–RBP complex. This complex, released from the liver, provides a fairly constant blood retinol level. The plasma level of the other component of total vitamin A, the retinyl ester fraction, increases after intestinal absorption of vitamin A and/or carotene (Branstetter et al., 1973; Tomlinson et al., 1974; Surynek et al., 1976) and in hypervitaminosis (Goodman, 1984). This is confirm-

Table 4

Significant differences between calves and dams

		Total carotene		Total vitamin A		Retinol		Total protein		Albumin		Total globulin	
		C	D	C	D	C	D	C	D	C	D	C	D
Group 1 (n = 27)	pp	xxx		xxx		xxx		xxx		xxx		xxx	
	24 h	xxx		xx		x		NS		xxx		x	
	72 h	xxx		xxx		xxx		NS		xxx		NS	
Group 2 (n = 11)	pp	xxx		xxx		xxx		xxx		xxx		xxx	
	24 h	xxx		NS		x		NS		xxx		NS	
	72 h	xxx		NS		NS		NS		xx		NS	

Symbols and abbreviations:
C: calves
D: dams
pp: immediately post partum
24 h: 24 hours after calving
72 h: 72 hours after calving
NS: not significant; x $P<0.05$; xx $P<0.01$; xxx $P<0.001$

Table 5

Correlations ($r \geq .5$) between the investigated plasma parameters of calves

		BLOOD PLASMA							
		Retinol				Globulin			
		pp	24h	72h		pp	24h	72h	
BLOOD PLASMA	Total vitamin A	pp	+.83			Total protein	+.92			
		24 h		+.81				+.98	+.55	GROUP 1
		72 h			+.81			+.57	+.98	
		pp	+.82	+.56			+.94			
		24 h		+.95				+.99	+.66	GROUP 2
		72 h			+.67			+.69	+.96	

ed here by the small difference between total vitamin A and retinol plasma levels at birth and the gradual increase of the retinyl ester fraction after the commencement of suckling (Table 2), consistently with previous reports (Bouda et al., 1980; Vlcek et al., 1980).

Plasma albumin level showed significant difference between calves and dams at every sampling time in both groups. After the first day of life, new-

born calves in both groups recovered from a relative hypoproteinaemia. Their values approximated to the adults as described by Tennant et al. (1969). As neonatal plasma albumin remained near to the birth time value (approximately 25 g/l), the increase of total protein could be attributed to the increase in the globulin fraction (Table 3). In the early days of life, globulin appears in neonatal blood mainly due to colostral absorption providing the so-called maternal immunity. The highest total daily output of immunoglobulin G (IgG) by the dam's mammary gland was found in the first 24 h after calving (Pethes et al., 1987).

According to the correlation matrix (Table 5), the elevation of retinol and globulin resulted in the increase of total vitamin A and total protein levels (Tables 2 and 3).

Although neonatal acid–base changes greatly influence the rate of perinatal mortality (Eigenmann et al., 1981; Held et al., 1985; Szenci, 1985; Szenci and Taverne, 1988), they exert no effect on the specific changes of plasma vitamin A, plasma total carotene (with the exception of significantly lower ($P < < 0.05$) total carotene level in group 2 at 24 h after birth) and certain plasma proteins during the first three days of life, which are similarly very important factors in postnatal life.

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LIPID COMPOSITION OF PULMONARY SURFACTANT IN BREEDING FOXES WITH CARDIOPULMONARY INSUFFICIENCY

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The lipid composition of the pulmonary surfactant of breeding foxes was examined. The surfactant isolated from healthy polar and common foxes did not show significant strain differences in lipid composition. On the other hand, in the surfactant isolated from the lung of common foxes that died with the symptoms of cardiopulmonary insufficiency the quantity of phosphatidylcholine and phosphatidylglycerol was lower and that of sphingomyelin and lysophosphoglycerides was higher than in the surfactants prepared from healthy animals. Moreover, in the phosphatidylcholine and phosphatidylglycerol fractions of the surfactant isolated from the diseased animals the level of palmitic acid was significantly lower.

Key words: Fox, respiratory insufficiency, lung surfactant, lipids

Pulmonary surfactant is a phospholipid-rich material lining the lung alveoli of vertebrates. Due to its surface tension-reducing properties, it prevents sticking of alveoli together during expiration (Goerke, 1974). Primary deficiency of the surfactant, caused by the immaturity of the type II pneumocytes, is the cause of the so-called infant respiratory distress syndrome (Hallman et al., 1983). Perturbations in its lipid composition cause adult respiratory distress syndrome (Simmons et al., 1979).

Studies on the pulmonary surfactant in animals have been mostly concerned with its physiological aspects (Keough et al., 1985; King and Clements, 1972; Yu et al., 1983). Little attention has been paid so far to the disturbances in the lipid composition of surfactant in diseased animals.

In the years 1984–1988, young common foxes (aged mostly from 3 to 8 months) in Poland experienced a disease with the symptoms of pulmonary oedema, rapidly increasing dyspnoea, disorders in the circulatory system, and sudden death. The aetiology pathogenesis of the disease, which initially was called "cardiopulmonary insufficiency syndrome", have not been understood so far.

The aim of the present study was to isolate the lung surfactant from breeding foxes, and to investigate its lipid composition in animals that died with the symptoms of cardiopulmonary insufficiency.

Materials and methods

Animals

The lungs of 23 common foxes that died with the symptoms of cardiopulmonary insufficiency were examined. The control group consisted of the lungs of 12 healthy common foxes and 14 polar foxes obtained during the planned slaughter of animals. The time interval from the death of the animals to starting the isolation of the surfactant did no exceed 2 h.

Isolation of surfactant from bronchoalveolar lavage

The surfactant was isolated by the modified method of Frosolono et al. (1970). Lungs were weighed and, after cannulation of the trachea, washed 7 times with 50 ml of 0.154 mol/l NaCl every time. The bronchoalveolar lavage (BAL) thus obtained was centrifuged for 30 min at 1000 *g*. The supernatant was discarded and the residue suspended in 0.01 M Tris-HCl buffer of pH 7.4, containing 0.154 mol/l NaCl and 1 mmol/l EDTA. The residue, after thorough mixing, was spread on a non-linear gradient of 0.68 mol/l and 0.25 mol/l sucrose prepared in the same buffer and centrifuged at 65,000 *g* for 60 min. The interphase formed at the border of sucrose layers was transferred to another tube with a Pasteur pipette, diluted with Tris-HCl buffer and centrifuged again at 1000 *g* for 30 min. The dilution of the primary suspension caused the formation of a deposit on the bottom of the tube (Oulton et al., 1986). The supernatant was discarded again and the residue, after being suspended in 0.01 mol/l Tris-HCl buffer, was spread on a non-linear sucrose gradient and centrifuged as described above. The interphase between the two sucrose layers was collected and centrifuged at 1000 *g* for 30 min. The pellet was washed three times with 0.01 mol/l Tris-HCl buffer and suspended in 0.154 mol/l NaCl for lipid extraction.

Isolation of surfactant from post-lavaged lung tissue

The washed lungs were weighed to determine the quantity of liquid trapped in the tissue. Then 0.01 mol/l Tris-HCl buffer was added to obtain a 10% w/v homogenate. The homogenate was centrifuged at 1000 *g* for 30 min and the supernatant was rejected. The residue was suspended in 0.01 mol/l Tris-HCl buffer and it was spread on a non-linear gradient of sucrose. The further purification of the surfactant was the same as in the case of the bronchoalveolar lavage.

Determination of surfactant purity

To determine the degree of contamination in both types of surfactant, the activity of marker enzymes was assayed.

Succinate dehydrogenase activity was assayed as described by Pennington (1961), NADPH: cytochrome c reductase activity was estimated by Omura and Takesue's method (1971), and 5'-nucleotidase activity was estimated as described by Avruch and Wallach (1971).

Pulmonary tissue mitochondria and microsomes were obtained by differential centrifugation according to Harding et al. (1983). Protein content was estimated according to Lowry et al. (1951) with bovine serum albumin as standard.

Extraction of lipids

Lipids were extracted according to Folch et al. (1957). Crude lipid material was separated on silica gel G plates (Merck, Darmstadt, Germany) in the solvent system hexane–diethyl ether–acetic acid 90 : 10 : 1 v/v/v. Phospholipids were separated by two-dimensional thin-layer chromatography (Gray, 1967). Chromatograms were visualized in iodine vapours. The spots of individual lipid classes were scraped off the plate and extracted with 5 ml of a chloroform–methanol 2 : 1 v/v mixture. The extracts were evaporated to dryness in vacuum, and phosphorus was determined according to Bartlett (1959). The composition and quantity of fatty acids in phosphatidylcholine

Table 1

Distribution of marker enzyme activities (nmole of substrate $\times$ min^{-1} $\times$ mg $protein^{-1}$) in subcellular fractions of fox lung (Mean value $\pm$ S. D. BAL = bronchoalveolar lavage; PLLT = post-lavaged lung tissue. Student's t test for unpaired data: a: $P<0.01$ II vs. I; b: $P<0.01$ IV vs. III)

	Succinate dehydrogenase		NADPH: cyt.c reductase		5'-nucleotidase	
	Silver fox	Blue fox	Silver fox	Blue fox	Silver fox	Blue fox
Healthy						
Mitochondria	16.7±1.1	17.2±1.2				
Plasma membranes					88.7±2.6	89.6±2.7
Microsomes			31.2±1.4	32.6±1.5		
BAL surfactant (I)	0.2±0.1	0.2±0.1	0.6±0.1	0.7±0.1	2.7±0.3	2.8±0.3
PLLT surfactant (II)	0.4±0.1[a]	0.3±0.1	1.1±0.2[a]	1.2±0.2[a]	4.4±0.4[a]	4.6±0.5[a]
Respiratory insufficiency						
Mitochondria	17.0±1.0					
Plasma membranes					88.2±2.5	
Microsomes			30.8±1.5			
BAL surfactant (III)	0.2±0.1		0.5±0.1		2.6±0.3	
PLLT surfactant (IV)	0.5±0.1[b]		1.0±0.2[b]		4.3±0.4[b]	

and phosphatidylglycerol fractions were analysed by the gas-liquid chromatography method in Perkin Elmer F 30 gas chromatograph as described elsewhere (Michalak et al., 1988).

Statistical significance of differences was analysed by Student's *t* test for unpaired data.

Table 2

Phospholipid composition of surfactant isolated from bronchoalveolar lavage (BAL) and post- values ± S.D. Abbreviations: PE = phosphatidylethanolamine; PS = phosphatidylserine; = sphingomyelin; LpC = lysophosphatidylcholine; LpE = lysophosphatidylethanolamine.

	PE	PS	PI	PC
BAL surfactant from healthy foxes				
Silver (I)	4.1±0.2	2.9±0.2	4.3±0.3	76.3±1.6
Blue (II)	4.0±0.2	3.0±0.1	4.4±0.3	76.2±1.8
BAL surfactant from silver foxes with respiratory insufficiency (III)	4.3±0.2[a]	4.2±0.2[a]	6.5±0.4[a]	67.0±2.0[a]
PLLT surfactant from healthy foxes				
Silver (IV)	4.5±0.3	1.9±0.2	7.0±0.6	80.1±1.3
Blue (V)	4.6±0.2	1.8±0.2	7.1±0.7	79.5±1.4
PLLT surfactant from silver foxes with respiratory insufficiency (VI)	4.4±0.2	1.9±0.2	9.2±1.0[b]	71.7±2.0[b]

Results

After the first centrifugation of bronchoalveolar lavage and post-lavaged lung tissue surfactant on the non-linear gradient of sucrose, a phospholipid-rich material was obtained in the sucrose interphase. The second centrifugation of surfactant, using identical conditions, caused an additional loss of about 15% of phospholipids. After further centrifugations no phospholipid loss was found. Therefore, this material was considered the final surfactant preparation, which was contaminated by mitochondria (1–3%), microsomes (2–4%) and plasma membranes (3–6%) (Table 1). It should be noted that the surfactant isolated from pulmonary tissue homogenate was contaminated to a greater extent with subcellular fractions than that isolated from bronchoalveolar lavage.

The phospholipid composition of pulmonary surfactant of healthy polar and common foxes did not show considerable strain differences. On the other hand, in the surfactant isolated from the lungs of common foxes that died with the symptoms of cardiopulmonary insufficiency, the phosphatidylcholine and phosphatidylglycerol fractions were substantially lower, whereas the sphingomyelin and lysophosphoglyceride fractions were higher than in the surfactant prepared from healthy foxes (Table 2). The fatty acid composition of phosphatidylcholine fraction of both types of surfactant is illustrated in Table 3. The major acids of this fraction were palmitic and oleic, and then

linolenic and arachidonic acids. The fatty acid composition of this fraction was similar in both types of surfactant, both within strain and between the strains of healthy animals under study. In the phosphatidylcholine fraction isolated from the lungs of diseased animals, distinct decreases in the quantity of palmitic acid and of other saturated acids were observed.

lavaged lung tissue (PLLT) of silver and blue foxes (% of total phospholipid phosphorus) (Mean PI = phosphatidylinositol; PC = phosphatidylcholine; PG = phosphatidylglycerol; Sph = Student's *t* test for unpaired data: a: <0.01 III vs, I; b: <0.01 VI vs. IV)

	PG	Sph	LpC	LpE
BAL surfactant from healthy foxes				
Silver (I)	7.4±0.3	0.4±0.1	2.1±0.2	2.5±0.2
Blue (II)	7.2±0.3	0.3±0.1	2.2±0.2	2.7±0.3
BAL surfactant from silver foxes with respiratory insufficiency (III)	3.6±0.2[a]	6.2±0.3[a]	4.9±0.5[a]	3.3±0.4[a]
PLLT surfactant from healthy foxes				
Silver (IV)	4.4±0.6	1.2±0.2	0.4±0.1	0.5±0.1
Blue (V)	4.2±0.7	1.3±0.2	0.6±0.2	0.9±0.4
PLLT surfactant from silver foxes with respiratory insufficiency (VI)	1.9±0.3[b]	8.7±0.7[b]	1.3±0.2[b]	0.9±0.2[b]

Table 3

Fatty acid composition of phosphatidylcholine fraction of surfactant isolated from bronchoalveolar lavage (BAL) and post-lavaged lung tissue (PLLT) of foxes (mole %) (Mean values ± S.D. Student's *t* test for unpaired data: a: P<0.01 II vs. I; b: P<0.01 III vs. II; c: P<0.01 V vs. IV; d: P<0.01 VI vs. IV)

Fatty acid	BAL surfactant			PLLT surfactant		
	Silver		Blue (III)	Silver		Blue (VI)
	Healthy (I)	Diseased (II)		Healthy (IV)	Diseased (V)	
12 : 0	2.8±0.3	2.6±0.3	2.6±0.3	2.5±0.2	2.1±0.2[c]	2.6±0.2
14 : 0	0.6±0.1	0.5±0.1	0.5±0.1	0.6±0.1	0.5±0.1	0.5±0.1
16 : 0	66.4±1.2	51.2±1.3[a]	65.6±1.4	64.4±1.3	52.8±1.2[c]	65.7±1.4
16 : 1	4.2±0.3	5.6±0.4[a]	4.0±0.4	4.3±0.3	4.8±0.4	4.1±0.3
18 : 0	2.0±0.2	1.4±0.2[a]	2.1±0.2	2.2±0.2	2.0±0.2	2.3±0.2
18 : 1	9.4±0.6	10.8±0.8	9.2±0.6	10.6±0.7	10.6±0.7	9.6±0.6
18 : 2	5.4±0.4	6.2±0.5	5.0±0.5	4.2±0.3	5.9±0.4[c]	5.4±0.4[d]
18 : 3	0.4±0.1	0.4±0.1	0.5±0.1	0.5±0.1	0.4±0.1	0.6±0.1
20 : 0	1.6±0.2	2.1±0.2[a]	1.5±0.2	2.2±0.2	2.4±0.2	2.3±0.2
20 : 3	0.7±0.1	0.9±0.1[a]	0.6±0.1	0.6±0.1	0.9±0.1[c]	0.7±0.1
20 : 4	2.6±0.2	3.1±0.3[a]	2.8±0.2	2.7±0.2	3.5±0.4[c]	2.5±0.2
Others*	3.9±0.4	15.2±1.2[a]	5.6±0.4[b]	5.2±0.4	14.1±1.2[c]	3.7±0.3[d]

* Others include: 20 : 1, 20 : 2, 20 : 3, 20 : 5, 22 : 0, 22 : 1, 22 : 4, 22 : 5, 22 : 6, 24 : 0

The phosphatidylglycerol fraction of both types of surfactant isolated from the lungs of healthy foxes had similar fatty acid composition, but it was poorer in palmitic and stearic acids than in the phosphatidylcholine fraction. The quantitative changes of phosphatidylglycerol fatty acids isolated from the lungs of dead common foxes were less intense than the changes found in the phosphatidylcholine fraction (Table 4).

Table 4

Fatty acid composition of phosphatidylglycerol fraction of surfactant isolated from bronchoalveolar lavage (BAL) and post-lavaged lung tissue (PLLT) of foxes (mole %) (Mean values ± S.D. Student's *t* test for unpaired data: a: $P<0.01$ II vs. I; b: $P<0.01$ V vs. IV; c: $P<0.01$ VI vs. V)

Fatty acid	BAL surfactant			PLLT surfactant		
	Silver		Blue (III)	Silver		Blue (VI)
	Healthy (I)	Diseased (II)		Healthy (IV)	Diseased (V)	
12 : 0	0.6±0.1	0.8±0.1[a]	0.5±0.1	0.5±0.1	0.6±0.1	0.6±0.1
14 : 0	0.4±0.1	0.5±0.1	0.5±0.1	0.5±0.1	0.7±0.1[b]	0.5±0.1
16 : 0	51.6±2.2	48.3±2.0[a]	52.4±2.1	49.2±2.3	47.3±2.1	49.8±2.2
16 : 1	6.2±0.4	6.4±0.4	6.4±0.5	6.1±0.4	6.2±0.5	6.0±0.5
18 : 0	7.1±0.5	7.0±0.5	6.7±0.5	6.8±0.5	7.1±0.6	6.6±0.5
18 : 1	24.1±1.1	24.9±1.0	23.5±1.2	21.5±1.3	20.3±1.4	22.6±1.2
18 : 2	4.5±0.4	4.3±0.3	4.6±0.4	4.2±0.4	4.4±0.4	4.1±0.4
18 : 3	0.5±0.1	0.6±0.1	0.6±0.1	0.5±0.1	0.5±0.1	0.6±0.1
20 : 0	0.8±0.1	0.9±0.1	0.7±0.1	0.8±0.1	0.7±0.1	0.7±0.1
20 : 3	1.4±0.2	1.5±0.2	1.2±0.2	1.0±0.2	1.2±0.2	1.1±0.2
20 : 4	1.6±0.2	1.8±0.2	1.5±0.2	1.4±0.2	1.5±0.2	1.3±0.2
Others*	1.2±0.2	3.0±0.4[a]	1.4±0.2	7.5±0.6	9.5±0.7[b]	6.1±0.5[c]

*Others include: 20 : 1, 20 : 2, 20 : 3, 20 : 5, 22 : 0, 22 : 1, 22 : 4, 22 : 5, 22 : 6, 24 : 0

Discussion

Density gradient centrifugation methods for pulmonary surfactant, both from bronchoalveolar lavage and from post-lavaged lung tissue, have been described by several authors (Peleger and Thomas, 1971; Yu et al., 1983; Shelley et al., 1982; Pawlowski et al., 1971). Katyal et al. (1977) and Oulton et al. (1986) detected two surfactant pools in the lung: an extracellular pool which can be washed out through the respiratory tract, and an intracellular one, stored in the form of lamellar bodies in type II pneumocytes. Our research proved the existence of both types of pulmonary surfactant in breeding foxes as well, and we described a method for their isolation.

It was found that the surfactant isolated from bronchoalveolar lavage is contaminated with subcellular fractions to a lesser degree than that isolated from post-lavaged lung tissue. These data confirm the results obtained by others (Katyal et al., 1977; Oulton et al., 1986). According to Hubert et al. (1966) and Reidbord (1967), the smaller extent of extracellular surfactant contamination is the result of undisturbed integrity of bronchial and alveolar cells during washing them with saline. On the basis of the results of the authors cited and of our own work, one can assume that the surfactant isolated from the bronchoalveolar lavage constitutes a better material for studies as (*i*) it is easy to obtain, (*ii*) it is less contaminated with subcellular fractions, and (*iii*) the method with it can be applied intravitally.

While the pulmonary surfactant was purified in a two-step sucrose gradient, the loss of the phospholipid material from the pool obtained primarily did not exceed 15%. Similar results have been obtained by Frosolono et al. (1970), Katyal et al. (1977) and Oulton et al. (1986) with surfactants of dog, rat and rabbit origin, respectively.

The lipid composition of pulmonary surfactant in different mammalian species is similar. Phosphatidylcholine and phosphatidylglycerol constitutes 80–87% and 8–11% of the total phospholipid fraction, respectively. Our own studies have proved that phosphatidylcholine constitutes 76% of the total surfactant phospholipids isolated from bronchoalveolar lavage, and 80% in the case of surfactant from post-lavaged lung tissue. On the other hand, palmitic acid constitutes ca. 65% of phosphatidylcholine fatty acids, and ca. 52% of fatty acids in the phosphatidylglycerol fraction. The data thus obtained indicate that the lipid composition of pulmonary surfactant of breeding foxes is similar to that of other mammalian species.

The surfactant isolated from the lungs of healthy common and polar foxes did not reveal substantial species differences in lipid composition. On the other hand, it differs from the surfactant isolated from the lungs of common foxes that died with the symptoms of cardiopulmonary failure. The differences consisted of a substantial decrease in the phosphatidylcholine and phosphatidylglycerol levels, by 13% and 53%, respectively, and of an increase in phosphatidylserine, sphingomyelin and lysophosphatidylcholine by 41%, 1425% and 128%, respectively.

For lack of studies concerned with this subject, the results of our study are difficult to compare with other data. Similar changes have been described by Hallman et al. (1982) in human patients with pneumonia. They included decreased phosphatidylcholine and phosphatidylglycerol levels and increased phosphatidylethanolamine, phosphatidylserine and lysophosphatidylcholine contents.

Our own study has shown substantial changes in the lipid composition of pulmonary surfactant of common foxes that died with the symptoms of

cardiopulmonary failure. The results of our work show that independently of the primary cause (bacteriological, virological, mycological and parasitological studies gave negative results), the changes in the lipid composition of the surfactant constituted an additional cause of the symptoms' intensification in diseased animals. Recent studies (Catanzaro et al., 1988; Wilsher et al., 1988) concerned with the suppressive action of certain lipid fractions of pulmonary surfactant upon immunity, should also be stressed. The authors cited above stated that high concentrations of certain lipid fractions of pulmonary surfactant caused retardation of lymphocyte proliferation and reduced the cytotoxicity of NK cells.

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EFFECT OF SOME ELECTROLYTES ON *IN VITRO* RUMEN MICROBIAL PROTEIN SYNTHESIS IN BUFFALOES

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In vitro studies were carried out to examine the effect of $MnCl_2$, $MgCl_2$, $CoCl_2$ and $CaCl_2$ on protein synthesis by rumen microorganisms obtained from fistulated buffalo calves (*Bubalus bubalis*). The concentration of the electrolytes in strained rumen fluid (SRF) ranged from 1 to 20, 1 to 20, 0.25 to 5 and 1 to 20 mM, respectively. $MnCl_2$ (15 mM), $MgCl_2$ (10 mM), $CoCl_2$ (2.5 mM) and $CaCl_2$ (20 mM) increased the protein content in the incubation mixture (IM) by 501, 230.8, 537.6 and 84% and the per cent incorporation of ^{35}S from $(NH_4)_2\,^{35}SO_4$ into microbial protein by 125.6, 108.5, 113.4 and 40.3, respectively, over the control values, when the incubation lasted 8 h. The NH_3-N content in IM decreased by 8, 14, 43 and 16% when 10 mM $MnCl_2$, 20 mM $MgCl_2$, 1 mM $CoCl_2$ and 1 mM $CaCl_2$, respectively, was added and the incubation lasted 6 h. The reasons for increased protein biosynthesis by rumen microorganisms in the presence of the above electrolytes are discussed.

Key words: Protein synthesis, rumen microbes, ruminants, buffaloes, electrolyte

In ruminants, most of the dietary proteins and other nitrogenous compounds are degraded by microorganisms in the reticulo-rumen with ammonia as the main end product. Ammonia constitutes the major source of nitrogen for microbial protein synthesis (Pilgrim et al., 1970; Salter et al., 1979). Microbial protein of rumen origin meets most of the amino acid requirement of the host animal. It may be, therefore, desirable to enhance microbial protein synthesis in the rumen. Our earlier studies (Saini and Singh, 1987, 1988a, 1988b) demonstrated that certain electrolytes, like $MnCl_2$, $MgCl_2$, $CoCl_2$ and $CaCl_2$, enhanced the activity of various ammonia-utilizing enzymes from mixed rumen bacterial flora of buffaloes and cattle. It is possible that an increased activity of these enzymes may result in an elevated microbial protein synthesis in the rumen. This study deals with *in vitro* effects of the above electrolytes on rumen microbial protein synthesis in buffaloes.

Materials and methods

Two rumen-fistulated buffalo calves (*Bubalus bubalis*) of 200 kg average body mass were fed a growth ration of 3 kg wheat straw, 2 kg green lucerne,

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0.6 kg mustard cake and 0.4 kg crushed maize grains per day. The animals were fed this diet for 3 months.

The animals were fasted for 12 h and samples of rumen fluid were taken before feeding in the morning by suction through a perforated plastic tube from various parts of the rumen. An equal volume of samples from both animals was pooled and the pool was strained through four layers of cheese-cloth into a prewarmed thermos (39 °C).

Salts ($MgCl_2$ 0.01–0.20 mmole, $MnCl_2$ 0.01–0.20 mmole, $CoCl_2$ 0.0025–0.05 mmole or $CaCl_2$ 0.01–0.20 mmole; starch, 160 mg; urea, 17.4 mg; and an amino acid mixture (18 amino acids mixed on an equal molar basis), 17 mg) were dispersed in 10 ml buffer (pH 6.8). The suspension was saturated with nitrogen gas (Sharma et al., 1981) in conical flasks fitted with stopper and Bunsen valves (Johnson, 1966). Thirty μCi $(NH_4)_2\ ^{35}SO_4$ was added to each flask for determining microbial protein synthesis. The flasks were incubated at 39 °C in an atmosphere of CO_2 for 20 min. SRF (10 ml, saturated with CO_2) was then added to each incubate and the contents were gassed with CO_2 again; they were stoppered and incubated as above for different periods in a temperature-controlled water bath with a shaking platform. Zero-hour and incubated controls were run simultaneously in each experiment. All the incubations were done in duplicate. At the end of the incubation period, fermentation was stopped in each flask by adding 1 ml saturated mercuric chloride solution. Since the normal buffer used for incubation contained $MgSO_4$ and $CaCl_2$, these salts were omitted from the buffer used for $MgCl_2$ and $CaCl_2$ incubations.

Proteins were precipitated from the incubation mixture (IM) with ethanol (McLaren et al., 1976), dissolved in 0.1 N NaOH, cell debris was removed by centrifugation, and in the supernatant the radioactivity was measured by Liquid Scintillation Spectrometer using scintillation fluid as suggested by Bray (1960). The protein content was estimated by Lowry's method (Lowry et al., 1951). IM was also analysed for ammonia-nitrogen (Conway, 1950).

Results

In the present investigation, the extent of ^{35}S-incorporation from $(NH_4)_2\ ^{35}SO_4$ into protein is taken as an index of protein synthesis by rumen microorganisms. The protein content and the per cent ^{35}S incorporation into microbial proteins proved to increase when $MnCl_2$, $MgCl_2$, $CoCl_2$ and $CaCl_2$ concentrations were increased up to 0.15, 0.10, 0.025 and 0.20 mmole/10 ml SRF, respectively, and the incubation was done for 8 h (Table 1). At these optimum concentrations of $MnCl_2$, $MgCl_2$, $CoCl_2$ and $CaCl_2$ the protein content increased by 501, 230.8, 537.6 and 84% and per cent incorporation of ^{35}S into microbial protein increased by 125.6, 108.5, 113.4 and 40.3, respectively, over the res-

Table 1

Effect of different electrolytes on the synthesis of protein by rumen microorganisms from buffalo calves (n = 2)

Electrolyte (mmole/10 ml RF) added	Protein content (mg/10 ml RF)	^{35}S incorporation into protein (%)
$MnCl_2$		
0.00	3.68	20.7
0.01	3.38	20.9
		(0.9)
0.05	9.83	28.7
	(167)	(38.6)
0.10	18.68	42.5
	(407)	(105.3)
0.15	22.13	46.7
	(501)	(125.6)
0.20	16.43	38.5
	(346)	(86.0)
$MgCl_2$		
0.00	3.14	20.0
0.01	2.59	22.4
		(12.0)
0.05	7.23	34.8
	(130.4)	(74.0)
0.10	10.38	41.7
	(230.8)	(108.5)
0.15	10.23	41.7
	(226)	(108.5)
0.20	6.96	35.3
	(121.7)	(76.5)
$CoCl_2$		
0.00	4.65	21.6
0.0025	8.85	26.0
	(90.3)	(20.4)
0.005	14.85	28.0
	(219.3)	(25.6)
0.01	20.10	33.0
	(332.2)	(52.8)
0.025	29.65	46.1
	(537.6)	(113.4)
0.050	29.65	43.9
	(537.6)	(103.2)
$CaCl_2$		
0.00	3.41	21.6
0.05	3.86	28.10
	(14)	(30.1)
0.15	5.51	29.0
	(62)	(34.3)
0.20	6.26	30.3
	(84)	(40.3)

Incubation for 8 h. The figures represent net protein formation after subtracting zero-hour control (42.75, 37.7, 52.3 and 51.9 mg for $MnCl_2$, $MgCl_2$, $CoCl_2$ and $CaCl_2$, respectively). Figures in parentheses represent per cent increase over the corresponding control value.

Table 2

Effect of different electrolytes on the content of ammonia (mg NH_3-N/100 ml rumen fluid) in the incubation mixture (n = 2)

Electrolyte (mmole/10 ml RF) added	Incubation time (h)		
	2	4	6
$MnCl_2$			
0.00	45.5	60.2	55.3
0.01	37.8	53.2	55.3
	(—16.9)	(—11.7)	(0.0)
0.05	35.6	53.2	53.2
	(—21.8)	(—11.7)	(—3.8)
0.10	30.1	56.0	51.1
	(—33.8)	(—7.0)	(—7.6)
0.15	38.2	56.0	57.4
	(—16.0)	(—7.0)	(+3.8)
0.20	37.8	58.8	59.5
	(—17.0)	(—2.3)	(+7.6)
$MgCl_2$			
0.00	52.5	69.3	66.5
0.01	55.3	75.2	66.5
	(+5.3)	(+8.5)	(0.0)
0.05	52.4	69.6	63.7
	(—0.2)	(+0.4)	(—4.2)
0.10	41.3	63.7	60.2
	(—21.3)	(—8.1)	(—9.5)
0.15	49.1	65.4	60.2
	(—6.4)	(—5.7)	(—9.5)
0.20	52.5	63.7	57.0
	(0.0)	(—8.1)	(—14.3)
$CoCl_2$			
0.00	51.8	69.0	65.6
0.0025	65.3	68.6	57.4
	(+26.0)	(—0.6)	(—12.8)
0.005	64.4	65.2	60.2
	(+24.3)	(—5.4)	(—8.5)
0.01	60.5	47.9	37.8
	(+16.9)	(—30.6)	(—42.5)
0.025	54.6	68.6	67.2
	(+5.4)	(—0.6)	(+2.1)
0.050	58.8	70.0	68.6
	(+13.5)	(+1.4)	(+4.2)
$CaCl_2$			
0.00	53.2	67.2	63.0
0.01	60.2	63.7	52.6
	(+13.2)	(—5.2)	(—16.4)
0.05	63.0	68.6	61.6
	(+18.5)	(+2.1)	(—2.2)
0.10	62.3	68.6	61.6
	(+17.1)	(+2.1)	(—2.2)
0.15	56.0	65.8	64.4
	(+5.3)	(—2.1)	(+2.2)
0.20	58.8	65.8	64.4
	(+10.5)	(—2.1)	(+2.2)

The figures represent net ammonia nitrogen content after subtracting zero-hour control (11.2, 13.3, 7.0, 9.8 mg NH_3-N/100 ml rumen fluid for $MnCl_2$, $MgCl_2$, $CoCl_2$ and $CaCl_2$, respectively. Figures in parentheses represent per cent increase (+) or decrease (—) compared to the control value.

pective control values. An addition of further $MnCl_2$ and $MgCl_2$ (up to 0.20 mmole/10 ml SRF) decreased microbial protein synthesis, though the values remained much higher than the control values.

NH_3-N content at a particular time of incubation may depend upon the rate of NH_3-N production and utilization by rumen microorganisms. The effect of different concentrations of $MnCl_2$, $MgCl_2$, $CoCl_2$ and $CaCl_2$ on the content of NH_3-N in the IM incubated for 2, 4 and 6 h was, therefore, also investigated (Table 2). $MnCl_2$ and $MgCl_2$ decreased the content of NH_3-N at almost all the incubation periods and at all the concentrations used. $MnCl_2$ and $MgCl_2$ decreased the content of NH_3-N maximally (by 33.8 and 21.3%) at 0.1 mmole/10 ml SRF concentration, over the control values, when incubation lasted 2 h. $CoCl_2$ and $CaCl_2$ increased NH_3-N content in IM at all the concentrations used, as compared to control values obtained after 2-h incubation. Maximum increase occurred at 0.0025 and 0.05 mmole/10 ml SRF concentration of $CoCl_2$ and $CaCl_2$ respectively. However, NH_3-N content was decreased in most of the cases when the period of incubation was prolonged. Maximum reduction (over the control values) occurred in the presence of 0.01 mmole/10 of $CoCl_2$ when incubation was done for 4 h (30.6%) or 6 h (42.5%). The corresponding figures for per cent decrease in the NH_3-N content in the presence of 0.01 mmole/10 ml SRF of $CaCl_2$ were 5.2 and 16.4.

Discussion

It was reported earlier (Saini and Singh, 1987, 1988a, 1988b) that the main ammonia-utilizing enzymes in the rumen bacteria from buffaloes and cattle were NADH-dependent glutamate dehydrogenase (GDH), glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), glutamine synthetase (GS) and asparagine synthetase (AS). It has also been observed by the same authors that $MgCl_2$ (10 mM) enhanced the activities of NADH-dependent GDH, GOT and GPT by 20, 88 and 62%, respectively; $MnCl_2$ (4 mM for GOT and GPT and 10 mM for GS and AS) increased the activities of GOT, GPT, GS and AS by 88, 360, 16 and 30%, respectively; $CoCl_2$ (1 mM) increased the activity of GS by 117%; $CaCl_2$ (10 mM) increased the activities of GPT and AS by 38 and 25%, respectively. It is possible that the substantial increase in rumen microbial protein synthesis in the presence of $MnCl_2$, $MgCl_2$, $CoCl_2$ and $CaCl_2$ (Table 1) in this study may be due to enhanced activities of the above-mentioned ammonia-utilizing enzymes leading to an increased utilization of NH_3-N for microbial protein synthesis. This view is supported by the decrease in NH_3-N content in IM in the presence of these electrolytes (Table 2). However, the possibility of more energy production and more DNA

and RNA synthesis in the presence of these electrolytes and a consequent enhanced proliferation of rumen microorganisms and increased rumen microbial protein synthesis can neither be ruled out.

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IMMUNIZATION OF DAY-OLD CHICKS HAVING MATERNALLY DERIVED ANTIBODIES AGAINST INFECTIOUS BRONCHITIS: DEGREE OF PROTECTION AS MONITORED BY CILIARY ACTIVITY AFTER INTRATRACHEAL CHALLENGE

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One-day-old chicks with maternally derived antibodies were vaccinated against infectious bronchitis (IB) with 3000 EID_{50} of the IB vaccine virus designated H120. The degree of protection induced by intranasal–eye drop (IE) vaccination was compared to that achieved by spray (S) vaccination.

The protection afforded by vaccination was monitored by intratracheal challenge with IBV strain M-41 (clinical signs, ciliary activity in tracheal explants, virus isolation) and by serological tests (ovoneutralization, microneutralization in cell culture, haemagglutination inhibition (HI) test, ELISA).

Intranasal–eye drop vaccination provided protection against intratracheal challenge. Immunity developed around 31 days of age. Spray vaccination failed to give protection against challenge by the same route.

No difference was demonstrable in effectiveness between the two routes of vaccination by serological tests. No elevation of the antibody level occurred in either group. The level of maternally derived antibodies declined with age.

Key words: Infectious bronchitis, immunization, day-old chicks, maternal antibodies, protection, intratracheal challenge, ciliary activity, tracheal explant

There are dissenting views in the literature about the protective value of maternally derived immunity against infectious bronchitis (IB) of chickens. Specific antibodies are demonstrable by serological tests up to the age of 28–35 days (Jungherr and Terrel, 1948; Davelaar and Kouwenhoven, 1977; Andrade et al., 1982; King, 1986; Mockett et al., 1987; Al-Tarcha, 1990). The serological results are, however, not always consistent with the success of challenge (Stumpel, 1980). Maternal antibodies give only very weak, or no, local protection against infection (Mockett et al., 1987). The degree of maternal immunity has been found to depend on the route of challenge (Al-Tarcha, 1990). Based on ciliary activity in tracheal explants, protection against intranasal challenge lasted up to 7–10 days of age. Maternally derived antibodies failed to protect birds against intratracheal challenge; they only delayed the onset and reduced the severity of clinical symptoms and shortened the course of the disease.

The dubious value of maternally derived immunity prompted several authors to protect young chicks by vaccination at day old. Interpretation of

the results was, however, hampered by the dissimilar routes of vaccination (intranasal–eye drop, virus spray, intratracheal inoculation) and challenge (intranasal–eye drop, intratracheal inoculation) used by the different authors.

All three routes of vaccination (intranasal–eye drop, spray, intratracheal) afford protection against challenge at 4 weeks old, but only against homologous virus strains (Andrade et al., 1982). There is no major difference between these routes of vaccination in the level of induced protection (Gough and Alexander, 1979). Eye drop immunization protects the birds only against challenge by the same route but not against intratracheal challenge (Davelaar and Kouwenhoven, 1981). Intratracheal immunization provides satisfactory protection (Raggi and Lee, 1958). In day-old chicks eye drop vaccination gives better protection than the virus spray (Stumpel, 1980; Davelaar and Kouwenhoven, 1980). In chicks with maternal antibodies stronger immunity can be induced by vaccination at 2 weeks old than at day old (Darbyshire and Peters, 1985).

The purpose of the experiments described here was to determine the level of protection induced by intranasal–eye drop or virus spray vaccination of one-day-old broilers against intratracheal challenge.

Materials and methods

Chicks

One-day-old broiler chicks derived from "Hybro" layers immunized at one day old with a spray containing IB vaccine strain H-120 (Intervet) and at 16 weeks old with a combined IB–IBD–ND inactivated, oil-adjuvant vaccine were used.

Vaccine

The chicks were vaccinated with the commercial IBV vaccine Bronchovac I (PHYLAXIA, Budapest) containing strain H120. The immunizing dose was 3000 EID_{50} per bird. PBS was used as diluent.

Challenge virus

IBV strain M-41 (8th chicken embryo passage) was used as challenge strain. Its titre was $10^{7.5}$ (0.1 ml. The challenge strain was inoculated intratracheally at a dose of 1000 EID_{50}/0.1 ml.

Design of the experiments

Immunization. Forty-five chicks were vaccinated by the intranasal–eye drop (IE) route. 0.1 ml vaccine was divided into four parts and dropped into the two eyes and two nostrils.

Forty-five chicks were immunized by spraying (S). The vaccine was diluted according to the number of birds to be vaccinated and the vaccine titre, so that 0.5 ml vaccine per bird, containing 3000 EID_{50}, was sprayed onto the birds placed in a transporting box, from a distance of about 50 cm, at an angle of 50°, using a portable sprayer. Particle size (about 1/10 mm) was checked by microscopy.

Forty-five chicks served as unvaccinated controls.

The groups of chicks were placed in isolators. Before vaccination blood samples were taken from 10 birds, pooled, and used as "baseline" serum.

Test for vaccination reaction. On days 3, 6 and 10 after vaccination (at 4, 7 and 11 days of age) 5 birds per group were examined for the presence of clinical signs and ciliary activity in tracheal explants.

Tests for protective immunity

The birds were tested for protective immunity by intratracheal challenge and by serological methods.

Intratracheal challenge. On postvaccination days 10, 21 and 31 (at 11, 22 and 32 days old), 10 birds per group were challenged intratracheally with 1000 EID_{50} (0.1 ml) of strain M-41.

Examination of tracheal explants for ciliary activity. Ciliary activity in tracheal explants was checked on postchallenge days 4 and 7, using 5 birds each. The chicks were sacrificed, the trachea of each bird was removed and transverse sections, approximately 0.5 to 1.0 mm thick, were cut from the upper (5 explants), mid- (5) and lower (5) portions, making a total of 15 explants from each bird. The individual explants were examined for ciliary activity by low-power microscopy at a magnification of $\times 100$. Ciliary activity was expressed in per cent for each explant and the mean value for the given bird (trachea) was calculated. Group averages were also calculated. Individual birds and groups with 50% or more ciliary activity were considered to have been protected by the vaccine against the challenge strain (Darbyshire, 1980).

Virus isolation. On postchallenge day 4 (simultaneously with the examination of tracheal explants) an about 5 mm thick explant was cut from the mid–portion of the trachea, placed in Minimal Essential Medium–Hanks (MEM–H) containing 200 mg/ml gentamicin and stored at −70 °C until used. SPF embryonated hen's eggs preincubated for 10–11 days were used for virus isolation. The tracheal explants were thawed, cut up, shaken thoroughly in the medium, and 5 eggs were inoculated into the allantoic sac with 0.2 ml of each sample. The eggs were incubated at 37 °C. Embryos that died within 24 h were removed. The eggs were candled twice daily. At 30 h of incubation the allanto-amniotic fluid of 3 eggs each was collected for further passage, and stored at −20 °C. The remaining 2 eggs per sample were incubated further

up to day 7 when the pathological changes of the embryos were recorded. A second or third passage was done if in the first passage no embryonic lesions typical of IB had been observed.

Serological tests

Blood sampling. Before intratracheal challenge, blood samples were taken from the birds' cubital vein and pooled by group. The sera were inactivated at 56 °C for 30 min and stored at —20 °C.

Ovoneutralization test. The beta method was carried out with strain M-41 as described by Picault et al. (1986).

Virus neutralization (VN) test in cell culture. Cell cultures were prepared and the VN test was done by a micro-method as described by El-Zein et al. (1972), on Linbro plates using strain M-41. The test was read on the 3rd day.

Haemagglutination inhibiton (HI) test. The method described by Alexander et al. (1983) was used.

ELISA. Anti-IBV antibodies were measured by indirect ELISA using Dynatech 129A plates. M-41 virus purified in three steps (total protein: 12.7 mg/ml) was used as antigen, diluted 1 : 5000 in carbonate buffer of pH 9.6. The plates were incubated at 4 °C for 16 h. A fourfold dilution series, starting with the 1 : 20 dilution, was made from the serum samples, using phosphate buffer (2000 ml)–Tween 20 (1 ml) + 0.5 M NaCl as diluent, and added to the wells. The plates were incubated at room temperature for 60 min. The conjugate used was a peroxidase-labelled goat anti-chicken IgG diluted 1 : 3000 in phosphate buffer–Tween 20 + 0.5 M NaCl + 1% bovine serum albumin. The substrate was 34 mg ortho-phenylenediamine + 20 μl H_2O_2 in 100 ml of citric acid–phosphate buffer, pH 5.25. The reaction was stopped using 2M sulphuric acid. The results are expressed in extinction extrapolated to undiluted serum (E_0) after subtracting the value obtained for the negative serum. Extinction was measured with a TITERTEK MULTISKAN instrument at a wavelength of 492 nm.

Results

Test for vaccination reaction

Ciliary activity in tracheal explants and clinical signs found on postvaccination days 3, 6 and 10 (when the birds were 4, 7 and 11 days old, respectively) showed that by postvaccination day 3 IE vaccination produced 14% reduction in ciliary activity in tracheal explants. On days 6 and 10 ciliary activity was practically the same in the three groups.

On pressure exerted on the nasal region, serous exudate appeared in one bird vaccinated by the IE route and in one spray-vaccinated chick, on postvaccination day 10.

Tests for protective immunity

Results of intratracheal challenge. The group means of ciliary activity in tracheal explants, determined on day 4 and 7 after intratracheal challenge on postvaccination days 10, 21 and 31, were as follow.

On day 4 after challenge performed on postvaccination day 10, there was no ciliary activity in tracheal explants from the vaccinated birds, whereas in the control chicks mean ciliary activity was 8% (ranges: 0–23%). On day 7 after challenge, mean ciliary activity in the group vaccinated by the IE route (further on: group IE), in the spray-vaccinated (S) and control (C) group was 54% (47–64), 7% (0–19) and 7% (0–23), respectively.

On day 4 after challenge carried out on postvaccination day 21, mean ciliary activity obtained for group IE, S and C was 30% (0–51), 0% and 8% (0–40), respectively. On day 7 after challenge, mean ciliary activity in group IE, S and C was 58% (0–93), 0% and 4% (0–20), respectively.

On day 4 after challenge on postvaccination day 31, mean ciliary activity in group IE, S and C was 51% (0–90), 0% and 0%, respectively. On day 7 the respective values were 85% (69–93), 4% (0–13) and 0%, respectively.

The individual means of ciliary activity, the results of virus isolation and the clinical signs are summarized in Table 1. On day 4 after challenge carried out on day 10 following IE vaccination there was complete absence of ciliary activity in explants from all birds, the virus was reisolated from the tracheas, and 3 out of 4 chicks showed rather severe clinical signs.

After challenge on postvaccination day 21, one chick was free from clinical signs, two showed mild while another two severe respiratory symptoms. Ciliary activity was demonstrable in four out of five birds. The virus was reisolated from the tracheas of three chicks.

After challenge on postvaccination day 31, four out of the five birds were symptomless and one showed severe clinical signs. Ciliary activity was observed in tracheal explants from four chicks: its mean value was higher than that found after challenge on postvacination day 21. Virus reisolation was successful from two chicks.

In group S, one symptomless chick and two chicks showing mild response were found only after challenge on day 10. All other birds showed severe clinical signs. Challenge on day 10, 21 or 31 after vaccination caused complete loss of ciliary activity, and reisolation of the virus from the trachea was consistently successful.

In the unvaccinated control group, challenge on postvaccination day 10 was followed by the loss of ciliary activity. Virus isolation was positive and clinical signs including lachrymation and serous nasal discharge occurred. Five birds responded to challenge on postvaccination day 21 with total loss of ciliary activity. The challenge strain was reisolated from all tracheas. From the nostrils of all birds a serous exudate could be squeezed out. Challenge on day 31 also resulted in complete loss of ciliary activity, all birds showed clinical signs, and the virus was consistently reisolated from their tracheas.

Serological tests. The results of the serological tests are summarized in Table 2. With the exception of one ELISA value (day 10 serum of spray-vaccinated chicks), the groups vaccinated by the two different routes gave identical or very similar results by the three serological tests.

Discussion

Intranasal–eye drop or spray vaccination with IBV strain H120 of one-day-old chicks with maternally derived immunity elicited a mild vaccination reaction. No overt clinical signs occurred, only very slight serous exudate could be squeezed out from the nostrils of one chick in each group on day 10. A slight (14%) reduction in ciliary activity was observed only in the group vaccinated by the intranasal–eye drop route, at postvaccination day 3.

After vaccination by the eye drop route at one day old, Davelaar et al. (1983) could not detect the vaccine virus by immunofluorescence in the trachea of chicks with maternally derived antibodies.

In the present experiments the intratracheal route of challenge was chosen for two reasons. The trachea is the main target organ of IBV infection (Darbyshire et al., 1976); therefore, protection against challenge by that route is of unquestionable value. The other reason is that by inoculating the challenge strain directly into the trachea we can rule out the factors of uncertainty associated with intranasal or eye drop challenge (e.g. that a certain part of the challenge dose is lost because of sneezing, lachrymation and head shaking).

The mean values of ciliary activity clearly indicate the superiority of intranasal–eye drop vaccination. Taking as a basis the values found on post-challenge day 4 (as is the usual procedure), birds vaccinated by that route become protected against intratracheal challenge by day 31, showing a mean ciliary activity of 51%. Taking as a basis the values obtained on postchallenge day 7, the mean ciliary activity of 54% indicates protection already at day 10. The level of protection (i.e. ciliary activity) is practically the same at day 21 and increases to 85% at day 31.

The values found for spray-vaccinated chicks consistently indicated a lack of protection, similarly to those of the controls.

Table 1

Ciliary activity, virus isolation results and clinical signs on day 4 after challenge in chicks vaccinated against infectious bronchitis at one day old by the intranasal — eye drop route or by spray, and challenged intratracheally on postvaccination days 10, 21 and 31

Route of vaccination	Challenge on postvaccination day	Parameter	Results: Chick no. 1	2	3	4	5	Total: CA	VI	CS
Intranasal-eye drop	10	Ciliary a., %	0	0	0	0	NT	0/4*		
		Virus isol.	p.	p.	p.	NT	NT		3/3*	
		Clinical s.	+++	—	+++	+++	NT			3/4*
	21	CA	51	32	30	33	0	4/5		
		VI	n.	n.	p.	p.	p.		3/5	
		CS	+	—	+++	+	+++			4/5
	31	CA	32	0	90	86	45	4/5		
		VI	p.	p.	n.	n.	n.		2/5	
		CS	—	++	—	—	—			1/5
Spray	10	CA	0	0	0	0	NT	0/4		
		VI	p.	p.	p.	p.	p.		5/5	
		CS	+++	—	+	+	NT			3/4
	21	CA	0	0	0	0	0	0/5		
		VI	p.	p.	p.	p.	p.		5/5	
		CS	++++	+++	+++	++++	++++			5/5
	31	CA	0	0	0	0	NT	0/4		
		VI	p.	p.	p.	p.	NT		4/4	
		CS	+++	+++	++	+++	++++			5/5
Control	10	CA	0	0	0	0	NT	0/4		
		VI	p.	p.	p.	p.	NT		4/4	
		CS	++++	++++	++++	++++	NT			4/4
	21	CA	0	0	0	0	0	0/5		
		VI	p.	p.	p.	p.	p.		5/5	
		CS	+	+	++	++++	++			5/5
	31	CA	0	0	0	0	0	0/5		
		VI	p.	p.	p.	p.	p.		5/5	
		CS	+	++++	+	+	+			5/5

CA: ciliary activity; VI: virus isolation; CS: clinical signs; 0: no ciliary activity; NT: not tested; x: no. of positive birds/no. of birds tested; p.: successful virus isolation; n.: unsuccessful virus isolation; + to ++++: severity scores of clinical signs; —: no clinical signs

From ciliary activity values found in tracheal explants on day 4 after challenge (Table 1) it appears that challenge on postvaccination day 10 resulted in total loss of ciliary activity in all birds of the two groups. The situation was different in the case of challenge on postvaccination day 21. While four out of the five chicks of group IE showed ciliary activity in tracheal explants, in group S there was total loss of ciliary activity. Following challenge on postvaccination day 31, the difference between the two groups increased further, as the mean ciliary activity of group IE chicks rose. Although mean ciliary activity showed rather large variation by individual, the group means can be considered a reliable indicator as group size was sufficiently large.

The results of virus isolation and the clinical signs showed a similar tendency. Reisolation of the challenge strain was successful from the trachea of 8 out of the 13 birds of group IE. The virus was reisolated from all 14 birds of the spray-vaccinated group. Virus isolation usually failed from tracheas showing high ciliary activity.

As regards the clinical signs, especially their severity (Table 1), intranasal–eye drop vaccination was superior to spray vaccination (in group IE 8 out of the 14 while in group S 13 out of the 14 chicks showed clinical signs).

The individual means of ciliary activity and the virus isolation results are worthy of analysis. The individual means show rather high deviation but the group means give reliable information especially if group size is sufficiently large. Virus isolation usually failed from tracheas showing high ciliary activity.

The results presented here allow us to conclude that the intranasal–eye drop route is more effective than spray vaccination for immunizing one-day-old chicks with maternally derived antibodies against IB. This is consistent with Stumpel's view (1980): in his opinion, a big drawback of spray vaccination is that its technical implementation is difficult and the vaccine virus often fails to get to the target site in a sufficiently large dose. Davelaar and Kouwenhoven (1980) also achieved better protection by conjunctival vaccination than by spraying.

The results of serial challenge after intranasal–eye drop vaccination inform us about the time of the development of protection. At day 10 after vaccination there is no vaccine-induced immunity yet but maternal antibodies afford some protection for that period against eye drop challenge (Al-Tarcha, 1990). At day 21 a modest active immunity can be demonstrated the level of which rises by day 31. Further studies are needed to establish whether this protection lasts until the end of broiler rearing (the age of 49–56 days). According to Davelaar and Kouwenhoven (1980), conjunctival immunization at one day old elicits immunity to challenge by the same route by the age of 3 weeks, and this protection still exists when the birds are 7 weeks old.

Intranasal–eye drop immunization induced protection despite the presence of maternal antibodies. We cannot give an explanation for the inef-

Table 2

Serological results obtained with blood samples taken on postvaccination days 10, 21 and 31 from chicks vaccinated against infectious gronchitis at one day old by the intranasal–eye drop route or by spraying

Serum	Route of vaccination								Control			
	Intranasal–eye drop				Spray							
	HI	VN		ELISA	HI	VN		ELISA	HI	VN		ELISA
		eggs	cell c.			eggs	cell c.			eggs	cell c.	
Baseline serum	8*	1 : 330**	1 : 256	3.044***	8	1 : 330	1 : 256	3.044	8	1 : 330	1 : 256	3.044
Day 10	5	1 : 32	1 : 8	1.644	5	1 : 32	1 : 16	2.623	6	1 : 32	1 : 16	1.940
Day 21	4	1 : 2	1 : 4	1.036	5	1 : 3	1 : 4	1.054	3	1 : 5	1 : 4	0.883
Day 31	4	0	1 : 2	0.686	3	0	1 : 2	0.621	3	0	1 : 2	0.591

*: exponent in log 2 (starting dilution: 1 : 2).
**: the highest dilution which still neutralizes.
***: extrapolated extinction of undiluted serum, minus the value obtained for the negative serum (E_0).

fectiveness of spray vaccination in this study and, hence, cannot take a stand as regards its value in vaccination practice. (The preliminary results of our studies currently in progress indicate that spray vaccination protects the chicks against intranasal–eye drop challenge).

The serological testing of sera taken before intratracheal challenge gave identical or very similar results in all three groups, with the exception of one ELISA value (day 10 serum of group S; Table 2). The serum titres gradually declined with time, which is consistent with data reported on the changes in maternal antibody titres (Jungherr and Terrel, 1948; Andrade et al., 1982; Davelaar and Kouwenhoven, 1981; Hofstad, 1984; King, 1986; Mockett et al., 1987; Al-Tarcha, 1990).

The results of the serological tests, unlike those obtained after intratracheal challenge, did not indicate differences between the groups. It is known, however, that in IB the serological results are not always consistent with the success of challenge (Raggi and Lee, 1965; Davelaar and Kouwenhoven, 1977).

The fact that IE vaccination protected the chicks against challenge and that the serological tests demonstrated very low antibody levels, or no antibodies at all, leads us to conclude that the observed protection was first of all due to local immunity. This is in good harmony with Davelaar and Kouwenhoven's (1977) view, i.e. that it is not necessary for virus to reach deeper situated immunocompetent cells to stimulate protective immunity against IB. Gomez and Raggi (1974) discussed the local protection of the trachea against IB. Mentioning the possible role of interferon, antibodies of the IgA class, reduced viability of the tracheal epithelium after infection, and secretion of aspecific antiviral antibodies, they concluded that the mode of action was unknown. In their view, changes of the tracheal mucosa cells were responsible for preventing complete manifestation of IBV infection.

To sum up: chickens having maternally derived antibodies can be protected against intratracheal challenge by vaccination at one day old by the intranasal–eye drop route. The vaccine administered by the spray route does not protect chicks against intratracheal challenge. This must be considered when results are compared with those from vaccination trials described in the literature in which a large variety of challenge routes was used (Winterfield et al., 1978; Davelaar and Kouwenhoven, 1977, 1980, 1981; Gough and Alexander, 1979; Darbyshire, 1980).

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CROSS-PROTECTION STUDIES WITH VACCINE STRAIN H-120 OF INFECTIOUS BRONCHITIS VIRUS USING CILIARY ACTIVITY

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Forty 3-day-old chickens were immunized intratracheally and another 40 intranasally-intraocularly with vaccine strain H-120 of infectious bronchitis virus (IBV). The chickens were divided into groups of five and each group was challenged intratracheally or intranasally-intraocularly with one of 8 different heterologous strains of IBV 4 weeks after vaccination. The vaccinated chickens were protected against challenge with three heterologous strains (Massachusetts M41, B and ÁEG), showing 89, 86 and 89% ciliary activity, respectively, but were not protected against challenge with strains D-3896 and D-3128.

Clinical signs and/or a reduction in ciliary activity was observed neither for the vaccinated nor for the unvaccinated groups challenged with strains Sz, D-207 and D-212. This suggests that these isolates have no primary pathogenic role in broiler flocks. The remaining 5 strains (M41, B, ÁEG, D-3896 and D-3128) caused mild respiratory signs and ciliostasis in birds of the unvaccinated groups.

Key words: Infectious bronchitis virus, cross-protection, ciliary activity

Infectious bronchitis virus (IBV) produces a highly contagious viral disease of chickens characterized by a short incubation period and rapid spread through a susceptible flock. It produces more severe respiratory signs in young chickens than in older birds.

The extensive use of IBV vaccine has provided a generally effective control of the infection in chickens. Many investigators (Winterfield, 1973; Winterfield and Fadly, 1975; Rosenberger et al., 1976) have indicated that the Holland H-120 strain of IBV provides a wide range of protection against different variant strains of IBV occurring in the field.

Neutralization tests have produced sufficient evidence of the existence of antigenic variation among IBV strains (Hofstad, 1958; Berry and Stokes, 1968; Dawson and Cough, 1971; Hopkins, 1974; Cowen and Hitchner, 1975; Johnson and Marquardt, 1985; Darbyshire et al., 1979).

However, there are also indications that the serological relationships determined *in vitro* are not always reflected by the result of cross-immunity studies in chickens (Winterfield and Hitchner, 1962; Hitchner et al., 1964; Raggi and Lee, 1965; Winterfield and Fadly, 1972).

Different methods have been used to interpret the immune status to IBV (Hofstad, 1967; Lucio and Hitchner, 1970; Lukert, 1969; Darbyshire,

1980). Results based on detection of respiratory symptoms and virus recovery from tracheal swabs are variable and subjective.

Cross-neutralization tests in embryonated hen's eggs have been used for differentiating immunological variants of IBV (Hitchner et al., 1964; Hofstad, 1967). Discrepancies are evident between this method and cross challenge (Chomiak et al., 1963).

This study was done to determine the ability of the Holland strain (Bronchovac H-120, Phylaxia) of IBV to induce protection against challenge with strain M41, four Dutch variant strains (D-207, D-212, D-3896 and D-3128) and three Hungarian isolates (B, Sz and ÁEG).

Materials and methods

Viruses

The following IBV strains were used: H-120 (vaccine strain, Phylaxia); Massachusetts M41, 8th passage, kindly supplied by Dr. Márta Csermelyi, Veterinary Medical Research Institute of the Hungarian Academy of Sciences; three Hungarian isolates of IBV designated ÁEG, B and Sz; and four lyophilized Dutch (Doorn) variants (D-207, 62nd chicken embryo passage; D-212, 60th chicken embryo passage; D-3896, 23rd chicken embryo passage; and D-3128, 66th chicken embryo passage). The strains were passaged twice, propagated and titrated in SPF embryonated eggs before use.

Infectivity titres were calculated according to Reed and Muench (1938) and expressed as embryo infective dose (EID_{50}) per 0.1 ml.

Chickens

One hundred and sixty one-day-old SPF chickens were obtained from an SPF flock (Dabas, Phylaxia) and were reared in isolators before and throughout the experiments.

Immunization

Forty 3-day-old chickens were inoculated intranasally-intraocularly with 10^4 EID_{50}/bird of IBV strain H-120. Another 40 birds were inoculated with the same dose of strain H-120 by the intratracheal route. Eighty unvaccinated birds served as controls.

Challenge

Challenge was carried out by one of two different routes 4 weeks after immunization.

Experiment 1. Forty intratracheally immunized and 40 unvaccinated chickens were divided into 8 groups (5 birds in each) and the groups were challenged intratracheally with one of the challenge strains M41, B, Sz, ÁEG, D-207, D-212, D-3896 and D-3128. Each bird was challenged with approximately 10^4 EID_{50} of the specified virus.

Experiment 2. Forty intranasally-intraocularly immunized chickens and 40 unvaccinated birds were challenged by the same route with the same strains as in Experiment 1. Each bird was challenged with approximately 10^4 EID_{50} of the specified virus.

Examination of tracheal rings

Four days after challenge all birds were killed, their tracheas were removed with minimum injury, and rings, approximately 1–1.5 mm thick, were cut from the upper, mid- and lower part of the trachea (3 rings from each, making a total of 9 rings from each bird).

Each tracheal ring was examined under low-power microscope for evidence of ciliary activity as described by Darbyshire (1980).

To simplify the interpretation of the results, individual birds were considered protected by the vaccine against the challenge strain if their tracheal rings showed 50% or higher ciliary activity.

Clinical signs

All birds were checked carefully for clinical signs of infectious bronchitis (IB) daily for 4 days after challenge.

Results

The results of experiment 1 are summarized in Table 1. The definite presence or absence of ciliary activity in each ring affords possibility to quantify the response to the challenge virus.

Chickens vaccinated with strain H-120 were clearly protected against challenge with strains M41, B and ÁEG, but not against challenge with strains D-3896 and D-3128.

Tracheal rings from the unvaccinated control chickens challenged with M41, B or ÁEG showed no evidence of ciliary activity. Tracheal rings from chickens challenged with isolates Sz, D-207 and D-212 showed 86 (72–100), 85 (61–96) and 81% (50–100%) ciliary activity, respectively.

In experiment 2, protection was challenged intranasally-intraocularly with the same strains as in experiment 1. The results are presented in Table 2.

Table

Ciliary activity four days after challenge in chickens vaccinated intratracheally with IBV (results of

Virus*	Ciliary activity (%) in immunized chickens					
	1	2	3	4	5**	Average (%)
M41	85	100	95	75	89	89
B	90	84	91	80	85	86
Sz	84	96	81	87	85	86
ÁEG	82	97	86	89	90	89
D-207	74	83	ND	71	78	77
D-212	76	86	83	78	83	81
D-3896	0	0	0	0	0	0
D-3128	0	0	0	0	0	0

* challenge virus 10,000 EID_{50}/0.1 ml; ** 5 birds per group; + clinical signs; ND = not

Table

Ciliary activity four days after challenge in chickens intranasally-intraocularly vaccinated with (results of

Virus*	Ciliary activity (%) in immunized chickens					
	1	2	3	4	5**	Average (%)
M41	75	85	95	80	90	85
B	100	100	85	90	90	93
Sz	100	100	100	100	85	96
ÁEG	100	100	90	90	85	93
D-207	91	89	84	79	91	87
D-212	80	84	72	84	78	87
D-3896	79	81	84	62	86	79
D-3128	84	86	90	93	92	89

* challenge virus 10,000 EID_{50}/0.1 ml; ** 5 birds per group; + clinical signs

In the control groups, ciliary activity was not affected by challenge with the above strains except strain M41 (ciliary activity: 0). In the vaccinated groups there was complete ciliary activity after challenge with any of the challenge strains including M41.

Clinical signs like sneezing, rales, head shaking and slight nose blowing were observed in the unvaccinated groups (except groups challenged with Sz, D-207 and D-212), especially after intratracheal challenge (Table 1).

1

strain H-120 and challenged by the same route with heterologous strains four weeks later experiment 1)

Virus*	Ciliary activity (%) in control chickens					
	1	2	3	4	5**	Average (%)
M41	0	0	0	0	0	0+
B	16	15	0	23	0	10+
Sz	76	72	92	100	90	86
ÁEG	9	10	10	15	18	13+
D-207	96	ND	94	61	93	85
D-212	100	88	50	89	ND	81
D-3896	0	0	0	0	0	0+
D-3128	0	0	0	0	0	0+

determined

2

IBV strain H-120 and challenged by the same route with heterologous strains four weeks later experiment 2)

Virus*	Ciliary activity (%) in control chickens					
	1	2	3	4	5	Average (%)
M41	0	0	0	0	0	0+
B	100	80	80	90	100	90
Sz	100	90	100	100	80	94
ÁEG	100	90	85	80	90	89
D-207	87	80	86	100	92	89
D-212	77	89	88	80	94	86
D-3896	86	87	74	59	90	79
D-3128	78	100	74	77	78	83

Discussion

In cross-protection tests with most respiratory viruses, objective interpretation of the clinical response may be difficult, if not impossible.

In similar experiments with IBV, the problem of assessment of immunity has been approached by reisolation of the challenge virus within a limited period (Hitchner et al., 1964; Hofstad, 1967; Winterfield and Fadly, 1972).

Such reisolation attempts have been made from tracheal swabs of chickens challenged after immunization.

The primary target organ of IBV infection in chicken is the trachea (Cunningham, 1976; Darbyshire et al., 1976) and uncomplicated IB is clinically an acute, brief and self-limiting disease of that organ (Purcell and Clarke, 1972). A demonstrable resistance to infection at that site would suggest a more generalized immunity in an individual chicken. Geilhausen et al. (1973) observed that during the first seven days after challenge with a virulent strain of IBV no ciliary activity could be detected in the tracheal rings, while infection with an avirulent strain had no such effect. In this study, we observed that isolates Sz, D-207 and D-212 did not infect chickens of the unvaccinated groups by either route of challenge, and ciliary activity was 85, 85 and 81%, respectively, after intratracheal challenge (Table 1) and 94, 89 and 86%, respectively, after intranasal-intraocular challenge (Table 2). No clinical signs were observed after challenge by either route.

According to the statement made by Geilhausen et al. (1973), this suggests that isolates Sz, D-207 and D-212 alone do not play any role in infection of broiler flocks.

In other unvaccinated groups challenged intratracheally with strains B, ÁEG and M41 (Table 1) ciliostasis and respiratory signs were detected. Intranasal challenge with strains B and ÁEG gave no evidence of infection in the control groups (Table 2), indicating that these isolates are not able to infect chickens when given through the intranasal route. This result is consistent with that obtained by us earlier (Al-Tarcha and Kojnok, 1990).

Earlier we found (Al-Tarcha and Kojnok, 1990) that intratracheal infection of unvaccinated chickens leads to ciliostasis and appearance of respiratory signs. In this study, we observed ciliostasis in unvaccinated chickens, but the vaccinated groups showed ciliary activity and were protected against challenge with these isolates.

Ciliary activity was 79 and 83% after intranasal challenge of unvaccinated chickens with isolates D-3896 and D-3128, respectively (Table 2), but there was ciliostasis in both the vaccinated and the unvaccinated groups when they were challenged with these strains intratracheally (Table 1). These results indicate that vaccination with strain H-120 does not protect chickens against intratracheal challenge. Therefore, we suggest using a homologous vaccine against these viruses if they occur in broiler flocks.

Challenge with strain M41 by either route caused infection and acute respiratory signs in the unvaccinated chickens. Chickens immunized with vaccine strain H-120 were protected against challenge with strain M41.

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CONTENTS

Pathophysiology

Physiology

Acta Veterinaria Hungarica 39 (3—4), pp. 103—107 (1991)

SEDATIVE EFFECT OF DETOMIDINE IN INFANT CALVES

P. K. PESHIN, A. P. SINGH*, Jit SINGH, D. B. PATIL and Davood SHARIFI

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(Received November 2, 1990)

Detomidine administered intramuscularly at a dose of 10, 20 or 40 μg/kg body mass was evaluated for its sedative effects in 15 unfasted infant calves (age: 15–20 days; body mass: 18–33 kg). The drug produced dose-dependent sedation. At a dose of 10 μg/kg detomidine produced effective sedation for 30 to 45 min without any observable analgesia. At doses of 20 or 40 μg/kg it caused deep sedation, sternal recumbency, and moderate analgesia of the trunk. Hyperglycaemia was recorded at all dose levels. The changes in respiratory rate, rectal temperature, haemoglobin, packed cell volume, total erythrocyte count and plasma concentration of total protein were not significant.

Key words: Detomidine, sedative effects, infant calves

Detomidine, an alpha-2 agonist has been shown to possess good sedative and analgesic properties in horses (Stenberg, 1986; Jochle and Hamm, 1986). Clinical experience for treating horses and cattle has also been described (Clarke and Taylor, 1986; Alitalo, 1986). We have recently evaluated different low doses of detomidine for sedative effect in 6-month-old to 1-year-old calves (Peshin et al., 1990). Encouraging results were obtained. These favourable experiences prompted us to evaluate three different doses of detomidine in infant calves. The results are reported in this paper.

Materials and methods

Fifteen infant calves (age: 15–20 days; body mass: 18–33 kg) were used. The calves were fed skimmed milk and were not fasted before administration of the drug. Three different doses of detomidine, *viz.* 10, 20 and 40 μg/kg were administered intramuscularly to 5 calves each. The variables observed were sedation and analgesia, heart rate, respiratory rate, rectal temperature, haemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC), plasma concentration of total protein (TP), and blood glucose (Folin and Wu, 1920).

For sedation, the position of the head, the position and expression of calves, the response to noise and approach, and the presence or absence of

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ataxia were recorded. Sedation was graded as mild (animal calm but responds to noise, approach and touch), moderate (calm with sluggish response to noise, approach and touch), and deep (calm with no response to noise, approach and touch). For analgesia, the pin-prick method was used at three points on the trunk (neck, shoulder and hindquarter) and two spots each on the forelimbs and hindlimbs (coronary border, carpal and tarsal area). Analgesia of the limbs and that of the trunk were graded separately. Miscellaneous clinical signs were also recorded. Statistical analysis of the data was done by Student's t test at a $p < 0.05$ level of significance.

Results

Intramuscular administration of detomidine produced dose-dependent sedation in the calves (Table 1). Induction was quicker at higher doses. The

Table 1

Onset, total duration of sedation, duration of effective sedation and complete recovery time (mean ± SE) in min after intramuscular administration of detomidine in infant calves at three dose levels (μg/kg)

Dose	Onset of sedation	Total duration of sedation	Duration of effective sedation	Complete recovery time
10	18.2 ± 1.4	100 ± 3.0	37 ± 2.6	158 ± 2.3
20	6.8 ± 0.8	122 ± 9.0	67 ± 7.5	183 ± 7.5
40	5.2 ±	167 ± 8.5	115 ± 8.5	223 ± 7.2

dose of 10 μg/kg produced effective sedation for 30 to 45 min. Complete recovery from sedation at this dose took 140–170 min. Elevation of the dose prolonged the duration of effective sedation as well as the recovery time. After the 20 μg dose, calves were deeply sedated and had a sleepy look. Their eyes were closed with relaxed upper eyelids. The 40 μg/kg dose produced a very strong sedation. After 10 μg, 3 animals were in a fixed standing position, with a weak response to noise during the period of effective sedation. Ataxia was mild. Two calves went into sternal recumbency 15 min after the injection. At the higher doses (20 and 40 μg) all calves went into either sternal or lateral recumbency during the period of effective sedation. In sternal recumbency, either the jaw was resting on the ground or the neck was turned on to the shoulder. The jaw was relaxed 30 min after the injection. The degree of lower jaw relaxation was higher after the 40 μg dose, and in most animals the tongue protruded. Salivation was mild (10 and 20 μg) or mild to moderate (40 μg). At higher doses, most of the animals did not respond to noise at all.

There was no observable analgesia of the trunk or limbs at the dose of 10 μg. Higher doses produced mild to moderate analgesia. The duration of analgesia was less than that of effective sedation (Table 2). Frequent urination occurred at all the three dose levels.

Table 2

Duration (mean ± SE) of moderate analgesia of trunk and limbs in minutes after intramuscular administration of detomidine in infant calves at three different dose levels (μg/kg)

Dose	Trunk	Limbs
10	—	—
20	49 ± 7.0	34 ± 8.0
40	74 ± 9.4	46 ± 4.3

Detomidine caused bradycardia in the calves (Fig. 1). The maximum per cent decrease in heart rate was 18, 20 and 25% with the doses of 10, 20 and 40 μg, respectively. There was no effect on the respiratory rate after the 10 μg dose. The 20 μg dose slightly depressed respiration, while the 40 μg dose induced its mild stimulation. The decrease in rectal temperature was not significant. The changes in Hb, PCV, TEC and TP were minor and non-

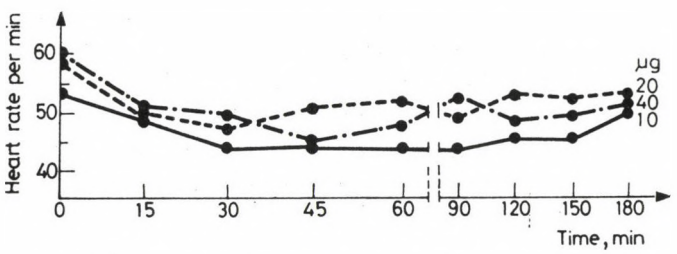

Fig. 1. Effect of detomidine on the heart rate of young calves after intramuscular administration at different doses

Table 3

Effect of three different dose levels of intramuscularly administered detomidine on blood glucose level (mg/100 ml) at different intervals (mean ± SE)

Dose (μg/kg)	Time (h)			
	0	1	3	24
10	70.6 ± 2.05	126.8 ± 2.45	119.6 ± 5.60	76.8 ± 0.95
20	62.0 ± 2.83	129.2 ± 4.75	140.0 ± 4.91	65.2 ± 3.51
40	67.8 ± 5.02	131.6 ± 5.01	152.8 ± 5.87	74.4 ± 5.56

significant. Hyperglycaemia occurred at all the three dose levels (Table 3). The maximum per cent increase in plasma glucose concentration was 80% with 10 μg and 125% with 20 and 40 μg. Glucose concentration returned to near initial values at 24 h.

Discussion

In combination with local analgesic agents, a safe and effective sedative can be used in infant calves for minor surgical procedures. The results of the present study show that an intramuscular dose of 10 μg/kg produces effective sedation of sufficient duration. If required, the depth and duration of sedation can be increased by using the 20 μg/kg dose. However, the use of local analgesia for painful procedures will be mandatory as detomidine did not produce good analgesic effect. The dose of 40 μg may not be desirable because of strong sedation and prolonged recovery time.

The sufficient dose of detomidine for adult cattle was reported to be in the range of 30 to 60 μg/kg (Alitalo, 1986). We previously evaluated detomidine in calves (six months to one year old) older than used in the present study and found that doses of 5 μg intravenously and 10 μg intramuscularly were sufficient to produce effective sedation. An intravenous dose of 10 μg and higher doses of 20 and 40 μg both iv. and im. caused deep sedation. Sedation obtained with the higher doses (20 and 40 μg) in the infant calves of this study was much deeper than that induced in the older calves. Although a dose of 40 μg produced strong sedation with prolonged recovery, there were no observable harmful effects. Therefore, the efficacy of low detomidine doses in infant calves is accompanied by a good margin of safety.

Detomidine causes characteristic bradycardia in horses (Clarke and Taylor, 1986) which is related to its central depressant action. The minimum heart rate after detomidine administration (10–40 μg/kg) was 28–43% of the initial values in foals (Oijalo and Katila, 1988) and 30–50% in horses (Clarke and Taylor, 1986). In infant calves of this study minimum heart rate was 25–28% as compared to 29–33% in older calves (Peshin et al., 1990) after the same dose and route of administration. Hyperglycaemia as observed in the present study has also been reported in horses (Short et al., 1986), sheep (Koichev et al., 1988; Singh et al., 1990) and older calves (Peshin et al., 1990). The mechanism of the hyperglycaemia-inducing effect of detomidine has not been elucidated so far.

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Acta Veterinaria Hungarica 39 (3—4), pp. 109—114 (1991)

EVALUATION OF DETOMIDINE AS A SEDATIVE IN GOATS

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(Received November 2, 1990)

Intramuscular (i.m.) and intravenous (i.v.) administration of detomidine at doses of 10, 20 and 40 μg/kg body mass was evaluated for its sedative and analgesic properties in 15 goats *(Capra hircus)*. The drug produced dose- and route-dependent sedation. The 10 μg/kg dose was effective only when administered i.v. There was no observable analgesia at this dose. Higher doses produced effective sedation and moderate analgesia of the body with either route of administration. Severe ataxia and sternal recumbency were seen in all the animals after the dose of 40 μg/kg. Other effects of detomidine in these goats included mild to moderate salivation, depressed respiratory rate, decreased rectal temperature, bradycardia and hyperglycaemia. Plasma concentrations of total protein, sodium, potassium and chloride were not affected.

Key words: Detomidine, goat, sedative effects

The alpha-2 agonist detomidine is quite a new sedative which has yet to be marketed in many countries. The drug has proved useful in horses (Vainio, 1985; Clarke and Taylor, 1986; Short et al., 1986). Clinical trials in cattle have been reported from Finland (Alitalo, 1986). We have recently evaluated this drug as a sedative in cattle, sheep and buffaloes, given at different doses intramuscularly (i.m.) as well as intravenously (i.v.). The results of these studies are under publication.

Goats are agile in nature, and a good sedative is always useful in the handling and clinical examination of this species. Since the effects of detomidine in goats have not been investigated so far, we evaluated three dose levels of this drug with two routes of administration.

Materials and methods

Experiments were conducted on 15 goats weighing 15 to 20 kg. Three different doses of detomidine, viz. 10, 20 and 40 μg/kg were tried out in five animals each. Two routes of administration, viz. i.m. and i.v. (jugular vein), were used for each dose at 10-day intervals. The goats were clinically healthy

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and had been kept under the same managemental conditions before the experiments. The variables observed included degree of sedation and analgesia, heart rate, respiratory rate, rectal temperature, blood glucose level (Folin and Wu, 1920), plasma concentration of total protein (biuret method), sodium and potassium (flame photometer) and chloride (chloride meter). For sedation, the position of head, drooping of upper eyelid, position and expression of animal, response to noise and approach, and presence or absence of ataxia were recorded. Since the total duration of sedation included mild effects too, effective sedation time was recorded separately. Sedation was graded as mild (animal calm but responds to noise, approach and touch), moderate (calm with sluggish response to noise, approach and touch), or deep (calm with no response to noise, touch and approach). The duration of effective sedation was taken as the sum of moderate and deep sedation. For analgesia, the pin-prick method (Oijalo and Katila, 1985) was used at three points on the trunk (neck, shoulder and hindquarters) and at two spots each on the forelimbs (coronary border and carpal area) and hindlimbs (coronary border and tarsal area). The limb and the trunk were graded separately. Urination, salivation and lacrimation during the effect of drug were also recorded. The statistical analysis of data was done by Student's t test at $p < 0.05$ significance.

Results

The signs of effective sedation in these goats were calm, uninterested expression, fixed standing position or sternal recumbency, moderate relaxation of the upper eyelids, weak response to noise, approach and touch, and minimal restraint required for clinical examination. Lowering of head was not a reliable indicator of sedation. The dose of 10 μg i.m. failed to produce satisfactory results: only one goat developed effective sedation of 20 min duration. In the remaining animals sedation was mild and they responded quickly to the approach of the investigator. The induction time, duration of effective sedation and complete recovery time after detomidine administration are shown in Table 1. The depth and duration of effective sedation were dose as well as route dependent. I.v. administration produced quick induction and better depth of sedation. The recovery time was also dose dependent. Ataxia was mild to moderate after 10 to 20 μg and moderate to severe after 40 μg. Sternal recumbency was recorded in a few animals after 20 μg and in all after 40 μg.

The development of analgesia in the animals was not satisfactory. Moderate analgesia of the trunk and mild analgesia of the forelimbs developed at doses of 20 and 40 μg. Analgesia of the hindlimbs did not develop and most animals responded to stimuli exerted on the coronary border. Salivation was mild after 10 to 20 μg and mild to moderate after 40 μg detomidine.

Table 1

Onset and duration of effective sedation and complete recovery time (mean ± SE) in minutes after detomidine administration at three dose levels

Route and dose (μg/kg)	Onset of sedation	Duration of effective sedation	Complete recovery time
Intramuscular			
10	—	—	—
20	12 ± 2.5	50 ± 6.0	170 ± 5.5
40	8.4 ± 1.5	78 ± 7.0	199 ± 5.7
Intravenous			
10	1.6 ± 0.2	43 ± 4.5	124 ± 5.0
20	1.3 ± 0.3	62 ± 5.8	152 ± 9.3
40	1.2 ± 0.2	107 ± 6.4	220 ± 5.5

The change in respiratory rate was not significant. However, detomidine caused a general decrease in respiratory rate at all dose levels used. A hypothermic effect of the drug was also recorded in these goats. The decrease in rectal temperature ranged between 0.8 and 2.0 °F. The decrease in rectal temperature was neither dose nor route dependent. Rectal temperature returned to the initial values within 3 h after drug administration. Significant bradycardia occurred in all the goats after the administration of detomidine (Fig. 1). Minimum heart rate was observed 30 to 45 min after i.m. injection and within

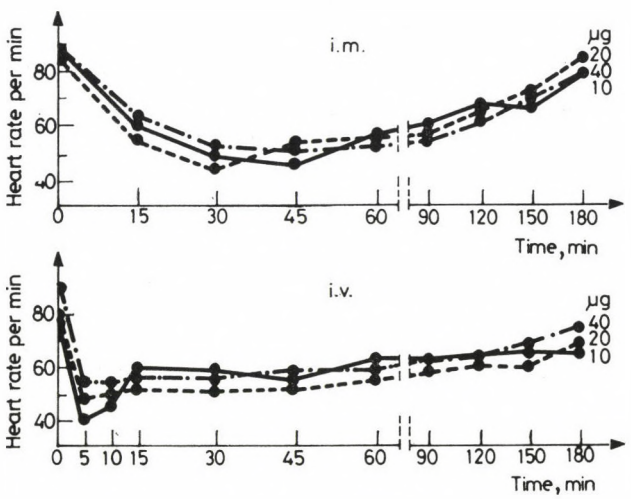

Fig. 1. Effect of detomidine on the heart rate of goats after intravenous and intramuscular administration at different doses

10 min after i.v. injection. The decrease in heart rate was statistically significant up to 90 to 150 min in the different groups. The heart rate remained below the initial values even 3 h after i.v. administration of the drug. The development of hyperglycaemia was significant at all the dose levels (Table 2). Changes in the plasma concentration of total protein, sodium, potassium and chloride were not significant.

Table 2

Effect of detomidine administered at three dose levels on blood glucose levels (mg/100 ml) at different intervals after administration (mean ± SE)

Route and dose (μg/kg)	Time (h)				Maximum increase, %
	0	1	3	24	
Intramuscular					
10	64.4 ± 3.41	108.0 ± 6.52	108.6 ± 6.83	66.2 ± 2.16	69
20	61.8 ± 3.75	120.0 ± 7.00	110.4 ± 2.89	71.0 ± 4.84	94
40	62.0 ± 3.74	120.0 ± 7.67	126.0 ± 4.93	88.6 ± 5.26	103
Intravenous					
10	90.4 ± 4.92	103.4 ± 7.23	157.0 ± 8.75	135.8 ± 7.01	74
20	86.6 ± 4.65	124.2 ± 12.71	165.6 ± 8.10	121.0 ± 10.54	91
40	80.4 ± 3.55	140.0 ± 14.26	166.2 ± 11.1	115.4 ± 7.28	107

Discussion

The goats used in this study were highly agile in nature. Before sedation it was very difficult to catch them as they ran away when any approach was made. Therefore, after sedation the goats' response to the approach of the investigator provided a measure of the degree of sedation. Mild response in these goats was always accepted even after deep sedation, unless the animals were in sternal recumbency. The results of this study show that detomidine produces excellent sedative effects in the goat. The sedative effects of the drug were dose as well as route dependent. The depth and the duration of effective sedation were better at higher doses and after i.v. administration.

The dose of 10 μg/kg i.v. was not sufficient to produce effective sedation. It appears that doses of 20 to 40 μg/kg should be sufficient to produce satisfactory sedation. The 40 μg/kg dose caused deep sedation of longer duration and recovery time was much prolonged. When i.m. doses of 10 and 20 μg were used, restraint of the animal's head was essential during clinical examination. The results did not show satisfactory analgesia in these goats after detomidine administration. Only the highest dose (40 μg) produced moderate

analgesia of the trunk. Clarke and Taylor (1986) did not observe analgesia in horses at the dose range between 5 and 20 μg. Other investigators reported analgesic effects of detomidine in horses and cattle (Vainio, 1985; Jochle and Hamm, 1986; Stenberg et al., 1986; Oijalo and Katila, 1988).

Bradycardia, hypothermia, depressed respiratory rate and hyperglycaemia were observed in the goats after detomidine administration. A slight depression of the respiratory rate was observed with detomidine in cattle (Vainio, 1985). In goats, a significant reduction in respiratory rate was reported after xylazine (0.22 mg/kg) administration (Kumar and Thurmon, 1979). Inhibition of sympathetic tone in the CNS by alpha-2 agonist was stated to be the cause of bradycardia, hypothermia and sedation (Virtanen, 1986). In horses, A–V and S–A block was reported to cause bradycardia after detomidine administration (Clarke and Taylor, 1986). Hyperglycaemia in connection with detomidine administration has been observed in horses (Short et al., 1986), sheep (Koichev et al., 1986; Singh et al., 1990) and cattle (Peshin et al., 1990). The exact mechanism by which this hyperglycaemia develops remains to be investigated. Inhibition of insulin production may be one of the factors involved (Hsu and Himmel, 1981; Trim and Hanson, 1986).

The results show that a 20 μg/kg dose of detomidine should be sufficient to cause effective sedation of goats for clinical examination. The 10 μg/kg dose, given i.v., may be useful in relatively docile animals, but i.m. administration of this dose may not produce satisfactory results. The highest dose tested, 40 μg/kg, might be useful in combination with local anaesthetic solutions for minor surgical procedures, but recovery time is prolonged.

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SLEEP AND ACTIVITY OF PIGLETS WEANED INTO CAGES

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The duration and type of sleep and activity were monitored in a group of 9 Duroc piglets weaned at 7 weeks of age and placed into a cage of 1.2×1.2 m. Average air temperature in the cage ranged between 20 and 23 °C and relative humidity was around 70%. The piglets were fed and watered *ad libitum*. The measurements were performed one week after the pigs had been transferred to the cages, in the period between 8 a.m. and 1 p.m.

Approximately half of the 5-hour period of observation was occupied by sleep. There were, however, marked individual differences (ranges: 66 and 24%). Non-REM sleep occupied 79% while REM sleep 21% of the whole sleep time. REM episodes lasted, on the average, 3.8 + 0.58 min.

The other half of the period studied was occupied by wakefulness which was devoted to movement, lying and, also, massaging and sucking each other. In some animals this activity was high and was the cause of unrest in the whole group.

Differences in respiratory rate between non-REM and REM sleep recorded in heavier animals were the result of their heat load.

Key words: Pig, cage breeding, activity, massaging, sucking, REM sleep, non-REM sleep

Sleep is essential for the development and growth of young piglets. Under optimal conditions the time spent sleeping may reach 90% of the daytime (Pokorná et al., 1988). During rearing, however, there are periods when sleep is strongly affected by external factors such as the weaning of piglets from their mothers. In that period not only the environment but the nutrition and, frequently, also the composition of the group change. All this brings about problems which usually cause a transient decrease in body mass gain and in some animals even a prolonged retardation in growth. After weaning the animals' activity decreases to the detriment of the resting period and sleep. The establishment of a new wake–sleep rhythm, which gradually increases the time spent sleeping in the circadian cycle, indicates that the animal is becoming adapted to the new conditions.

Sleep is known to be accompanied by reduced sympathetic and increased parasympathetic tone. As a result of the elimination of movement, sleep significantly contributes to decreasing the level of energy metabolism (Zepelin and Rechtschaffen, 1974). In addition, REM sleep was found to reduce the postprandial increase in the piglets' heat production, suggesting that this sleep contributes to a more economical use of feed energy (Kotrbáček, 1988;

Kotrbáček and Hönig, 1990). From the above it follows that investigations into the sleep of pigs are undoubtedly very important because they inform us about the animals' ability to adapt to different breeding measures and various technologies. The aim of this study was to contribute to elucidating these problems.

Materials and methods

The investigations reported here were aimed at determining the duration of vigilance and sleep in a group of weaned piglets kept in cages. In addition, the type of their activity as well as the duration and course of the individual sleep–wake stages were studied A group of 9 Duroc piglets, weaned at the age of 7 weeks and placed into an experimental grated-floor cage 1.2×1.2 m in size, was used. The walls and ceiling were made of plexiglass and could be moved and used for closing the cage. Fans with automatic regulation of incoming air temperature were used for ventilation. Average air temperature ranged between 20 °C and 23 °C, and relative humidity of air was around 70%. The piglets were fed and watered *ad libitum*. The measurements were performed in the period between 8 a.m. and 1 p.m., one week after the pigs had been transferred to the experimental cages. The piglets' behaviour was observed and recorded at 20-second intervals on paper of a recording instrument equipped with a chart drive of 1 cm per 20 seconds. Activity was spoken of when the piglets moved in the cage, were eating, drinking water, or when a piglet communicated with the others. Mutual massaging and sucking were studied separately. The active phase also included peaceful lying down on the grated floor with eyes open.

The period of peaceful lying down with eyes closed, lasting at least 2 min, was termed peaceful non-REM sleep. The active sleep stage, the so-called REM sleep, was identified according to the typical twitches of the rostral and mimic muscles spreading to the legs with a simultaneous visible reduction in muscle tone (Kotrbáček, 1988). The respiratory rate was determined in both stages of sleep by counting the movements of the chest. These data were obtained from 6 out of the 9 piglets and analysed statistically by Student's t test. This method of observation was simple but very accurate and required full concentration. Its advantage was the elimination of disturbing moments arising from contact with the animals, e.g. when taking EEG or when using other instruments.

Results

Approximately half of the 5-hour period of observation was occupied by sleep. There were, however, marked individual differences within the experimental group. Peaceful non-REM sleep occupied 79% of the whole period,

while 21% was active REM sleep. The frequency of REM sleep was approximately 3 episodes per h and was characterized by slackening of the skeletal musculature, regular twitches of the mimic muscles and later, also of the muscles of the legs. REM episodes, lasting 3.8 $\pm$ 0.58 min on the average, were observed after a shorter or longer period of non-REM sleep and mostly appeared simultaneously in several piglets. A very important change during REM sleep was the decreased respiratory rate (Fig. 1), which was statistically significant in piglets of higher live body mass ($p < 0.01$).

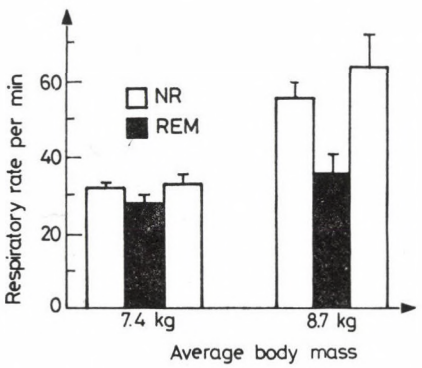

Fig. 1. Changes in respiratory rate in piglets of different body mass in REM sleep (NR = non-REM sleep)

The other half of the period of study was occupied by wakefulness. The longer part of wakefulness was occupied by lying, either completely quiet or accompanied by small movements of the head and legs. This movement was devoted first of all to contacts among the piglets, mostly playful movements but also fights. The piglets were also observed to massage each other's belly and to suck each other. Some animals performed this activity only sporadically while others very frequently and regularly. For example in piglet no. 396 this activity occupied more than one-third of the period of wakefulness, that is 76% of the whole period of observation. Feed uptake was, to a certain extent, synchronized and usually did not take much time. The piglets drank immediately after they had consumed the feed, but also during the quiet period, and in the limited space available to them this was the cause of mutual disturbance.

Discussion

Although the period of observation used in this study was short and concerned only few piglets, the results are consistent with our previous findings, i.e. that sleep occupied approximately half of the whole period of ob-

servation (Kotrbáček et al., 1990). The international literature also gives this proportion (Ruckebusch and Morel, 1968; Robert and Dallaire, 1986). Pokorná et al. (1988) found that the percentage of sleep was much higher in piglets during the first two weeks of life. However, these piglets were kept individually in thermal comfort and were fed *ad libitum*. Sleep occupied as much as 90% of the period of observation, suggesting that juvenile piglets are capable of considerably increasing the proportion of sleep under favourable conditions.

Table 1

Sleep–wake states, types of sleep and activities during wakefulness in the experimental piglets

Piglet no.	Total sleep, %	Non-REM sleep, % of total	REM sleep, % of total	Total wakefulness, %	Lying, % of total wakefulness	Movement, % of total wakefulness	Massaging and sucking, % of total wakefulness
389	53	82	18	47	62	28	10
384	60	78	22	40	27	64	9
385	63	75	25	37	51	48	1
383	66	88	12	34	45	55	0
395	39	73	27	61	53	43	4
396	24	76	24	76	15	50	35
Average	50.8	78.7	21.3	49.2	42.2	48.0	9.8
±SD	16.3	5.5	5.5	16.3	17.7	11.8	13.0

Marked individual differences were found in the duration of sleep in this experiment (Table 1). In piglet no. 395 sleep occupied 39% while in piglet no. 396 it took only 24% of the period of observation. This means that these piglets continued to be active even when the other piglets were falling asleep or were already sleeping. Piglet no. 396 devoted more than one-third of the time to sucking, and massaging the belly of, other animals. This ritual was so intensive that the piglet could not fall asleep without sucking another piglet. Regular massaging was also observed in two other piglets, though it was less frequent. This activity was an important source of disturbance in the group. This could be said to be one of the main reasons for the comparatively big difference between the time of lying (i.e. about 70% of the entire period of investigation) and sleep which occupied only half of this period.

The causes of massaging and sucking are not quite clear. Algers et al. (1988) associated these activities with the quick weaning of piglets and their transition to solid feeds. This change may affect piglets within a litter differently due to the dissimilar milk production of the sow's teats. Algers et al. (1988) suggest that the transition from an environment rich in external stimuli to keeping in cages (i.e. an environment poor in such stimuli) contributes to

the development of the sucking and massaging behaviour. The reduction of sleep, especially REM sleep, might play a certain role. Experiments proved that the deprivation of experimental rats of REM sleep led to increased locomotion and to a gradual increase of exploratory as well as aggressive behaviour (Hicks et al., 1979). Longer periods of the massaging ritual affected the piglets themselves as well as the whole group. Its common concomitant phenomena included general discomfort and disturbance of the lying and dozing, or already sleeping, piglets. The question arises whether these situations can be prevented in the practice, e.g. by isolating the piglets or rearing them in separate cages. Rearing on litter is known to reduce the incidence of these phenomena to a considerable extent.

As far as the type of sleep, i.e. the percentage of non-REM/REM cycles and the duration and frequency of REM episodes, is concerned, the piglets of this experiment virtually did not differ from the 1- to 14-day-old experimental piglets which had been kept individually under comfortable thermal and nutritional conditions in a previous experiment (Kotrbáček, 1988). It was interesting to note, however, that in some piglets the change of the type of sleep was accompanied by alteration of the respiratory rate. This was the case with piglets of higher live body mass which consistently had considerably elevated respiratory rate in the non-REM cycle. On the basis of what we know about REM sleep we can attribute this finding to a commencing thermal load. In accordance with our previous findings, during REM sleep the thermoregulating mechanisms are of reduced intensity, and polypnoea is also a thermoregulating response to heat (Kotrbáček, 1988). The decrease in the necessary output during REM sleep must then be balanced by an elevated respiratory rate in the following non-REM cycle (Fig. 1). The particular REM episodes may then gradually be reduced or even eliminated. In our experimental piglets this did not occur. In spite of this fact the above findings show that the thermal conditions of the cage suited piglets of lower live body mass much better than piglets of higher live body mass. It is evident that such a simple physiological observation can warn us of the danger of overheating the piglets. This is especially important if piglets are reared in cages as they cannot compensate elevated temperature by thermoregulating behaviour, e.g. by seeking cool, wet places, by cooling themselves in the drain near the walls, etc. This is one of the reasons why special attention should be paid to the thermal regimen of the cage.

From the results it can be concluded that not all piglets weaned into cages are capable of a quick adaptation to the new conditions and of establishing a physiological sleep–wake pattern. Some animals are characterized by highly active behaviour evoked especially by massaging and sucking reflexes. As this leads to unrest in the whole group, such animals should be isolated from the others.

Differences in respiratory rate between the non-REM and REM cycles are a manifestation of increased heat output and could be used for timely signalization of the piglets' excessive thermal load.

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HAEMAGGLUTINATING AND ADHESIVE ACTIVITY OF *BORDETELLA BRONCHISEPTICA* TREATED WITH SUB-MINIMAL INHIBITORY CONCENTRATIONS OF GENTAMICIN

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The effect of sub-minimal inhibitory concentrations of gentamicin on the haemagglutinating activity of *Bordetella bronchiseptica* was studied.

Gentamicin exerted dissimilar effect on the production of haemagglutinin to calf and dog erythrocytes. The drug significantly reduced the haemagglutinating titres of calf-negative strains with dog red blood cells while it caused only a slight decrease in the haemagglutinating titres of calf-positive strains with bovine erythrocytes.

The results support the view that bovine haemagglutinin is an adhesin of *B. bronchiseptica*.

Key words: Gentamicin, *Bordetella bronchiseptica*, haemagglutination, adherence

Bordetella bronchiseptica is a respiratory pathogen implicated in the aetiology of atrophic rhinitis (AR) of swine (Goodnow, 1980). It produces a range of potential virulence determinants: haemagglutinins, an adhesin to nasal epithelial cells (Semjén and Magyar, 1985), mouse lethal factor, cytotoxin (Collings and Rutter, 1985; Magyar, 1990), adenylate cyclase (Weiss and Hewlett, 1986) and haemolysin (Goodnow, 1980). Although their role is not clearly understood yet, colonisation of the nasal cavity by toxigenic *B. bronchiseptica* seems to be a prerequisite for its contribution to the development of AR.

The ability of *Bordetella bronchiseptica* to agglutinate bovine red blood cells (calf-positive strains) seems to be a virulence factor that correlates well with its adherence to nasal epithelial cells *in vitro* and *in vivo* (Yokomizo and Shimizu, 1979; Semjén and Magyar, 1985). Strains of *B. bronchiseptica* that fail to agglutinate bovine erythrocytes but maintain haemagglutinating activity (HA) to canine, lapine and porcine red blood cells are called calf-negative variants.

Growth in sub-minimal inhibitory concentrations (sub-MICs) of antibiotics has been shown to alter the attachment of bacteria to host cells (Shibl, 1985). Deneke et al. (1985) found that sub-MIC of tetracycline reduced the attachment of $K88^+$ enterotoxigenic strains of *Escherichia coli* to porcine small intestinal cells. However, the literature contains no data on whether HA of *B. bronchiseptica* or its adhesion to the target cells can be modified by sub-MIC of antimicrobial agents.

The present study was undertaken to determine if subinhibitory concentrations (sub-MICs) of gentamicin have any influence on the HA and *in vitro* attachment of *B. bronchiseptica* strains.

Materials and methods

Bacterial strains

B. bronchiseptica strains designated B58, CE, CF, BIII/4/4 and 5052 were isolated from different pig herds affected by clinical AR. These strains showed the characteristics of phase I isolates (Magyar, 1990), including the ability to agglutinate calf red blood cells (calf-positive strains). *B. bronchiseptica* strains B65, CEp27 and CFp61 were derived from the fresh isolates by repeated subculture on MacConkey agar. These strains did not agglutinate calf erythrocytes (calf-negative strains) and showed phase III characteristics (Magyar, 1990).

Determination of MICs

The MIC values of gentamicin for the strains were determined by the agar diffusion method described by Ericsson and Sherris (1971).

Haemagglutination

The strains were cultured on 5% sheep blood agar plates with or without gentamicin at a level of one third MICs for 24 h at 37 °C. Bacterial growths were harvested in phosphate buffered saline (PBS), pH 7.2, and the concentration of the suspension was adjusted to approximately 5×10^9 colony forming units (cfu) ml^{-1} by photometry. Haemagglutinating activity was determined on microtitre plates as described by Bemis and Plotkin (1982) using 1% calf and dog red blood cell suspensions.

Adherence assay

In vitro adherence was tested on porcine ciliated nasal epithelial cells isolated from the turbinates. The test was performed as described earlier by Semjén and Magyar (1985).

Statistical analysis

The *t* test was performed by Clin-Stat programme using Apple II computer.

Results

The MICs of gentamicin for the strains ranged between 2 and 4 $mg \times l^{-1}$.

The haemagglutinating activity of calf-positive and calf-negative strains grown with or without gentamicin is shown in Table 1. The sub-MICs of the drug did not affect significantly the haemagglutinating activity (HA) of calf-positive strains with bovine red blood cells. However, gentamicin significantly reduced the HA of both calf-positive and calf-negative strains of *B. bronchiseptica* with canine erythrocytes. The rate of decrease in HA titres with dog red blood cells was higher in the group of calf-negative strains than in that of calf-positive isolates.

Table 1

Haemagglutinating activity of *B. bronchiseptica* strains cultured on blood agar without or with one third MIC of gentamicin

Strains	HA titres[1] with					
	n	Calf RBCs		n	Dog RBCs	
		Control	Gentamicin		Control	Gentamicin
Calf-positive (5)	17	6.12 ± 0.21	5.47 ± 0.32	16	6.12 ± 0.37	4.19* ± 0.55
Calf-negative (3)	9	0	0	17	5.82 ± 0.34	1.94** ± 0.44

[1] Log_2 mean ± SE, n: number of tests, *: $P < 0.05$, **: $P < 0.001$

Table 2

Adherence of *B. bronchiseptica* strains to nasal ciliated epithelial cells

Strains	Number of adhered bacteria[1]	
	Control	Gentamicin
Calf-positive		
B58	22.83 ± 4.67[2]	22.0 ± 6.43
5052	23.88 ± 5.95	22.0 ± 4.95
Calf-negative		
CEp27	0.99 ± 1.49	0.18 ± 0.38

[1] Number of bacteria attached to 20 epithelial cells were counted and the means were calculated; [2] mean ± SD

Irrespective of gentamicin treatment, all growths of calf-positive *B. bronchiseptica* strains adhered well to porcine ciliated nasal epithelial cells (Table 2) while calf-negative variants failed to attach.

Discussion

The adherence of bacteria to epithelial cell surfaces is considered to be the first step in bacterial colonisation and initiation of several infectious diseases (Beachey, 1981). Phase I strains of *B. bronchiseptica* also attach to swine nasal epithelial cells (Yokomizo and Shimizu, 1979; Semjén and Magyar, 1985; Jacques et al., 1988) and by damaging the mucosal surface, they allow the poorly adhering *P. multocida* to colonise the target tissue (Pedersen and Barfod, 1981). *B. bronchiseptica* phase I strains agglutinate erythrocytes of several animal species including calf, whereas phase III variants lack the ability to agglutinate calf red blood cells and also to adhere to ciliated epithelial cells (Semjén and Magyar, 1985). Based upon this observation it has been suggested that bovine haemagglutinin functions also as a ligand in the linkage between *B. bronchiseptica* bacteria and host cells.

Attachment of bacteria to target cells can be modified by several factors including environmental conditions (ionic strength, pH, temperature etc.), chemical analogs of the bacterial ligand or host cell receptors, antibodies against the adhesins, cultural conditions (nutrients, temperature, antimicrobials) which may influence the expression and synthesis of bacterial surface ligand (Semjén and Magyar, 1983).

Inhibition of the binding of bacteria (*Escherichia coli*, *Streptococcus pyogenes*, *Neisseria gonorrhoeae*, *Proteus vulgaris*, *Actinomyces viscosus*, etc.) by antibiotics was demonstrated in a variety of *in vitro* models (Shibl, 1985). Antimicrobials can affect the adhesive ability of bacterial cells to different extent.

In this study, which was the first in this line, gentamicin had no influence on the adherence of phase I strains of *B. bronchiseptica*. In harmony with this observation, the HA activity with calf RBCs was hardly modified by this drug. The observed decrease in HA titres with bovine erythrocytes was not significant. On the contrary, the drug added to the culture medium at one third MIC resulted in a significant reduction of HA titres with dog RBCs. The decrease was more pronounced with calf-negative strains than with calf-positive parent strains. These data add further evidence to our earlier suggestion that the bovine HA and the ligand responsible for attachment to host cells are closely related.

Gentamicin exerts its antimicrobial effect through the inhibition of protein synthesis (Tai and Davis, 1979). HA and adherence are mostly mediated by fimbriae which are of protein nature. Although data on gentamicin are very scanty in this respect, some evidence clearly showed that at 50% MIC this drug decreased the piliation and mannose-specific binding of *E. coli* to human buccal epithelial cells (Eisenstein et al., 1980). Since the influence of gentamicin on the HA with dog RBCs markedly differed from that obtained

with bovine RBCs, it seemed logical to assume that *B. bronchiseptica* phase I strains had at least two types of haemagglutinin.

The few studies done on the association of haemagglutination with adherence have led to conflicting results. Vosbeck et al. (1982) found good agreement when examining the effect of low levels of antibiotics on the haemagglutination and epithelial adhesion of *E. coli*. However, in another study (Deneke et al., 1985) inhibition of the adhesion of *E. coli* to porcine small intestinal cells by tetracycline was not accompanied by any change in its HA. Our results may give some explanation for this inconsistency by demonstrating the difference in the behaviour of gentamicin-treated bacteria towards calf and dog RBCs. Therefore, it can be concluded that HA should be considered merely a screening tool for revealing modifications caused by antimicrobials at subMICs in the adherence of bacteria to target cells.

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PREVENTION AND TREATMENT OF ATROPHIC RHINITIS IN PIGS WITH GETROXEL, CHLORQUINALDOL AND OXYTETRACYCLINE

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The sensitivity of ten *Bordetella bronchiseptica* and ten *Pasteurella multocida* strains, each isolated from cases of atrophic rhinitis (AR), was examined in tube dilution test. Getroxel, chlorquinaldol and oxytetracycline and the former two ones combined with trimethoprim inhibited the growth of both species *in vitro*. The minimum inhibitory and the minimum bactericidal concentration was less than 0.5 μg/ml. When efficacy was tested in SPF pigs in the group fed a combination of Getroxel, chlorquinaldol and oxytetracycline (60 mg, 240 mg and 360 mg/kg of feed, respectively), *P. multocida* disappeared from the nasal cavity by the end of a 30-day treatment. *B. bronchiseptica* was reisolated in low numbers from 2 out of 9 piglets. The daily body mass gain was by 7.9% higher and the feed conversion rate was by 19% better than in the control group. After slaughter, only mild signs of AR were seen in 3 out of 9 piglets treated with the above-mentioned drug combination, while in the control group severe lesions were observed in 8 out of 9 pigs.

In treated commercial herds *P. multocida* disappeared from the nasal cavity of the piglets by the end of the treatment (42nd day of life), but the *B. bronchiseptica* strains could not be completely eliminated. Due to the treatment, mortality between 2 and 6 weeks of age decreased by 0.8–7.6%. Daily body mass gain was, on the average, 16.4% higher, the amount of feed needed for 1 kg body mass gain was by 15.3% lower and the duration of fattening was by 30.8 days shorter than in the control groups,

Key words: *Pasteurella multocida*, *Bordetella bronchiseptica*, atrophic rhinitis. carbadox, chlorquinaldol, oxytetracycline

Atrophic rhinitis (AR) of swine is an infectious disease of great economic significance in all countries where pig rearing is intensive. Although the exact cause of AR is still debated, toxin-producing *Pasteurella multocida* and *Bordetella bronchiseptica* strains are recognized as the primary pathogens (Pedersen and Barfod, 1981; Rutter and Rojas, 1982). It is also accepted that the severity of the pathological lesions is greatly influenced by management and hygienic conditions (Giles and Smith, 1983; de Jong, 1981).

Vaccines have proved to be effective in preventing the disease (Barfod and Pedersen, 1984; Éliás and Török, 1984; Giles and Smith, 1983).

Chemoprophylaxis, viz. sulphonamides, trimethoprim. tylosin, carbadox, tetracyclines and their combinations by medication of weaner and grower rations, and sometimes sow rations, is another way of controlling AR (Farrington and Shively, 1979; Giles et al., 1981; Mefford et al., 1983; Rutter, 1981).

Since AR causes considerable losses to the Hungarian pig industry, our aim was to compose a drug combination capable of preventing the disease, and to try the new combination in the field.

Materials and methods

In vitro tests of drugs

Sensitivity of 10 *B. bronchiseptica* and 10 *P. multocida* strains, each isolated from cases of AR, was examined in tube dilution test using phenol red dextrose broth (DIFCO). The medium contained increasing concentrations (0.5, 1, 5, 10, 25, 50, 100 and 200 μg/ml) of Getroxel (a Hungarian-made equivalent of carbadox), chlorquinaldol, oxytetracycline or trimethoprim as well as certain combinations of these drugs. The combinations contained equal amounts of the components. The tubes were inoculated with 25 μl of a 16–18-h culture of the test organism and incubated at 37 °C for 4 days. The lowest concentration of the drug capable of preventing bacterial growth for 24 h was regarded as minimum inhibitory concentration (MIC) while that inhibiting bacterial multiplication for 4 days was the minimum bactericidal concentration (MBC).

Efficacy testing in SPF pigs

In an experiment using 5 groups of four-week-old SPF piglets with 10 animals in each group four combinations of the above-mentioned drugs were tested.

A:	Getroxel	60 mg/kg feed
	Chlorquinaldol	120 mg/kg feed
	Oxytetracycline	360 mg/kg feed
B:	Getroxel	60 mg/kg feed
	Chlorquinaldol	240 mg/kg feed
	Oxytetracycline	360 mg/kg feed
D:	Getroxel	60 mg/kg feed
	Chlorquinaldol	240 mg/kg feed
	Trimethoprim	360 mg/kg feed
E:	Getroxel	30 mg/kg feed
	Chlorquinaldol	60 mg/kg feed
	Oxytetracycline	360 mg/kg feed

The fifth group (C) served as infected but not treated control.

Piglets were inoculated intranasally with 0.5 ml (6×10^8 cfu/ml) of a broth culture of toxigenic *P. multocida* and *B. bronchiseptica* on the 30th day of life. Treatment with medicated food was started on the same day and lasted 30 days. Appearance of clinical signs, feed consumption, body mass gain, feed conversion rate and presence of *P. multocida* and *B. bronchiseptica* in the nasal cavity were monitored. Nasal swabs were taken at the beginning of the experiment and on the 15th, 30th and 60th days after infection. To reisolate *P. multocida*, samples were cultured on beef blood agar containing 0.5% yeast extract; for reisolation of *B. bronchiseptica*, MacConkey agar containing 10 μl/ml penicillin and 50 μl/ml nitrofurantoin (Éliás and Galgóczi, 1981) was used.

The pathologic lesions were examined at slaughter on the 60th day post infection after a transverse section was made at the level of the first premolar tooth (Miniats and Johnson, 1980). The lesions were scored as follows: (+): mild lesion of one turbinate; +: severe atrophy of one or mild lesion of two turbinates; ++: severe atrophy of two or mild atrophy of more turbinates; +++: severe atrophy of several turbinates; ++++: severe atrophy of all turbinates, distortion of the nose.

The antibacterial activity of muscle, liver, kidney, fat and lung samples on the 30th day after the end of the treatment was measured (Ács and Simonffy, 1984). Residue of Getroxel was checked by gas chromatography (Packard 419 EC type) (USDA, 1987).

During the experiment one piglet each from groups B, C and D died of an intercurrent disease.

Field trial for efficacy

Based on the results obtained in SPF piglets, efficacy of a combination of Getroxel, chlorquinaldol and oxytetracycline was tested in four commercial pig herds severely affected with AR. Several test groups together with control groups were formed from the offspring of 30–40 sows. On the four farms, a total of 3100 piglets received medicated feed. The dose of the drugs was as follows: 3 mg Getroxel/kg body mass, 12 mg chlorquinaldol/kg body mass, and 18 mg oxytetracycline/kg body mass.

A drug mixture containing the following compounds (1000 g) was prepared: 12 g Getroxel, 48 g chlorquinaldol, 72 g oxytetracycline, 424 g starch (amylum), 424 g dextrose and 20 g silicon dioxide. Piglets received medicated feed containing the above-mentioned mixture at 1.5% from the 14th to the 28th day (i.e. up to weaning) and thereafter 0.5% up to the 6th week of life. Nasal swabs were taken from 15 piglets of each medicated and control

group at the beginning and at the end of the treatment at the age of 2 and 6 weeks. Tests for the presence of *P. multocida* and *B. bronchiseptica* strains were done as above.

Examination of in vitro developed resistance

After the efficacy trials, development of induced antibiotic resistance was examined *in vitro* against the mixture of Getroxel, chlorquinaldol and oxytetracycline. One strain each of *B. bronchiseptica*, *P. multocida*, *Escherichia coli* and *Staphylococcus aureus* was serially passaged in phenol red dextrose broth containing rising concentrations of equal quantities of the drugs. The development of antibiotic resistance was examined through 20 passages.

Results

In vitro susceptibility

Getroxel, chlorquinaldol and oxytetracycline, and the former two ones combined with trimethoprim, inhibited the growth of both *P. multocida* and *B. bronchiseptica* strains *in vitro;* the minimum inhibitory concentration was less than 0.5 μg/ml (Table 1). The minimum bactericidal concentrations showed

Table 1

Minimum inhibitory concentration of selected chemotherapeutic agents and their combinations against ten *B. bronchiseptica* and ten *P. multocida* strains (μg/ml)

	B. bronchiseptica	*P. multocida*
Getroxel	200–>200	1–10
Chlorquinaldol	5–100	<0.5
Oxytetracycline	<0.5–1	<0.5
Trimethoprim	50–200	<0.5–25
Getroxel–Chlorquinaldol	25	<0.5
Getroxel–Chlorquinaldol–Oxytetracycline	<0.5	<0.5
Getroxel–Chlorquinaldol–Trimethoprim	<0.5	<0.5

a similar pattern (Table 2). The combination of Getroxel, chlorquinaldol and oxytetracycline in a concentration of less than 0.5 μg/ml not only inhibited but even killed strains of both species.

Efficacy tests with SPF piglets

The infected but not treated control piglets became ill within 2 weeks after infection: they had nasal discharge and all were sneezing. Mild clinical signs were seen among the piglets of group D; the other animals showed no

Table 2

Minimum bactericidal concentration of selected chemotherapeutic agents and their combinations against ten *B. bronchiseptica* and ten *P. multocida* strains (μg/ml)

	B. bronchiseptica	*P. multocida*
Getroxel	200–>200	1–10
Chlorquinaldol	50– 100	<0.5
Oxytetracycline	<0.5–5	<0.5–1
Trimethoprim	100–200	<0.5–25
Getroxel–Chlorquinaldol	25	<0.5
Getroxel–Chlorquinaldol–Oxytetracycline	<0.5	<0.5
Getroxel–Chlorquinaldol–Trimethoprim	<0.5	<0.5

clinical signs. The numbers of *B. bronchiseptica* and *P. multocida* carriers are shown in Table 3. In group B, *P. multocida* disappeared from the nasal cavity by the end of the treatment, but *B. bronchiseptica* could be isolated in low numbers from 2 out of 9 piglets. In groups A and E the bacteriological results were less expressed, the number of *P. multocida* and *B. bronchiseptica* carriers slightly decreased. In group D, the combination of Getroxel, chlorquinaldol and trimethoprim did not prove to be effective. Furthermore, the daily body mass gain was by 7.9% and the feed conversion rate was by 19% higher in group B than in the control. The incidence of pathological lesions in SPF pigs at slaughter is presented in Table 4. In the control group severe lesions were found in 6 out of 9 animals (Figs 1 and 2). In group E no lesions were found at all, while in group B 3 pigs showed very mild lesions (Fig. 3). In group D, where the combination with trimethoprim was used, 8 animals showed more or less pronounced lesions (Figs 4 and 5).

Table 3

Number of carriers of *B. bronchiseptica* (B. b.) and *P. multocida* (P. m.) among SPF piglets after infection

Group	0 day		15th day		30th day		60th day	
	B. b.	*P. m.*	*B. b.*	*P. m.*	*B. b.*	*P. m.*	*B. b.*	*P. m.*
A	0/10*	0/10	n.t.	n.t.	3/10	0/10	3/10	0/10
B	0/10	0/10	7/10	1/10	2/9	0/9	2/9	0/9
D	0/10	0/10	10/10	1/10	8/10	1/10	n.t.	n.t.
E	0/10	0/10	n.t.	n.t.	4/10	1/10	4/10	2/10
Control	0/10	0/10	10/10	4/10	4/9	4/9	n.t.	n.t.

* Number of carriers/number of animals examined; n.t.: not tested

Table 4

Pathologic lesions in SPF pigs after slaughter

Group	Lesion scores					
	Negative	(+)	+	+ +	+ + +	+ + + +
A	7	—	3	—	—	—
B	6	2	1	—	—	—
D	1	1	2	5	—	—
E	10	—	—	—	—	—
Control	2	–	1	2	2	2

Muscle, kidney, liver, fat and lung samples of the piglets slaughtered 30 days after the end of treatment had no antibacterial activity. No Getroxel residue could be detected.

Efficacy in farm experiments

The rate of carriers among 2-week-old piglets before beginning the experiments is given in Table 5. Piglets on farms C and D were heavily infected by both *B. bronchiseptica* and *P. multocida*. By the end of treatment *P. multo-*

Table 5

Number of carriers among 2-week-old piglets in farm experiments before treatment

Farms	*P. multocida* heavy	medium	mild	negative infection	*B. bronchiseptica* heaxy	medium	mild	negative infection	Free of both species
Farm A	2/15*	1/15	0/15	12/15	7/15	2/15	1/15	5/15	4/15
Farm B	0/15	0/15	0/15	15/15	4/15	0/15	1/15	10/15	10/15
Farm C	4/15	2/15	0/15	9/15	4/15	1/15	2/15	8/15	4/15
Farm D	3/15	5/15	0/15	7/15	8/15	3/15	0/15	4/15	0/15

* Number of carriers/number of animals examined

Table 6

Number of carriers among 6-week-old piglets in farm experiments after treatment

Farms	*P. multocida* heavy	medium	mild	negative infection	*B. bronchiseptica* heavy	medium	mild	negative infection	Free of both species
Farm A	0/15*	0/15	0/15	15/15	0/15	0/15	1/15	14/15	14/15
Farm B	0/15	0/15	0/15	15/15	0/15	0/15	0/15	15/15	15/15
Farm C	0/15	0/15	0/15	15/15	1/15	1/15	0/15	13/15	13/15
Farm D	0/15	0/15	1/15	14/15	1/15	1/15	1/15	12/15	11/15

* Number of carriers/number of animals examined

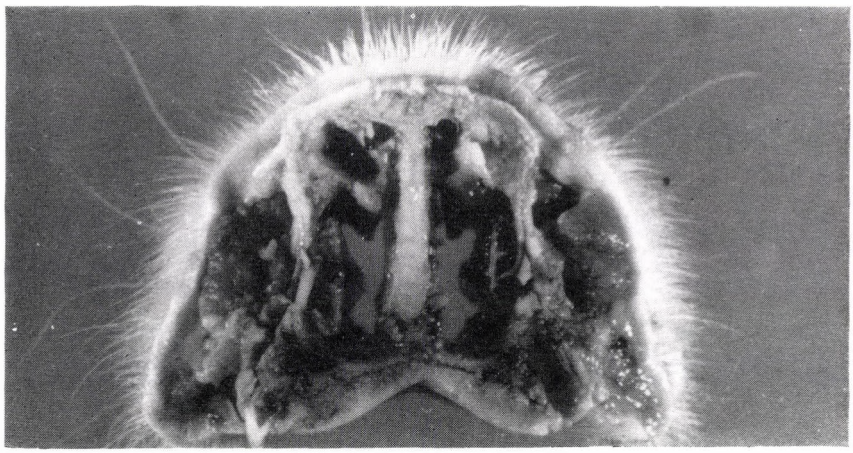

Fig. 1. Severe lesions in the nose of a control pig

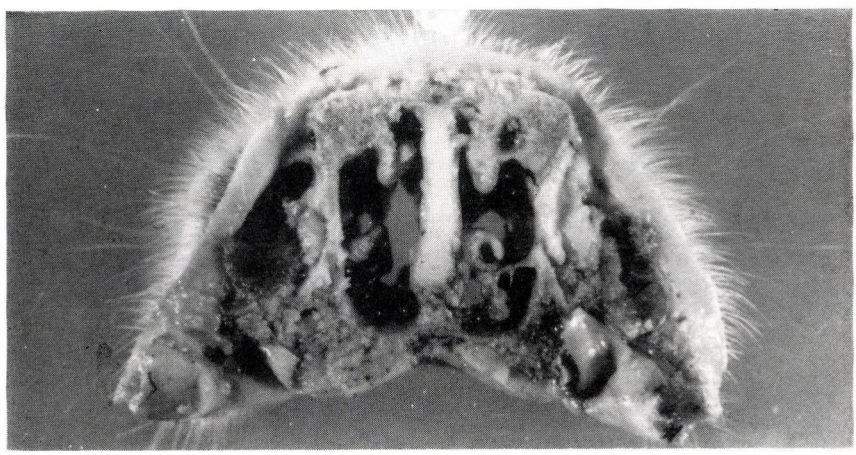

Fig. 2. Severe atrophy in the nose of a control pig

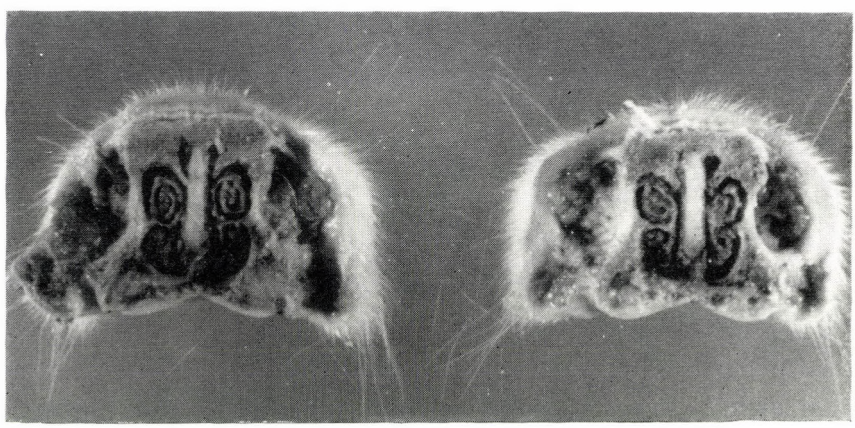

Fig. 3. Slight lesions in pigs of group B

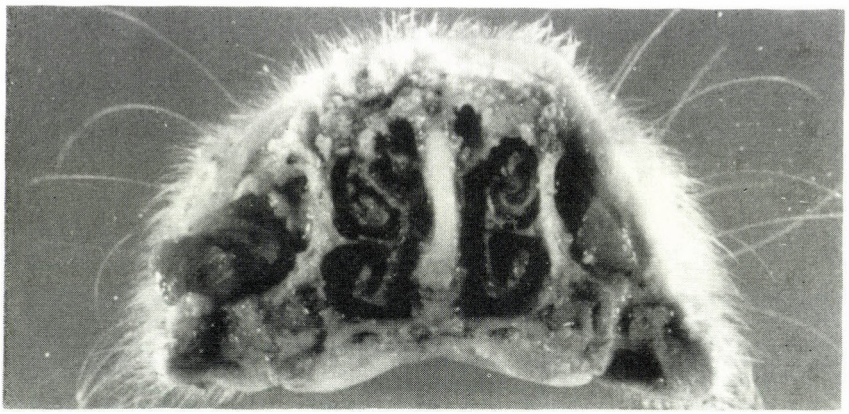

Fig. 4. Lesions in the lower turbinates (group D)

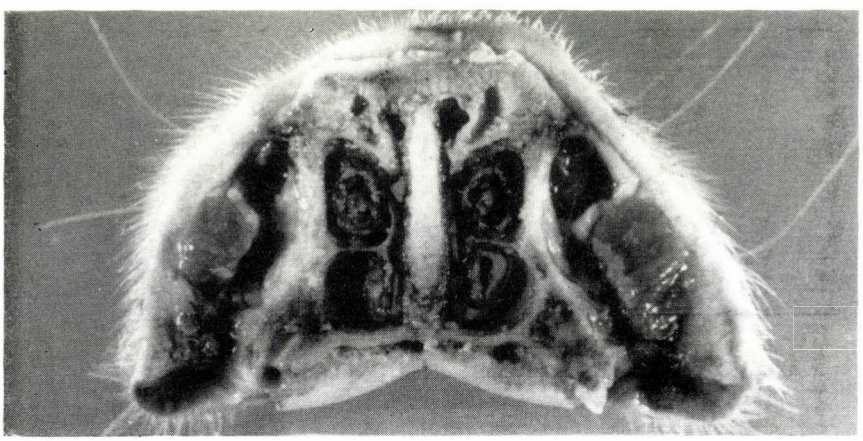

Fig. 5. Severe atrophy in the lower turbinates (group D)

cida disappeared from the nasal cavity of the piglets but *B. bronchiseptica* could not be completely eliminated (Table 6).

The economic aspects of the farm experiments are summarized in Table 7. The mortality of piglets during the first six weeks of life decreased by 0.8–7.6% (on the average by 3.7%) as compared to the controls. Daily body mass gain was, on the average, by 15.5% higher than in the control herds, the feed conversion rate was by 15.3% higher, and the duration of the fattening period was by 30.8 days shorter than in the control groups. The incidence of lesions in the nasal cavity of pigs at slaughter decreased by 54.7%.

Table 7

Economic aspects of the farm experiments

Variable	Treated group	Control group
Body mass, kg		
at 2 weeks old	3.60 ± 0.74	3.78 ± 0.86
at 6 weeks old	9.03 ± 1.96	10.47 ± 3.51
Mortality, % (0–6 weeks)	7.76	11.49
Duration of fattening, days	198.70 ± 6.65	229.50 ± 11.76*
Daily body mass gain, kg	0.52 ± 0.01	0.45 ± 0.03*
Feed conversion rate, kg/kg	3.47 ± 0.19	4.10 ± 0.21*
Animals with atrophy of the nose, %	17.5	72.2

* significant difference ($p < 0.001$)

In vitro antibiotic resistance

After 20 passages, the MIC of the combination for *P. multocida* showed no change, while that for *S. aureus* increased to 1 μg/ml, for *B. bronchiseptica* to 25 μg/ml and for *E. coli* to 100 μg/ml.

Discussion

Several experiments have been carried out on the chemoprophylactic control of AR. Sulphonamides alone or combined with trimethoprim, tylosin plus sulphadimidine were found to be effective; however, antibiotic resistance limited their use in the field (Giles et al., 1981; Rutter, 1981). Carbadox in combination with sulphamethazine reduced the prevalence of lesions of AR and improved growth rate and feed efficiency; however, in some field cases the proportion of animals with turbinate lesions and their score remained considerable (Farrington and Shively, 1979). Oxytetacycline therapy con-

sisting of three injections before weaning followed by feed medication or long-acting oxytetracycline injections prevented AR; these methods are, however, time consuming (Mefford et al., 1983; Rutter, 1981).

On the basis of *in vitro* antibiotic susceptibility tests, combinations of Getroxel, chlorquinaldol and oxytetracycline, and the former two drugs combined with trimethoprim, proved to be effective against *P. multocida* and *B. bronchiseptica* strains. The MIC and MBC were less than 0.5 μg/ml for both combinations.

Clinical signs of AR could be induced by inoculating 28-day-old SPF piglets with toxigenic *P. multocida* and *B. bronchiseptica* strains. At the age of 90 days, lesions of the turbinates and distortion of the nose were evident. In our experiment, drug mixture B proved to be the most effective in preventing AR and minimizing economic losses due to the disease. This combination was able to prevent colonization of the nasal mucous membrane of SPF piglets by a virulent *P. multocida* strain and it could repress *B. bronchiseptica* as well, thus reducing the risk of AR. Combination B helped to increase the daily body mass gain and improved the feed conversion rate. Reduction of the dose of the compounds (groups A and E) led to impaired efficacy.

The results of efficacy trials in SPF piglets were confirmed by the field trials. The moderate infection on farm A and the slight infection on farm B both were practically eliminated; on the heavily infected farm D *P. multocida* and *B. bronchiseptica* considerably decreased in number. The sporadic bacterium carriers were the smaller and weaker pigs which were surpassed at feeding so that they could not take up the drugs in effective doses.

Elimination of *P. multocida* and *B. bronchiseptica* strains from the nasal cavity or their reduction in number is important because piglets are susceptible to the agents of AR only in the first 6 weeks of life. If they get infected later, clinical signs and economic losses usually will not occur (de Jong, 1981).

The bacteriological results were confirmed by the clinical signs, by the lack of lesions in the nasal cavity at slaughter, and by the economic indices (daily weight gain, feed conversion rate, duration of fattening).

The combination of Getroxel, chlorquinaldol and oxytetracycline described above was capable of preventing AR and the consequent economic losses. We failed to develop *in vitro* antibiotic resistance to the drug mixture. Besides preventing AR, the combination can eliminate other infectious diseases of piglets at the age of 2–6 weeks (among others streptococcosis, *E. coli* diarrhoea, swine dysentery, *Haemophilus* infections, etc.).

Combination B has been registered in Hungary under the name of Vetricin® (EGIS Pharmaceuticals, Budapest).

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A NEW SEROTYPE OF *ERYSIPELOTHRIX RHUSIOPATHIAE* ISOLATED FROM PIG SLURRY

(SHORT COMMUNICATION)

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Since Watts (1940) observed that strains of *Erysipelothrix rhusiopathiae* could be divided into two antigenically different groups, numerous serotypes have been found.

Kucsera (1972), who examined and typed strains of *E. rhusiopathiae* from different parts of the world, discovered that some strains with the same designation were of different serotypes and other strains with different designation belonged to the same serotype. Kucsera, therefore, proposed to introduce a system using Arabic numerals instead of capitals and with reference strains within each serotype. By that time 15 different serotypes had been reported. By now 10 additional serotypes have been found and described: serotype 16 by Fábián et al. (1973), serotypes 17, 18, 19 and 20 by Wood et al. (1978), serotypes 21 and 22 by Nørrung (1979), serotypes 23 and 24 by Xu et al. (1984, 1986), and serotype 23 by Nørrung et al. (1987). At the time of publication of serotype 23 by Nørrung et al., the authors were not aware of two Chinese papers dealing with serotypes 23 and 24, since they were published in the Chinese language. Having received and typed the two Chinese strains, they proved to be of different serotypes and not identical with our serotype 23. For this reason, it has been decided to change designation of our strain, KS20A, to serotype 25.

Molin et al. (1989) found among strains of different serotypes one strain (L136) isolated from pig slurry which could not be typed by means of any of the type sera available (1 through 25).

Rabbits were immunized with antigen from the strain and the antisera produced were tested with antigen from all recognized type strains. The method of antigen production, immunization of rabbits and the agar gel diffusion test had been described by Nørrung et al. (1987).

Antisera produced with strain L136 did not react with any antigen produced from the recognized 25 serotypes. The only reaction observed was using

antiserum and antigen from strain L136, indicating that strain L136 is a serotype which had not been described earlier. The strain was designated serotype 26 and strain L136 is proposed as reference strain.

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IMMUNE REACTIONS AND IMMUNOPATHOLOGIC CHANGES INDUCED IN OVINE FETUSES AND LAMBS BY MAEDI-VISNA VIRUS

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Seventy- to 80-day fetuses of Merino ewes were inoculated intramuscularly *in utero* and 2-week-old lambs of the same breed intratracheally with $10^{6.3}$ $TCID_{50}$/0.1 ml of maedi-visna virus strain K1512 isolated in Iceland.

While no precipitins appeared in the serum of fetuses, such antibodies were demonstrable in the lambs from postinoculation (PI) day 30. Indirect immunofluorescence (IIF) revealed the presence of antibodies in samples from both fetuses and lambs; the detectability of these antibodies, however, varied even within a given animal during the experiment. The serologic results were inversely proportional to the kinetics of circulating immune complex (cIC) production.

By the lymphocyte stimulation test (LST), the blastogenic transformation of lymphocytes as measured by ^{3}H-TdR incorporation was 6–8% and 6–14% in the fetuses and lambs, respectively.

By antibody-dependent cell-mediated cytotoxicity (ADCC) test, cytotoxic capacity (10–14%) was only demonstrable in lambs inoculated at 2 weeks of age, in the 2nd month of life.

Histologic examination showed that in the lungs of both fetuses and lambs lympho-histiocytic infiltration developed from PI week 4. This was later joined by lymphoid hyperplasia in the peribronchial lymph nodes. T lymphocyte proliferation was dominant in these lesions as shown by a histochemical procedure (alpha-naphthyl--acetate-esterase, ANAE). By immunofluorescence (IF), deposited immune complexes (IC) were demonstrable in various organs (wall of cerebral ventricles, endothelium of blood vessels of the brain stem, cerebellum, lungs, kidneys). These IC may play an important role in the pathogenesis of maedi-visna.

Key words: Maedi-visna, virus, sheep, fetus, lamb, immune reactions, immunopathological changes

The pathogenesis of maedi-visna depends not only on the breed and age of sheep (Narayan et al., 1974; Petursson et al., 1976) but also on the virulence of the causative virus strain (Lairmore et al., 1988). The disease may manifest itself either as chronic interstitial pneumonia (Sigurdsson, 1954) or as progressive meningoencephalitis (Sigurdsson and Palsson, 1958). Owing to the "multiple tissue tropism" of the virus (Lairmore et al., 1988), these manifestations may be accompanied by arthritis and mastitis (Oliver et al., 1981; Narayan and Cork, 1985; Deng et al., 1986).

Immune responses to the virus may also vary depending on the virulence of virus strains (Thormar et al., 1966; Clements et al., 1988). However, neither precipitins nor virus-neutralizing antibodies can prevent the development of the pathological processes (Klein et al., 1985; Lairmore et al., 1988) as the virus is able to evade the host's defences (Haase, 1975; Nathanson et al., 1985)

and disseminate in the organism by a so-called "Trojan horse" mechanism (Narayan et al., 1977; Clements et al., 1980; Peluso et al., 1985). Antigenic variation has been invoked as an explanation for virus spread (Thormar et al., 1966; Clements et al., 1980; Lutley et al., 1983). Persistent viral infection may lead to the formation of immune complexes (Johnson et al., 1983; Cheevers and McGuire, 1985).

The aim of this work was to study the immune reactions and immunopathological changes induced by the virus in a model experiment.

Materials and methods

Animals. Twelve 70- to 80-day-old fetuses of Merino ewes (group A) and 12 two-week-old Merino lambs (group B) were used. A suspension of maedi-visna virus strain K1512 isolated in Iceland, containing $10^{6.3}$ $TCID_{50}$/ml virus, was used for experimental infection as follows. In group A, the fetuses of 6 laparotomized ewes were inoculated intramuscularly with 0.1 ml of the virus suspension, and the fetuses of 6 control ewes were treated, in the same way, with virus-free buffer solution. On postinoculation (PI) days 30 and 60 one fetus was extracted from one experimental and one control ewe, respectively. The lambs delivered by the other ewes were exsanguinated at 1 and 2 months of age, respectively. Blood and organ (spleen, liver, lungs, kidney, lymph node, brain, spinal cord) samples were taken to assess the humoral and the cell-mediated immune responses and the immunopathologic changes. In group B, 6 lambs were inoculated intratracheally with 0.3 ml of virus suspension and the control lambs were treated, by the same route, with virus-free buffer solution. Blood samples were taken from the experimental and the control lambs at 30-day intervals for a period of 6 months. At the time of blood sampling one experimental and one control lamb each was killed, and organ samples described for group A were taken from them.

Agar–gel precipitation (AGP) test. The sera were tested in Ouchterlony's system against an antigen prepared from maedi-visna virus of sheep (NADC, Ames, USA).

Indirect immunofluorescence (IIF) test. The viral antigens were adsorbed onto the surface of guinea pig erythrocytes and tested against different dilutions (1 : 2–1 : 6) of the test sera, then against an anti-sheep Ig FITC conjugate (MILES, Vienna).

Lymphocyte separation. Lymphocytes were separated from heparinized blood samples with Ficoll-Paque. Cell density was adjusted to 10^6/ml, then 1.5-ml aliquots of the cell suspension were measured into Leighton tubes.

Lymphocyte stimulation test (LST). After adding 0.1 ml viral antigen, the stimulated cell suspensions were incubated at 37 °C for 72 h. At 56 h of

incubation 10 μCi ^{3}H-TdR was added to each sample and the samples were covered with Ilford Nuclear (Ciba-Geigy) emulsion. The rate of blastogenic transformation was determined by autoradiography on the basis of ^{3}H-TdR incorporation.

Antibody-dependent cell-mediated cytotoxicity (ADCC). Guinea pig erythrocytes sensitized with maedi-visna virus were used as target cells. The reaction was carried out as described earlier (Kiss and Tuboly, 1981), with the only difference that 10 μl anti-maedi-visna serum (AGP titre: 1 : 2, dilution: 1 : 10) was added to the samples.

Determination of circulating immune complexes (cIC). Immune complexes present in the serum were determined by a polyethylene glycol (PEG)-6000 precipitation method (Haskova et al., 1978) as modified by Mayr et al. (1982). Spectrophotometry was carried out at 450 nm using 1% aggregated sheep IgG treated in a water-bath of 63 °C for 30 min as standard. The presence of viral antigens and IgG in the precipitate was determined by ELISA (Neel and Stevens, 1980) after washing the precipitate with 3.75% PEG-6000 solution and dissolving it in PBS (pH 7.2).

Anti-maedi-visna immune serum and anti-sheep IgG peroxidase conjugate (HUMAN Institute, Budapest) were used as immunological reagents.

Determination of deposited immune complexes (dIC). The sections prepared from organ samples fixed and embedded according to Sainte-Marie (1962) were examined by immunofluorescence using anti-sheep IgG and anti-sheep IgM conjugates. Evaluation was carried out in an NU2 Zeiss microscope.

Determination of T and B lymphocytes. This was done by the alpha-naphthyl-acetate-esterase (ANAE) procedure (Mueller et al., 1975) and by direct immunofluorescence using anti-sheep IgG and anti-sheep IgM conjugates in sections prepared from thymus, spleen, lymph node, lung and brain samples from ovine fetuses and lambs.

Histologic examination. Samples were embedded in paraffin and the sections were stained with haemalum as described in manuals.

Results

Differences were found in the appearance and persistence of antibodies detectable by AGP and IIF. In fetuses of group A, the IIF test demonstrated serum antibodies already on PI day 60, while precipitating antibodies appeared in the serum only 2 months after birth.

The sera of experimental animals of group B were positive both by the IIF and the AGP test on PI day 30, and seropositivity changed as shown in Table 1.

Table 1

Antibodies demonstrable in group B animals by IIF and AGP

No. of animal	Sampling times (PI months)						
	1	2	3	4	5	6	7
Infected animals							
1	—/—	+/—					
2	—/—	+/—	+/—				
3	+/+	+/—	—/—	+/+			
4	—/—	—/—	—/—	—/—	—/—		
5	—/—	+/—	+/—	+/—	—/—	+/—	
6	+/—	+/+	+/—	+/+	+/—	+/—	+/—
Control animals							
7	—/—	—/—	—/—	—/—	—/—	—/—	—/—
8	—/—	—/—	—/—	—/—	—/—	—/—	
9	—/—	—/—	—/—	—/—	—/—		
10	—/—	—/—	—/—	—/—			
11	—/—	—/—	—/—				
12	—/—	—/—					

+/ = positive by the IIF test (serum dilution: 1 : 2)
/+ = positive by the AGP test (serum dilution: 1 : 2)

After PI day 30, cICs were detectable in serum samples from both the fetuses (group A) and the lambs (group B, as shown in Table 2). Their amount was inversely related to the positivity of the serologic tests. Namely, if the amount of cICs exceeded a spectrophotometrically measured extinction value of 0.020, both the AGP and the IIF tests were negative.

Beyond PI day 30 the LST showed a blastogenic transformation rate of 6–8% and 6–14% in samples from group A and group B, respectively.

ADCC in xenogenous system was negative for samples from both fetuses and lambs. In the 2nd month after inoculation, lambs inoculated at 2 weeks of age showed a cytotoxic capacity (CTC) of 10–14% (except lamb no. 4). Subsequently the test became negative.

On PI day 60, dICs were observed in peribronchial areas of the lungs and in the wall of bronchi, in the renal glomeruli and in the wall of the cerebral ventricles of the fetuses by direct IF using IgM conjugate.

Using anti-IgG and anti-IgM conjugates, in samples from lambs dICs were demonstrable in the walls of blood vessels and bronchi in the lungs, in the glomeruli and tubules of the kidney, and around the central arteries of the liver. Deposited ICs were also detected in the endothelium of blood vessels of the brain stem in samples taken from two experimental animals

Table 2

Circulating immune complexes (cIC) in group B inoculated at lamb age

No. of animal	Sampling times (PI months)						
	1	2	3	4	5	6	7
Infected animals							
1	0.040	0.020					
2	0.080	0.010	0.015				
3	0.020	0.010	0.070	0.015			
4	0.075	0.080	0.060	0.080	0.085		
5	0.030	0.020	0.010	0.020	0.040	0.010	
6	0.010	0.020	0.015	0.020	0.010	0.010	0.020
Control animals							
7	0.000	0.000	0.000	0.000	0.000	0.000	0.000
8	0.000	0.000	0.000	0.000	0.000	0.000	
9	0.000	0.000	0.000	0.000	0.000		
10	0.000	0.000	0.000	0.000			
11	0.000	0.000	0.000				
12	0.000	0.000					

cIC: equivalent to mg/ml aggregated sheep IgG

in the 2nd and 6th month after inoculation, and in one animal such ICs were seen at the border of the grey and white matter of the cerebellum. Spleen, lymph node and thymus samples were consistently negative.

Histologic examination at PI week 4 revealed, in both the fetuses and the lambs, proliferative lymphohistiocytic infiltration in the interstitium of the lungs, mainly perivascularly. According to the histochemical examination (ANAE), T lymphocytes were dominant in these lesions. In the lambs, the above lesions were later joined by follicular and paracortical lymphoid hyperplasia in the peribronchial lymph nodes.

Discussion

In recent years numerous data have been obtained on immune reactions to lentiviruses and the pathogenesis of diseases caused by them (Brahic et al., 1981; Narayan et al., 1982; Haase et al., 1984; Gendelman et al., 1985; Narayan et al., 1985; Kennedy et al., 1985; Sharp and Jorgensen, 1985; Haase, 1986; Zink et al., 1987; Haas, 1987).

As regards the kinetics of humoral immune response and antibody production, several authors (Stowring et al., 1979; Clements et al., 1980; Dahlberg

et al., 1981; Johnson et al., 1983) reported the production of antibodies to viral glycoproteid antigens already at an early stage of infection. Precipitins were demonstrable as early as PI week 5 (Lairmore et al., 1988).

In this study, precipitins were demonstrated in the serum of lambs on PI day 30. No precipitins were produced in the fetuses; however, in the 2nd month after birth their presence was demonstrable in the serum. The kinetics of antibody production was studied also by IIF, a test which has been found to be sufficiently sensitive not only in maedi-visna (Dawson et al., 1985) but also in HIV infections (Gallo et al., 1986; Jackson and Balfour, 1988). The results of IIF indicate that antibodies to the virus are produced in both the fetuses and the lambs; however, their amount may vary even within a given animal at the different testing times and may be below the threshold of detection.

Several explanations exist for this "fluctuation", including reduced activity of antibodies to changing viral antigens (Gudnadottir, 1974; Kennedy-Stoskopf and Narayan, 1986), low level of antibody production or failure of antibodies to reach the threshold of detection (Lairmore et al., 1988). Our results show that the phenomenon is related to cIC formation. Formation of cICs was observed in both infected fetuses and infected lambs, and its kinetics was inversely proportional to the detectability of antibodies, i.e. at a cIC value exceeding an extinction of 0.20 the serologic tests gave negative results. Production of cICs has been observed in other lentiviral infections including infectious anaemia of horses and arthritis-encephalitis of goats (Johnson et al., 1983; Cheevers and McGuire, 1985), and the kinetics of their production was studied in HIV infections (Euler et al., 1985; Morrow et al., 1986; Lange et al., 1987). The detectability of antigen in the serum (Paul and Falk, 1986) was closely related to changes in antibody titres to *gag* (p24) antigen (Lange et al., 1986).

Few comparable data exist on *in vitro* reactions of the peripheral lymphocytes. In our experiments, the result of LST was 6–8% and 6–14% for the fetuses and the lambs, respectively.

ADCC was negative for samples from fetuses and lambs of group A. In samples of group B lambs, however, a cytotoxic capacity of 10–14% was measured in the 2nd month after infection. Later on ADCC was consistently negative. This is in harmony with the findings of Fauci and Lane (1987), namely that in HIV infections antibodies appearing in the serum are first active against both *gag* (p24) and *env* (p120) antigens by ADCC, but later, with the progression of the disease process, they may lose their responsiveness to *gag* and retain activity to the *env* (p120) antigen only.

At PI week 4, histologic examination revealed lympho-histiocytic infiltration containing mainly T cells in perivascular areas of the lungs. Later this was joined by lymphoid hyperplasia in the peribronchial lymph nodes.

In a study by Lairmore et al. (1988) lymphoproliferative processes were observed only after PI week 10.

No interpretable histopathologic changes were found in the brain of fetuses and lambs. This is consistent with the results of Narayan et al. (1977) who inoculated lambs intracerebrally with the American strain D1-2 and the Icelandic strain 1514. Although they could detect the virus in brain samples, no histopathologic lesions were seen. In this study, deposited ICs were demonstrated in the wall of the cerebral ventricles of the fetuses and in the endothelium of blood vessels of the brain stem as well as at the border of the gray and white matter in the cerebellum in lambs inoculated *in utero* or at 2 weeks of age.

IC formation due to persistent viral infection and the deposition of ICs in different tissues have been reported for other lentiviral infections, too (Cheevers and McGuire, 1985; Johnson et al., 1983). The dICs found in the brain and in other organs in this experiment suggest that formation and deposition of immune complexes play an important role in the pathogenesis of maedi-visna as well.

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SUBACUTE TOXICITY TESTING OF OCHRATOXIN A AND CITRININ IN SWINE

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Fourteen pigs were fed ochratoxin A and citrinin through a stomach tube at daily doses of 0.02 and 0.01 mg/kg body mass for 57 days. These toxin doses correspond to the average toxin contamination level of feeds in Central Europe. The clinical status of the pigs was monitored and clinical laboratory, haematological and mycotoxin-analytical examinations were performed throughout the trial. At the end of the experiment gross and histopathological examinations were carried out.

The results of ochratoxin A and citrinin determination in the blood, obtained by high-performance liquid chromatography (HPLC), are important from the food hygienic point of view. The sensitivity of the method was 2 and 10 ng/ml for ochratoxin A and citrinin, respectively. The recovery rate of the mycotoxins was above 60%.

Key words: Ochratoxin A, citrinin, toxicity, blood levels, HPLC, pig

Ochratoxin A and citrinin belong to the commonest mycotoxins produced in nature. Both toxins are produced by *Aspergillus* or *Penicillium* spp., mostly on barley, oat or wheat substrates. Both mycotoxins are nephrotoxic and hepatotoxic and may be important causes of mycotoxic porcine nephropathy (MPN). MPN is an endemically occurring disease in Scandinavia. It was first discovered in Denmark (Larsen, 1928). The renal disorder is characterized by degeneration of the proximal tubules and interstitial cortical fibrosis. Macroscopically the kidneys are enlarged and pale, and cortical fibrosis is distinctly observable on the cut surface in advanced cases, as reported from field surveys (Elling et al., 1973). MPN was identified also in Hungary (Sándor et al., 1982). Of the two toxins, especially ochratoxin A has importance in respect of residual toxicity. Therefore, numerous animal experiments with ochratoxin A have been reported in the literature. Citrinin is less toxic than ochratoxin A.

The aim of the experiment described here was to study the separate and combined effects of the two toxins on the average level of contamination occurring in nature, and to evaluate toxin concentrations demonstrable in the blood in respect of residual toxicity, animal health and diagnostics.

Materials and methods

Experimental animals

Fourteen pigs were used. At the beginning of the experiment the pigs were 8 weeks old and their average body mass was 16 kg. From 8 to 14 weeks

of age they were fed a piglet diet, while from the 15th week a fattening feed was given, twice daily. Drinking water was provided *ad libitum*.

Preparation and administration of toxin solutions

The solutions used for mycotoxin administration were prepared by dissolving crystalline ochratoxin A and citrinin in absolute ethanol and by subsequent dilution of the obtained solutions in water. The toxin concentration of the experimental solution was 0.04 mg/ml 10% ethanol. Fresh solutions were prepared every day and their concentration was checked by spectrophotometry before use. The test solution containing ochratoxin A + citrinin was a 1:1 mixture of the original solutions.

The toxin solutions were administered through a gastric tube 30 min–1 h after the morning feeding on every work-day. The control pigs received 10% ethanol solution by the same route. A total of 43 toxin treatments were performed during the 57-day experimental period.

Table 1

Design of the experiment

Group	Animal no.	Sex	Toxin dose, mg/kg body mass		Total
			Ochratoxin A (daily dose)	Citrinin (daily dose)	
Control	4	f	—	—	—
	9	f	—	—	—
	12	m	—	—	—
	13	m	—	—	—
	15	m	—	—	—
Group 1 (OA)	3	m	0.02	—	0.86
	11	f	0.02	—	0.86
	15	f	0.02	—	0.86
Group 2 (C)	1	f	—	0.02	0.86
	5	m	—	0.02	0.86
	6	m	—	0.02	0.86
Group 3 (OA + C)	2	f	0.01	0.01	0.43 OA + 0.43 C
	14	f	0.01	0.01	0.43 OA + 0.43 C
	17	m	0.01	0.01	0.43 OA + 0.43 C

f = female; m = male; OA = ochratoxin A; C = citrinin

The design of the experiment is shown in Table 1. The toxin dose was calculated on the basis of the feed intake. It corresponded to 0.2–0.9 mg toxin/kg of feed during the experiment.

The pigs were weighed once a week, before the morning feeding. The body mass served as a basis for calculating the subsequent 5 toxin doses.

Design of the experiment

Clinical examination. The pigs' general condition was checked daily.

Clinical laboratory examinations. Blood and urine samples for laboratory examination were taken on day 3 and 14 and at 4, 6 and 8 weeks after the beginning of the experiment. The variables tested included enzymes (aspartate aminotransferase, gamma glutamylpeptidase, creatine phosphokinase), metabolites (glucose, creatinine, urea), proteins (total protein and protein fractions such as albumin, alpha, beta and gamma globulins), electrolytes (Ca, Na, K, Cl^-, inorganic phosphorus), and haematological variables (haemoglobin, packed cell volume, white blood cell count and differential blood count). Urine samples were tested for pH, density, protein, glucose, haemoglobin, sediment, and net acid-base excretion.

Assay of blood samples for mycotoxins. Blood samples were taken before toxin treatment every day. The preslaughter blood sample was withdrawn 3 h after the last toxin treatment. After centrifugation the plasma samples were stored at −21 °C until assayed. Ochratoxin A and citrinin were assayed by the following procedure.

(i) Sample purification. Five ml of plasma sample was measured into a 250-ml screw-cap bottle, and 40 ml hydrochloric magnesium chloride solution was added. (Preparation of hydrochloric magnesium chloride solution: 0.1 M $MgCl_2$ was dissolved in 0.05 M HCl, i.e. 20.32 g $MgCl_2 \times 6\ H_2O$ + 50 ml 1 M HCl. After dissolution it was made up to 1,000 ml with distilled water.) The pH was checked and adjusted to a value less than 3 by adding some drops of 1 M hydrochloric acid. After adding 10 ml chloroform the cap of the bottle was screwed on and the sample was extracted by shaking it for 30 min. This was followed by centrifugation at 3,000 rpm for 10 min. Five ml of the lower chloroform phase was sucked out with a syringe and transferred into a 100-ml separating funnel. After extraction with 2×5 ml 0.1 M $NaHCO_3$ solution the upper, $NaHCO_3$ phases were pooled and adjusted to pH 2.5 with formic acid (appr. 0.5 ml formic acid was needed). The $NaHCO_3$ phase was extracted in a separating funnel with 2×5 ml chloroform, the lower chloroform phases were pooled and evaporated dry on Rotadest in a round-bottomed flask. If a substantial amount of emulsion was formed during extraction because of the lipid content of the sample, extraction was repeated with 5 ml chloroform. The chloroform phase was added to the previous ones in the round-bottomed flask and evaporated. The evaporated extract was washed into a vial with 4×1 ml chloroform and evaporated dry under nitrogen stream. For high-performance liquid chromatography (HPLC) the sample was dissolved in 250 μl eluent.

Table 2

The conditions of HPLC determination

	Ochratoxin A (OA)	Citrinin (C)
Standard concentration (μg/ml)	0.1	1.7
Excitation wavelength (nm)	330	335
Emission wavelength (nm)	460	500
Eluent	Flow rate ml/min	RT_{OA} (min)	RT_C
a) acetonitrile–water–acetic acid (99 + 99 + 2) (Fig. 2/a)	1	5.2 — 5.6	—
b) acetonitrile–water–acetic acid (57 + 41 + 2) (Fig. 2/b)	1.3	2.8	—
c) acetonitrile–isopropyl alcohol–0.08 M phosphoric acid (35 + 10 + 55) (Fig. 2/c)	2	4.7	2.7

RT = retention time

(ii) Instrument. High-performance liquid chromatograph: Waters 501 pump, controller model 680, U6K injector, C18 Guard-Pak column, μ Bondapak C18 150×3.9 mm, Shimadzu fluorescence detector RF 535, Waters Data Modul 745.

(iii) Measurement conditions. The conditions of chromatography are presented in Table 2.

Table 3

Average body mass gain of the pigs during the experiment

Group	Body mass gain (g/animal/day)
Control	555
Group 1 (0.02 mg OA/kg body mass/day)	491
Group 2 (0.02 mg C/kg body mass/day)	577
Group 3 (0.01 mg OA + 0.01 mg C/kg body mass/day)	506

Gross and histopathological examinations. On day 57 of the experiment the pigs were slaughtered and necropsied. The absolute and the relative mass of the liver, kidneys, thyroid gland and heart were determined. Samples from the following organs were examined histologically: liver, oesophagus, stomach, duodenum, jejunum, thyroid gland, spleen, inguinal lymph node, kidneys, ureter, urinary bladder and urethra. The organs were fixed in formalin (pH 7.2), embedded in paraffin, and the sections were stained with haematoxylin and eosin.

Results

Clinical observation

No clinical signs indicative of disease were observed during the experiment. Feed and drinking water intake, urination, defecation, motility and general behaviour were normal. The piglets' body mass gain during the experiment is shown in Table 3.

Table 4

Changes in blood beta globulin levels during the experiment (%)

Groups	Week 4	Week 8
Control (0)	18.1	18.2
Group 1 (0.02 mg OA)	15.3	19.3
Group 2 (0.02 mg C)	12.6	14.3
Group 3 (0.01 mg OA + 0.01 mg C)	16.0	20.0

Table 5

Changes in blood plasma chloride levels (mmol/l) during the experiment

Groups	Day 14	Week 4	Week 6	Week 8
Control (0)	105.9	122.7	125.9	121.9
Group 1 (0.02 mg OA)	100.9	112.3	111.0	104.2
Group 2 (0.02 mg C)	110.2	124.9	131.8	130.5
Group 3 (0.01 mg OA + 0.01 mg C)	105.2	126.2	129.3	123.7

Clinical laboratory examinations

The enzymes, metabolites, total protein, haematological and urinary variables tested showed no substantial deviation from the normal values. In group 2 (citrinin-treated pigs) beta globulin concentration was decreased at 4 and 8 weeks (Table 4). Serum chloride concentration of group 1 pigs decreased from day 14 of the experiment (Table 5). Blood potassium level increased in both groups 1 and 2 after day 3; subsequently, however, no further elevation occurred.

Mycotoxin assays. The mycotoxin concentrations of plasma samples, determined by HPLC, are presented in Tables 6 and 7. The mean values of ochratoxin A levels are illustrated in Fig. 1. Figs 2/a, b and c show HPLC chromatograms of serum ochratoxin while Fig. 3 illustrates serum citrinin determination.

Gross and histopathological examinations. No appreciable difference was found between the control and the experimental groups.

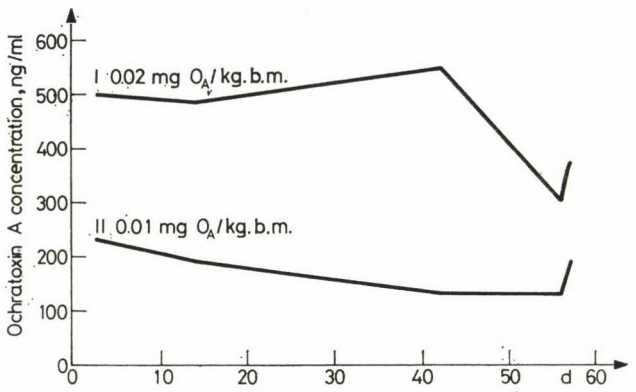


Fig. 1. Changes in the ochratoxin A concentration of plasma samples during the experiment

Table 6

Ochratoxin A concentration of the plasma samples (ng/ml)

Group	Animal no.	Sampling times					
		Day 3	Day 14	Day 28	Day 42	Day 56	Day 57
Control (0)	4	NT	NT	NT	11.4	3.5	5.1
	9	NT	NT	NT	10.1	5.5	5.9
	12	NT	NT	NT	3.7	5.1	4.0
	13	NT	NT	NT	10.6	6.6	6.2
	16	NT	NT	NT	3.3	5.1	5.5
	Mean:				7.8	5.2	5.3
Group 1 (0.02 mg OA)	3	NT	NT	384	537	310	409
	11	709	352	330	466	246	339
	15	289	620	638	634	350	361
	Mean:	499	486	517	545	302	370
Group 2 (0.02 mg C)	1	NT	NT	NT	NT	8	12
	5	NT	NT	NT	NT	3	5
	6	NT	NT	NT	NT	3	4
	Mean:					4.7	7
Group 3 (0.01 mg OA + 0.01 mg C)	2	307	189	153	143	118	150
	14	156	134	130	145	107	143
	17	NT	253	197	107	165	233
	Mean:	232	192	160	132	130	175

NT = not tested; sensitivity of the method for ochratoxin A: 2 ng/ml; detection limit: 45 pg; recovery rate: 67%

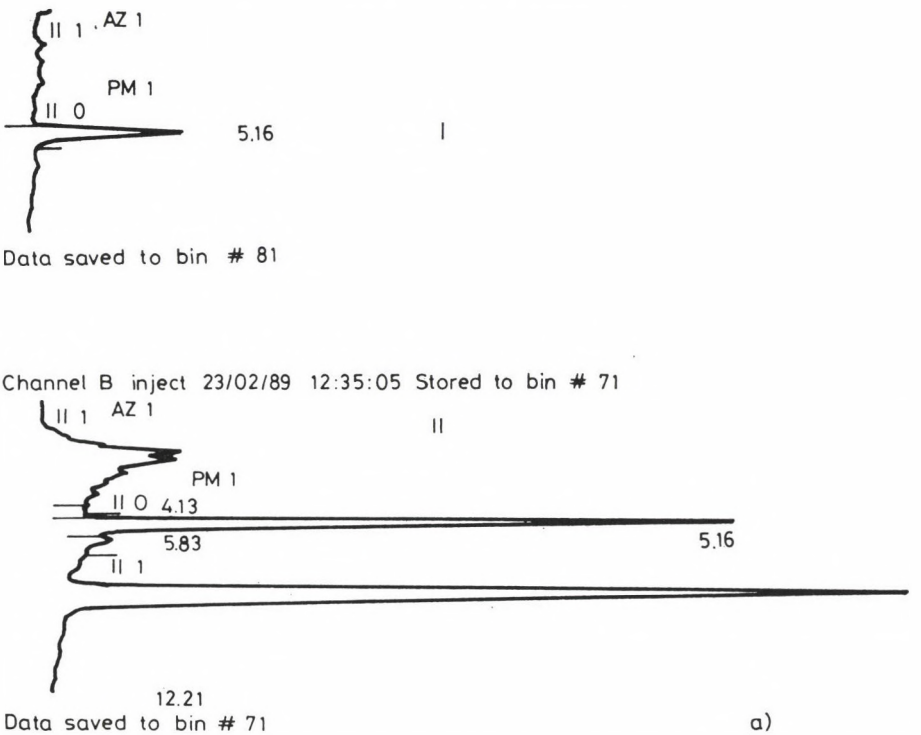


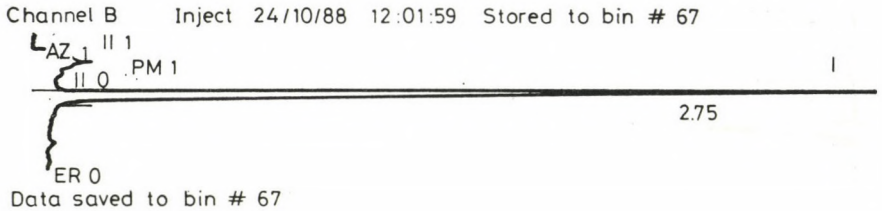
Fig. 2/a. Chromatograms of ochratoxin A standard (I) and ochratoxin A containing plasma sample (II) with eluent (a)

Table 7

Citrinin concentration of the plasma samples (ng/ml)

Group	Animal no.	Sampling times		
		Week 6	Week 8	Day 57
Group 2 (0.02 mg C)	1	127	131	121
	5	92	95	134
	6	33	48	79
	Mean:	84	91	111
Group 3 (0.01 mg OA + 0.01 mg C)	2	51	41	49
	14	48	28	33
	17	56	51	66
	Mean:	69	40	49

Sensitivity of the method for citrinin: 10 ng/ml; detection limit: 600 pg; recovery rate: 61%

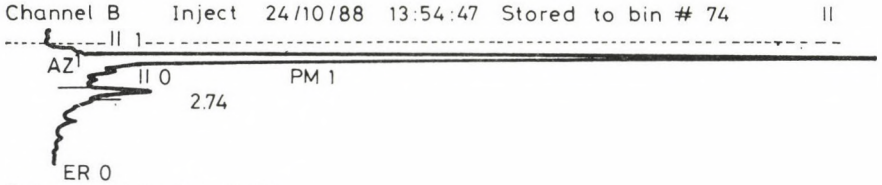


24/10/88 12:01:59 CH= "B" PS= 1.

File 1 Method 5 Run 3 Index 2 Calib bin 67

Analyst : SG

Name	PPB	RT	Area BC	RF
Ochra	110	2.75	207198 01	1883.618
Totals	110		207198	



24/10/88 13:54:47 CH= "B" PS =1.

File 1 Method 5 Run 10 Index 1 Bin 74

Analyst: SG

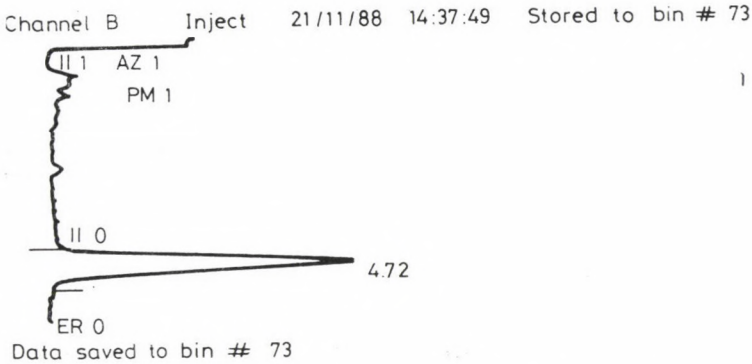
Sample 7 12 Bin 74 Name Brun0009

SA	IS	XF
1	0	0.25

Name	PPB	RT	Area BC	RF
Ochra	1.789	2.74	13421 01	1875.395
Totals	1.789		13421	

b)

Fig. 2/b. Chromatograms of ochratoxin A standard (I) and ochratoxin A containing plasma sample (II) with eluent (b)



21/11/88 14:37:49 CH = "B" PS = 1

File 1 Method 5 Run 1 Index 1 Calib Bin 73

Analyst: SG

Name	PPB	RT	Area BG	RF
Ochra	91	4.72	102968 01	1131.516
Totals	91		102968	

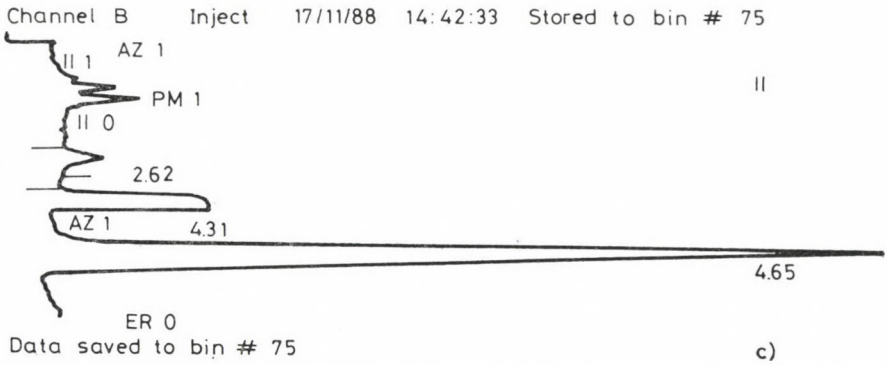
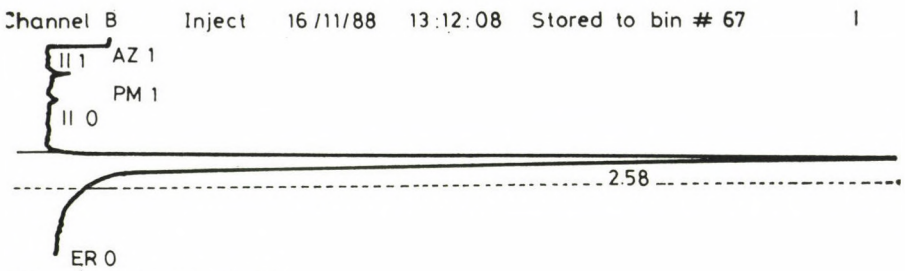


Fig. 2/c. Chromatograms of ochratoxin A standard (I) and ochratoxin A containing plasma sample (II) with eluent (c)

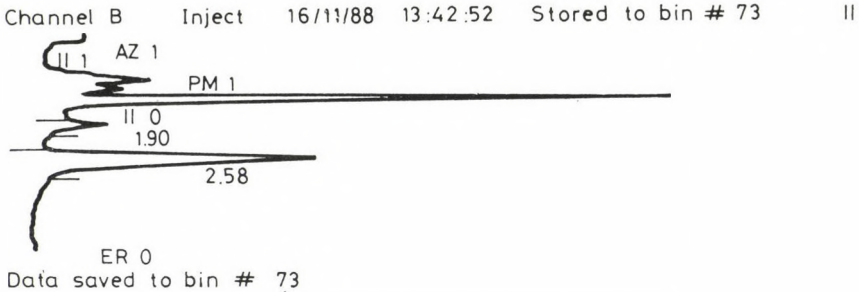


16/11/88 13:12:08 CH = "B" PS = 1.

File 1 Method 5 Run 3 Index 1 Calib Bin 67

Analyst: SG

Name	PPB	RT	Area BC	RF
Citrinin	860	2.58	182658 01	212.393
Totals	860		182658	



16/11/88 13:42:52 CH = "B" PS = 1

File 1 Method 5 Run 9 Index 1 Bin 73

Analyst: SG

Sample 5 5 Bin 73 Name Brun0009

SA	IS	XF
3	0	0.25

Name	PPB	RT	Area BC	RF
1	0	19	6208 01	
Citrinin	20.992	2.58	54853 01	217.759
--Totals--	20.992		61061	

Fig. 3. Chromatograms of citrinin standard (I) and citrinin-containing plasma sample (II)

Discussion

The toxin concentrations chosen for the experiment corresponded to the level of contamination detected in the Central European region. These toxin concentrations (0.86 mg ochratoxin A and citrinin separately and 0.43 mg ochratoxin A plus 0.43 mg citrinin) did not cause interpretable clinical or pathomorphological changes during the 57-day experimental period. A possible explanation for this is that low toxin doses were used and that toxin exposure was too short to result in detectable morphological changes. This is consistent with the practical observations. Although a certain retardation in growth did occur, because of the small group size it could not be evaluated statistically.

The significant drop in plasma chloride concentration in group 1 and beta globulin concentration in group 2, as well as the initial elevation of blood potassium level in both groups, may indicate renal dysfunction. To save space, the values of biochemical variables which did not significantly differ from the control are not shown here.

In developing a method for simultaneous determination of blood ochratoxin A and citrinin concentrations we made use of analytic experience gained by Bauer et al. (1984), Hult et al. (1979), Lepom (1986) and Nesheim et al. (1991). By modifying and improving the procedures used by them we have obtained a more sensitive method. This study is the first serial determination of blood mycotoxin concentrations in Hungary.

Blood ochratoxin A concentration was nearly identical in the first weeks. The concentration rise found on day 57 was virtual as sampling took place 3 h, and not 24 h, after toxin administration. Citrinin concentration of the blood was much lower than its ochratoxin A concentration. This may have been due to lower stability and possibly, more rapid elimination of citrinin. Low levels of ochratoxin A were found in the blood of control pigs and pigs of group 2 (animals given citrinin only) as well, probably because of ochratoxin A contamination of the feed. This finding, however, does not affect interpretation of the experiment even if these values are subtracted from results of the experimental animals.

As toxin administration via a tube differs from the natural route of toxin ingestion, a separate study should be devoted to the effects of this difference.

The important finding of this work is that toxin concentrations which fail to cause appreciable clinical or subclinical damage must not be neglected from the food hygienic and public health point of view. The toxins can easily be detected in the blood some hours after their ingestion. Their blood levels are dose dependent. This fact can be utilized in diagnostics. High-performance

liquid chromatography is especially suitable for assaying blood samples for toxin concentration.

The residue levels may have toxicological effects on man if the blood of toxin-contaminated animals is processed for food.

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INTESTINAL ABSORPTION OF GLYCYLPROLINE IN CHICKS INFECTED WITH *EIMERIA ACERVULINA*

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The absorption of glycine and proline through the mid-intestines of chicks infected with *Eimeria acervulina* was impaired when the amino acids were presented to the mucosal surface as the dipeptide, glycylproline.

Key words: *Eimeria acervulina*, coccidia, malabsorption, glycylproline, glycine, proline

Coccidiosis, a protozoan disease which is of major economic importance in poultry, causes intestinal tissue damage associated with decreased absorption of various nutrients including proteins and amino acids (Turk, 1978; Ruff, 1986). Studies on malabsorption of amino acids in infected chicks have used the compounds in a free form. However, amino acids can be absorbed from the gut in dipeptide form and hydrolysed to amino acids within the intestinal cells (Matthews, 1975). The present study was a preliminary examination of the absorption of a dipeptide across the intestinal tract of chicks infected with a coccidian. *Eimeria acervulina* was used because its later stages develop mainly in the proximal regions of the intestine and are confined to the surface epithelial cells of the villi. Glycylproline was used because the products of hydrolysis, the amino acids glycine and proline, can be distinguished by their different reactions with ninhydrin.

Materials and methods

White Leghorn cockerels reared in wire-floored cages were each inoculated when 18 days old with 5×10^6 sporulated oocysts (1 month old) of *E. acervulina* (Weybridge strain). Six days later, at the time of intestinal damage, absorption was assessed *in vitro* by using a modified version of the everted sac method of Wilson and Wiseman (1954) which is particularly sensitive to epithelial damage. Birds were killed by cervical dislocation. Mucosal concentrations of 20 mM glycylproline were used and sacs from 3 intestinal regions from infected and uninfected control birds were incubated for 20 min in oxygenated Krebs-Henseleit solution at 37 °C. The sacs were selected from

the duodenum, mid-intestine (halfway between duodenum and yolk stalk) and distal intestine (halfway between yolk stalk and the caecal junction). Infection was positively confirmed by microscopic examination of intestinal smears and sections stained with haematoxylin and eosin.

Results and discussion

The relative absorptive ability of the different segments of intestine and the effects of infection are given in Fig. 1. Serosal samples examined by thin-layer chromatography contained less than 0.5 mM of the dipeptide. As judged by the serosal concentration of amino acids, the mid-intestinal region is more effective at absorbing the dipeptide than the other regions in uninfected chicks. This result is analogous to the findings in rats in which the jejunum appeared to be the better intestinal site for intact uptake of glycylproline (Heading et al., 1977). Although monitored only on a single day, absorption of glycylproline and/or the passage of proline and glycine were impaired in the infected mid-intestinal region. The value of proline in infected chicks was also significantly

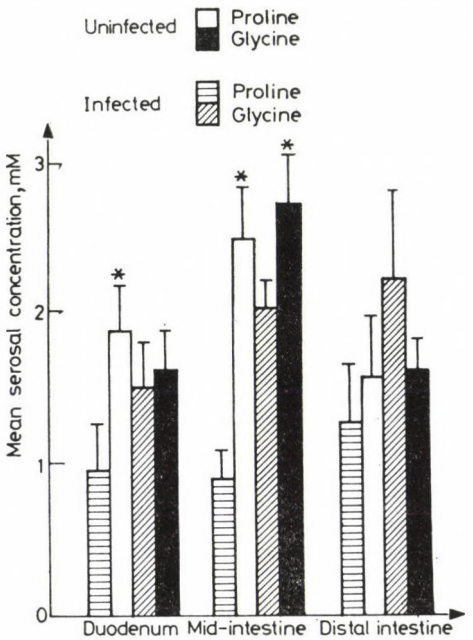

Fig. 1. Absorption of the dipeptide, glycylproline, when presented to the mucosal surface of the intestine of chicks infected with *Eimeria acervulina*. Concentration of proline and glycine detected in serosal fluid after 20 min. *Values significantly higher than corresponding infected values ($p < 0.05$). Vertical bars are standard errors of the mean. Each value is the mean of 4 replicates from 2 infected or 2 uninfected birds

less in the duodenum. All but one of the other values from infected birds were lower than from the corresponding uninfected controls, but were not statistically different. The increased glycine value in the distal intestine from infected birds might indicate a compensatory mechanism for the passage of this amino acid in a region of unparasitized tissue. In general, these results with *E. acervulina* in chicks are similar to those found with *E. nieschulzi* which causes intestinal coccidiosis in rats (Ball et al., 1980; Duszynski et al., 1982) and indicate that the measurement of glycine and proline transport across the gut wall is suitable for the study of dipeptide absorption in chicken coccidiosis.

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STUDY OF THE ACETYLCHOLINESTERASE ACTIVITY OF *ASCARIDIA GALLI*: KINETIC PROPERTIES AND THE EFFECT OF ANTHELMINTICS

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Acetylcholinesterase (EC 3.1.1.7) activity was demonstrated in whole worm homogenates of adult *Ascaridia galli* with acetylthiocholine as substrate. The pH optimum was not measurable because of an autohydrolysis of the substrate. The Michaelis constant (Km) was 4 mM with saturation by excess substrate. Optimum enzyme activity was noted at a protein concentration of 200 mg/ml assay medium and at a temperature of 37 °C. Arrhenius plot of temperature dependence of the enzyme activity showed an energy of activation (Δ Ea) of 28.962 K joule/mole above, and 25.448 K joule/mole below, the transition temperature (37 °C). Complete inhibition by eserine (physostigmine), a specific and classical acetylcholinesterase inhibitor, established the identity of the enzyme. A marginally higher enzyme activity was observed in females than in males as well as in homogenates from worms of mixed sexes. The enzyme was markedly activated by divalent metal cations such as Fe^{2+}, Mg^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+} and Ca^{2+}, while Co^{2+} and Mn^{2+} inhibited the activity. Piperazine adipate at a concentration of 10^{-3} M caused 45.5% and albendazole, a benzimidazole anthelmintic, 37.5% inhibition in the enzyme activity, while levamisole and mebendazole proved to be practically ineffective, causing an inhibition of 12 and 9%, respectively.

Key words: *Ascaridia galli*, acetylcholinesterase, kinetic properties, anthelmintics

Ascaridia galli is a pathogenic nematode occurring in the intestines of domestic fowl (Sadun, 1950). It causes haemorrhage, enteritis and severe anaemia to the host, imparting considerable economic loss by lowering egg and poultry production. Like in vertebrates, the importance of acetylcholinesterase in interneuronal and end-plate neurotransmitter release is well documented in nematodes (Lee and Hodsden, 1963) and has been the target of organophosphorous anthelmintic attack in chemotherapeutic control measures (Knowles and Casida, 1966). The enzyme may exert an interesting effect on host tissue also, in that acetylcholine secreted in large amounts by certain nematodes is thought to create a "biochemical holdfast" for the parasite by inhibiting the peristaltic action of the host's gut (Yeates and Ogilvie, 1976). Acetylcholinesterase has been studied in a number of nematodes (Hart and Lee, 1966), except *A. galli*. The enzyme showed wide variation in its kinetic behaviour, substrate utilization, sensitivity to inhibitors and anthelmintics, and other properties. Since the enzyme is already known to play an important role in studies of the pharmacology of the nematode nervous system and of the immunology of nematode infections, the present study was undertaken to

characterize the kinetic and other properties of the enzyme and its interaction with anthelmintics in *A. galli*, particularly in view of the extremely great economic importance of the parasite.

Materials and methods

Preparation of homogenates. Adult *A. galli* specimens were collected from the intestine of fowls procured from local poultry farms. The worms were thoroughly washed in cold 0.9% NaCl solution to remove intestinal debris and other adhering materials. A 20% (w/v) homogenate in distilled water was prepared in a motor-driven teflon-glass homogenizer according to the method of Probert and Lwin (1974). The homogenate was centrifuged at 1000 g in cold and the supernatant was used as the source of enzyme activity. Enzyme assays were carried out with freshly prepared homogenate, although there was no indication of a loss in enzyme activity during storage. Preparations were diluted, wherever needed, with distilled water to give the desired final concentration in terms of protein.

Enzyme assay. Acetylcholinesterase was routinely assayed by spectrophotometry at 25 °C and 412 nm following the method of Ellman et al. (1961) using acetylthiocholine iodide (ATChI) as substrate. Except where the test compounds were added, the routine assay medium contained 2.6 ml of 0.1 M phosphate buffer, pH 8.0, 0.1 ml of the homogenate, 0.1 ml of buffered Ellman's reagent (39.6 mg of dithiobis-nitrobenzoic acid and 15 mg of $NaHCO_3$ in 10 ml phosphate buffer) and 0.2 ml of 7.5 mM acetylthiocholin iodide. All reagents were incubated for 5 min prior to addition of the substrate. The increase in absorbance at 412 nm was recorded on a Baush and Lomb Spectronic 21 after 20 min against a blank of the above mixture prepared at the same time but in the latter case the 0.2 ml substrate (ATChI) was replaced with 0.2 ml of the buffer solution. Protein concentration was determined colorimetrically by the method of Lowry et al. (1951). Crystalline bovine serum albumin was used as standard.

Kinetic and thermodynamic analysis. For studying the kinetic parameters of the acetylcholinesterase of *A. galli*, Michaelis constant (Km) and maximum of the apparent initial enzyme velocity (V_{max}), the enzyme was assayed at substrate concentrations ranging from 0.1 to 50 mM. Lineweaver-Burk plot (Segel, 1975) was constructed from the double reciprocal expressions of the substrate concentrations and the enzyme velocities. The enzyme activities were also determined at a fixed substrate concentration but at different temperature points ranging from 2 to 60 °C, and $\log_{10}$ of the initial apparent velocity of the reaction was plotted against the reciprocal of absolute temperature (°K) to construct the Arrhenius plot (Segel, 1975). The slope of the curve

was determined by the least square procedure and the energy of activation (Ea) was calculated from the slope following the equation: Slope = = — Ea/2.303 × R, where R represented the gas constant. Transition temperature (T_0) was directly recorded from the plot.

Results

Acetylcholine acetylhydrolase (EC 3.1.1.7) activity was measured in *A. galli* homogenates with acetylthiocholine as substrate at 25 °C. The results on enzyme reaction velocity were expressed in terms of u moles of the substrate hydrolysed/mg protein/min. The enzyme activity was found to be linear with time of incubation up to about 60 min; further incubations failed to produce any change in activity (Fig. 1). The temperature dependence curve showed that the activity reached the maximum at 37 °C; however, the peak discernible was not particularly sharp (Fig. 2). Arrhenius plot constructed from the linear relationship of $\log_{10}$ apparent velocity maximum versus reciprocal of the absolute temperature showed typical nonlinearity with a break point (transition temperature) around 37 °C (Fig. 3). Energy of activation (Δ Ea) calculated from the slope of the line was found to be 28.962 K joule/mole and 25.448 K joule/mole for above and below the transition temperature, respectively (Table 1). The more usual bell-shaped pH curve as seen in other

Table 1

Thermodynamic parameters of acetylcholinesterase activity in *Ascaridia galli*

Transition temperature (T_0) (°C)	Energy of activation (Ea) K joule/mole	
	below T_0	above T_0
37	25.448	28.962

Transition temperature (T_0) was directly recorded from Arrhenius plot (Fig. 3). Energy of activation (Ea) was calculated from the slope of curve determined by least square procedure. Values are means of three different determinations.

enzyme systems was not present in our study. Beyond pH 9.0 and up to pH 10.5 an excessive colour was seen in the blank, indicating a rapid autohydrolysis of the substrate (Fig. 4). The pH optimum was, therefore, not measurable. Typical non-linear saturation kinetics was seen with enzyme protein concentrations (Fig. 5) and the amount of the acetylthiocholine (substrate) added (Fig. 6). Optimal activity was reached at 200 mg/ml incubation medium of protein and at 10 mM acetylthiocholine. No inhibition in enzyme activity was, however, seen at excess enzyme or substrate concentrations. Lineweaver-Burk plot showed that the substrate concentration at which the

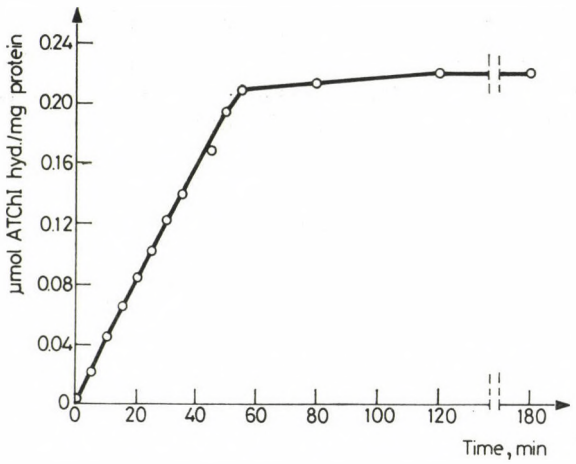

Fig. 1. Activity of acetylcholinesterase of *Ascaridia galli* in relation to time

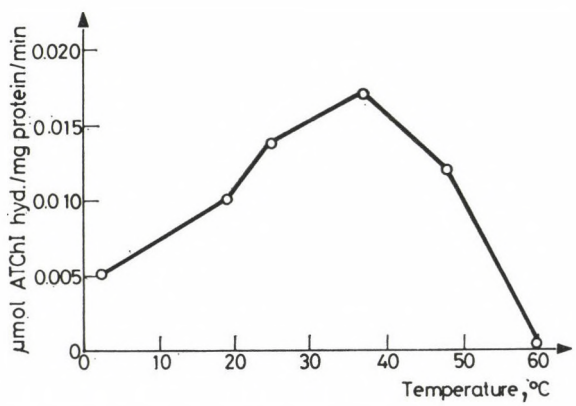

Fig. 2. Effect of temperature on the activity of acetylcholinesterase of *Ascaridia galli*

Table 2

Acetylcholinesterase activities of whole worm homogenates of different sexes of *Ascaridia galli*

Source of homogenate	Specific activity of acetylcholinesterase (µmoles ATChI hydrolysed/mg protein/min)
Male	0.0098
Female	0.0119
Mixed sexes	0.0101

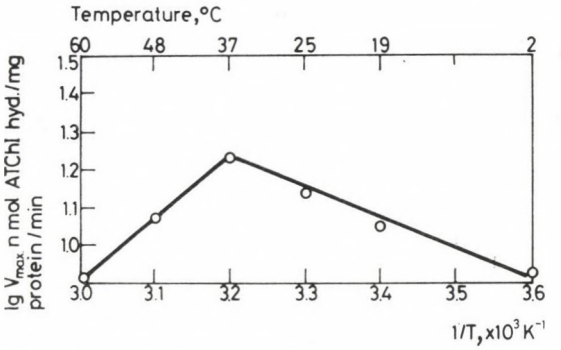

Fig. 3. Arrhenius plot of acetylcholinesterase activity in the homogenate of *Ascaridia galli*

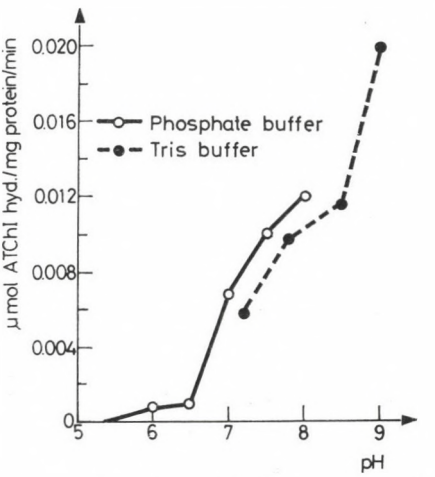

Fig. 4. Effect of pH on the activity of acetylcholinesterase in the homogenate of *Ascaridia galli*

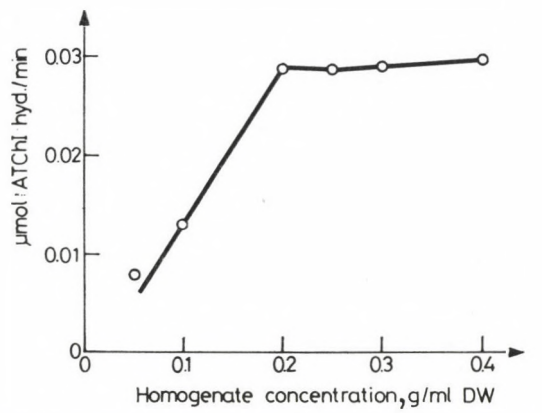

Fig. 5. Activity of acetylcholinesterase of *Ascaridia galli* in relation to homogenate concentration

half maximal velocity occurred (Km or substrate affinity constant) was 4.0 mM while the V_{max} (the maximum of apparent initial enzyme velocity) was calculated to be 0.0625 μ mole/mg protein/min (Fig. 7). A slightly higher enzyme activity was observed in female *A. galli* as compared to the males (Table 2) and even in mixed sex extracts higher specific activity was obtained than in the males. The enzyme activity showed particular preference for divalent metal ions (Table 3) with the exception of Mn^{2+} and Co^{2+} ions which

Table 3

Effect of various divalent metal cations on acetylcholinesterase activity of *Ascaridia galli*

Metal ions (1 mM)	% activation (+) or inhibition (—)
Co^{2+}	14.5(—)
Fe^{2+}	88 (+)
Mg^{2+}	10.6(+)
Cd^{2+}	129 (+)
Cu^{2+}	319 (+)
Zn^{2+}	48 (+)
Ca^{2+}	194 (+)
Mn^{2+}	27 (—)

Enzyme activity in control homogenate (without addition of metal ion) is 0.01262 μ moles ATChI hydrolysed/mg protein/min at 25 °C.

produced 27% and 14.5% inhibition, respectively. The enzyme was activated by Fe^{2+} (88%), Mg^{2+} (10.6%), Cd^{2+} (129%), Cu^{2+} (319%), Zn^{2+} (48%) and Ca^{2+} (194%) which showed that metal ions in trace concentrations can bring about remarkable changes in the activity of the enzyme.

The effect of drug treatment on acetylcholinesterase activity is presented in Table 4. At concentrations of 10^{-3} and 10^{-4} M eserine (physostigmine), a

Table 4

Percentage inhibition of acetylcholinesterase activity in *Ascaridia galli* caused by anthelmintics/inhibitors

Anthelmintic/Inhibitor	10^{-3} M	10^{-4} M
Eserine	100	100
Albendazole	37.5	—
Mebendazole	9.0	—
Piperazine	45.5	—
Levamisole	12	—

Enzyme activity in control homogenate (without inhibitor added) is 0.013 μ moles ATChI hydrolyzed protein/min at 25 °C.

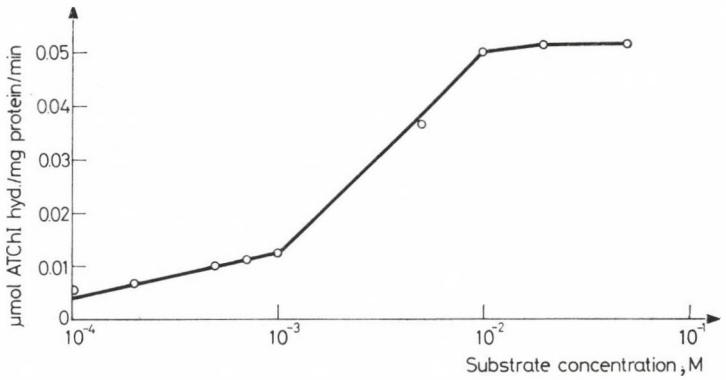

Fig. 6. Effect of substrate concentration on the acetylcholinesterase activity of *Ascaridia galli*

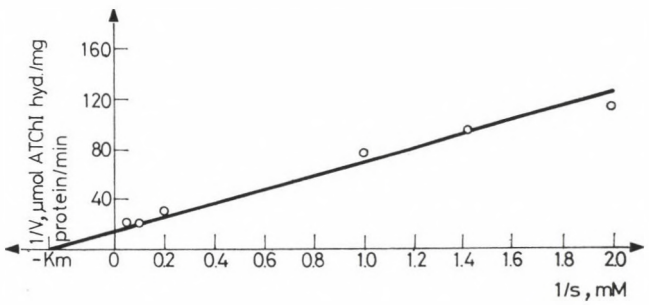

Fig. 7. Lineweaver-Burk plot of acetylcholinesterase activity in *Ascaridia galli*

known inhibitor of acetylcholinesterase, caused complete (100%) inhibition of *A. galli* acetylcholinesterase. Among the two benzimidazoles tested, viz. albendazole and mebendazole, the former proved to be effective with 37.5% inhibition, while the latter was practically ineffective (9% inhibition). Piperazine adipate produced, unexpectedly, 45.5% inhibition. Levamisole, a broad-spectrum anthelmintic against common helminth infestations of birds, cattle and man, also produced a moderate (12%) depression of the enzyme activity.

Discussion

The activity of the homogenates of *A. galli* to hydrolyse acetylcholinester has been demonstrated and the kinetic properties of the enzyme characterized. The inhibition of the enzyme by eserine, an acetylcholinesterase inhibitor at least in mammalian system, inhibition by benzimidazole and other anthelmintics, and the use of specific substrate put evidences beyond any reasonable doubt that the enzyme in question is indeed acetylcholinesterase, the enzyme responsible for neurotransmitter release.

The kinetic studies showed only a single peak of activity with varied substrate concentrations unlike the double locates reported by Hutchinson and Probert (1972) in *Ascaris suum*. These investigators also showed competitive inhibition of the enzyme with excess substrate which could not be confirmed in the present study. The excessively high blank yield in our assay system confirms that at high pH there is considerable non-enzymic hydrolysis of the substrate, acetylcholinester (Whittaker, 1984). Maximum enzyme activity was observed at 37 °C and like with *Haemonchus contortus* (Lee and Hodsden, 1963) or *A. suum* (Hutchinson and Probert, 1972) homogenates, inactivation of the enzyme was rapid above 40 °C and complete at about 60 °C but temperature dependence below the optimum activity point was not particularly critical. Whittaker (1984) showed that the assay of acetylcholinesterase was equally valid at 20, 25 or 37 °C, which, therefore, provides an added advantage to assay the enzyme at room temperature. Typical non-linear Arrhenius plot was observed of the thermal dependence of the enzyme activity when a plot was constructed between $\log_{10} V_{max}$ of the activity versus the reciprocal of absolute temperature. The discontinuity in Arrhenius expression is indicative of an effective denaturation of the enzyme protein and suggests that a partitioning of the active enzyme takes place between the fluid and ordered lipid domains of the membrane. The energy of activation (Ea), as noted, might have been needed for the binding and solubilization of the substrate to the enzyme molecule. The activity of the enzyme increased with the rise in substrate concentration, with a Michaelis constant of 4.0 mM. The activity increased from 0.1 to 10 mM, which agrees well with the observation of Lee and Hodsden (1963) in *H. contortus* and Knowles and Casida (1966) in *A. suum*. Enzyme activity was also estimated with increasing homogenate concentration and incubation time to choose assay conditions assuring linear kinetics and excess of substrate, i.e. the maximal velocity, in subsequent experiments. Optimum enzyme activity with a linear feature was obtained at enzyme incubation of 200 mg/ml assay medium and an excess of it did not produce any inhibition, which agrees well with the observation of Gunn and Probert (1981) in *Moniezia expansa* (Cestoda) homogenates. The activity was also essentially linear up to 60 min, then plateauing off but not inhibited when studied up to 180 min, thus confirming the results obtained earlier by Hutchinson and Probert (1972) in *A. suum* or Gunn and Probert (1981) in *M. expansa*.

The enzyme was also found to be activated to a great extent by a number of divalent metal ions, although rather unexpectedly. Activation was particularly marked with Cu^{2+}, Ca^{2+}, Cd^{2+}, Fe^{2+} and Zn^{2+}. There has been no report of the enzyme being activated by metal ions in any nematode or, for that matter, in other helminth species, although the requirement of Mg^{2+} for mammalian enzyme systems is well known (Nachmansohn and Wilson,

1955). A slight inhibition by Co^{2+} and Mn^{2+} as observed in the present study may not prove to be sufficient for further studies of their possible use in designing chemotherapeutic organo-metal complexes.

A 45% inhibition of the enzyme activity by piperazine as observed in the present study is well within the broad limits described by Hutchinson and Probert (1972) in *A. suum*, i.e. 63% inhibition with piperazine and 45% with phenothiazine. Knowles and Casida (1966) reported 52% inhibition of the enzyme in *Ascaris lumbricoides* using a concentration of 10^{-4} M piperazine citrate. Piperazine adipate also caused a moderate inhibition of enzyme activity in *A. suum* (Zukovic and Sir, 1960). The present result assumes particular significance since piperazine is not known to be effective in *A. galli* (Govorova and Polykova, 1971). Albendazole, but not mebendazole, produced a highly significant inhibition (37.5%), which is contrary to the findings of Gunn and Probert (1981) in *M. expansa*, where none of the benzimidazoles including albendazole, mebendazole, arecoline hydrobromide, praziquantel and piperazine adipate did cause any inhibition of enzyme activity. However, the concentration used in their study was 10^{-4} M as compared to the 10^{-3} M concentration used here. Haloxan, which is also known to be a cholinesterase enzyme inhibitor, produced a 97% inhibition at the dose level of 10^{-4} M in their assay system with a relatively high inhibition constant (Ki) of 4×10^{-5} M. Poor ability of benzimidazole compounds in enzyme inhibition has also been reported by Govorova and Polykova (1971). In their experiment, *A. galli* was sensitive to phenylbenzimidazole to a moderate extent (17% inhibition). Therefore, a suitable chemotherapeutic agent exploiting the acetylcholinesterase pathway of paralysis and cell death is yet to come which could be very effective in the control of intestinal nematodes including *A. galli*.

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THE INVOLVEMENT OF POLYMORPHONUCLEAR LEUKOCYTES IN THE PATHOGENESIS OF BRONCHOPNEUMONIA IN CALVES I. ACTIVATED GRANULOCYTE INDUCED LIPID PEROXIDATION IN RED BLOOD CELLS

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The influence of free radicals generated by polymorphonuclear leukocytes from bronchopneumonic calves on lipid peroxidation in red blood cells was studied. Incubation of granulocytes with red blood cells causes a rise in malondialdehyde production in these cells in linear dependence on the granulocyte number. Incubation of red blood cells with malondialdehyde causes a generation of adduct moiety of this compound with phosphatidylserine and phosphatidylethanolamine. This adduct is also present in erythrocytes which have been incubated with neutrophils of bronchopneumonic calves. Cytochrome c loaded erythrocyte ghosts showed a high reduction rate of this compound during incubation of ghosts with neutrophils of diseased calves.

It is suggested that neutrophils of bronchopneumonic calves are in an activated state in the blood-stream, and free radicals generated by these cells are capable of peroxidizing the red blood cell membrane lipids and causing cross-links between phospholipid moieties in erythrocyte membranes.

Key words: Calf, bronchopneumonia, neutrophil, erythrocyte, malondialdehyde, lipid peroxidation

Red blood cells are especially susceptible to peroxidation because of their haemoglobin content and abundance of polyunsaturated fatty acids in the cell membrane (Chiu et al., 1982; Pryor, 1976). Highly reactive oxygen species, which can initiate lipid peroxidation, may be generated endogenously in red blood cells during normal auto-oxidation of oxyhaemoglobin to methaemoglobin (Sutton et al., 1976), or may come from exogenous sources (Jaffe, 1981). These reactive oxygen species are capable of attacking the red blood cell membranes and altering their protein and lipid structure (Snyder et al., 1981; Stocks et al., 1972). The alterations may ultimately result in lysis of the cells (Carrell et al., 1978).

The activated neutrophils are known to produce oxygen radicals that may leak out of the cell and cause damage to the surrounding tissues (Sacks et al., 1978; Suttorp and Simon, 1982). Baehner et al. (1971) have shown that activated neutrophils can produce erythrocyte injury in patients with congenital glucose-6-phosphate dehydrogenase deficiency. Weiss (1980, 1982) showed that incubation of activated neutrophils with erythrocytes produced haemolysis which could be inhibited by free radical scavengers. The clinical

observation that infection enhances the rate of erythrocyte destruction is consistent with the possibility that similar damage may occur *in vivo* (Carson and Frischer, 1966; Lubin and Vichinsky, 1979).

The aim of this study was to investigate the effect of free radicals generated by polymorphonuclear leukocytes of bronchopneumonic calves on lipid peroxidation in red blood cells of healthy animals.

Materials and methods

The blood of 40 four-week-old calves affected with bronchopneumonia was used as the source of polymorphonuclear leukocytes. Red blood cells were obtained from the blood of 20 healthy animals of the same age. To obtain red blood cells, citrated blood was centrifuged at 1500 *g* for 10 min. The plasma and the buffy coat were removed, and the erythrocytes were washed with 0.154 M NaCl 3 times.

Malondialdehyde (MDA) was prepared by hydrolysis of malondialdehyde bis-dimethyl acetal (E. Merck, Darmstadt, Germany) with concentrated HCl.

A 5% erythrocyte suspension (in a buffer consisting of 7.808 g NaCl, 0.373 g KCl, 2.302 g Na_2HPO_4, 0.194 g NaH_2PO_4 and 2 g of glucose in 1000 ml; pH 7.4) was shaken with various concentrations (0.5–5.0 mM) of MDA in Erlenmeyer flasks in a water bath at 37 °C for various times. Erythrocyte lipids were extracted according to Rose and Oklander (1965). Phospholipids were analysed by thin-layer chromatography on silica gel G precoated plates (E. Merck, Darmstadt, Germany) as described by Michalak et al. (1988). Chromatograms were visualized with iodine vapours, and phosphorus was estimated in particular spots according to Bartlett (1959).

Since chromatograms of lipids of erythrocytes incubated with MDA show a new phospholipid spot, migrating between the phosphatidylserine (PS) and phosphatidylethanolamine (PE) fractions in a solvent system of chloroform–methanol–acetic acid–water (50 : 25 : 7 : 3 by volume), this phospholipid was assumed to be a reaction product of MDA and the above-mentioned phospholipids (Jain and Shohet, 1984). This fraction was scraped off the plate, and the gel was extracted with a 2 : 1 mixture of chloroform and methanol. The eluate was evaporated to dryness and the dry residue was dissolved in chloroform. A part of this extract was repeatedly evaporated to dryness, and the dry residue was hydrolysed with 6 M HCl in boiling water bath for 4 h. In control tubes, standards of serine and ethanolamine (Sigma, St. Louis, USA) were similarly treated with HCl. In the acid hydrolysate the amines were resolved on cellulose-precoated plates (E. Merck, Darmstadt, Germany) in a solvent system of butanol–pyridine–acetic acid–water (3 : 3 : 1 : 1 by volume). The amines were localized on the plate with ninhydrin reagent (Pataki, 1966).

The coloured complexes were eluted off the plate with phosphate buffer (50 mM, pH 7.4) and the amine concentration was estimated spectrophotometrically. In the remaining part of the chloroform extract, glycerol and phosphorus were estimated according to van Handel and Zilversmit (1957) and Fiske and Subbarow (1925), respectively.

Polymorphonuclear leukocytes were obtained by dextran sedimentation and gradient centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) according to Claster et al. (1984). Zymosan (Sigma,[1] St. Louis, USA) was opsonized in autologous serum according to Markert et al. (1984). Granulocytes were activated by adding 0.8 ml of opsonized zymosan (10 mg $\times$ ml^{-1}) to 1 ml of cell suspension (2×10^7 cells $\times$ ml^{-1}). The mixtures of erythrocytes (3 ml, 1×10^9 cells $\times$ ml^{-1}) and polymorphonuclear leukocytes (1 ml, 2×10^7 cells $\times$ ml^{-1}) were centrifuged at 2000 g for 5 min to induce cell-to-cell contact (Weiss, 1980) and then incubated for various times. MDA concentrations were measured as described by Ledwożyw et al. (1986). Haemoglobin content was estimated according to Drabkin (1946).

In some experiments, to identify the oxygen species that might be involved in peroxidative processes, the free radical scavengers superoxide dismutase (Sigma, St. Louis, USA, specific activity 3000 units $\times$ mg protein^{-1}) and/or catalase (E. Merck, Darmstadt, Germany, 5000 units $\times$ mg protein^{-1}) were added to the incubation mixture before zymosan addition. In these cases, the incubation mixture consisted of 1 ml of granulocyte suspension (2×10^9 cells $\times$ ml^{-1}), 0.8 ml opsonized zymosan (10 mg $\times$ ml^{-1}), 3 ml of erythrocyte suspension (1×10^9 cells $\times$ ml^{-1}), and 0.2 ml of superoxide dismutase (0.5 mg $\times$ ml^{-1}) and/or catalase (1 mg $\times$ ml^{-1}) in a total volume of 5.2 ml.

Erythrocyte ghost preparation. Erythrocytes were washed 3 times with 0.154 M NaCl containing 5 mM $MgCl_2$. The packed cells were suspended in 40 volumes of an ice-cold solution of 50 mM phosphate buffer (pH 8.0) containing 5 mM $MgCl_2$ and lysed for 30 min at 4 °C. The lysate was centrifuged for 20 min at 25,000 *g* and the pellet was washed twice with the same buffer. To remove residual haemoglobin, the pellet was washed with buffers of decreasing ionic strength: twice with 20 mM, twice with 5 mM, and twice with 0.5 mM phosphate buffers, all containing 5 mM $MgCl_2$ (pH 8.0). The centrifugations were performed at 17,000 *g* for 20 min at 4 °C. After the last centrifugation, the membranes were suspended in 0.5 mM phosphate buffer + 5 mM $MgCl_2$ + + 800 μM (Fe^{3+}) cytochrom c (Sigma, St. Louis, USA) + 100 units of catalase $\times$ ml^{-1} in a total volume of 20 ml. This suspension was rotated overnight at 4 °C. Afterwards, 1 volume of 770 mM NaCl was added to 4 volumes of membrane suspension, and the resulting mixture was incubated for 1 h at 37 °C (resealing of the ghosts). This suspension was centrifuged again (20 min, 17,000 *g*, 4 °C), and the fluffy layer of closed ghosts was decanted from the pellet of open membranes. The closed ghosts were washed with 0.154 mM

NaCl and decanted until the supernatant contained no more cytochrome c. The ghosts were suspended in 0.154 M NaCl to about 3×10^9 ghosts $\times$ ml^{-1} and stored at 4 °C.

The erythrocytes were incubated in a cuvette of a double-beam spectrophotometer (Specord UV-VIS, Karl Zeiss, Jena, Germany). Erythrocyte ghosts of free cytochrome c were present at a final concentration of 3 μM (3×10^8 ghosts $\times$ ml^{-1}). Polymorphonuclear leukocytes (1×10^6 cells $\times$ ml^{-1}), or xanthine (1.5 mM) plus xanthine oxidase (2 milliunits $\times$ ml^{-1}), were added as O_2^- generating systems. Catalase (100 units $\times$ ml^{-1}), was present in the ghosts and in the incubation medium to prevent reoxidation of (Fe^{2+}) cytochrome c by H_2O_2.

The reaction of (Fe^{3+}) cytochrome c was followed at 417 nm. This wavelength was chosen because it represents an isosbestic point in the spectra of (Fe^{2+})- and (Fe^{3+})-haemoglobin. Thus, at this wavelength the reduction of (Fe^{3+})-haemoglobin by O_2^- does not interfere with the assay of (Fe^{3+}) cytochrome c reduction by this radical.

Statistical significances of differences were analysed by Student's t test for unpaired data.

Results

Figure 1 shows the correlation between MDA production in red blood cells and the number of zymosan-activated granulocytes from healthy calves in the incubation mixture. As the number of white cells increases from 2×10^6 to 30×10^6 cells $\times$ ml^{-1}, there is a corresponding linear increase in MDA generation in red blood cells from 8.0 $\pm$ 1.7 to 31. $\pm$ 1.0 nM $\times$ g Hb^{-1}.

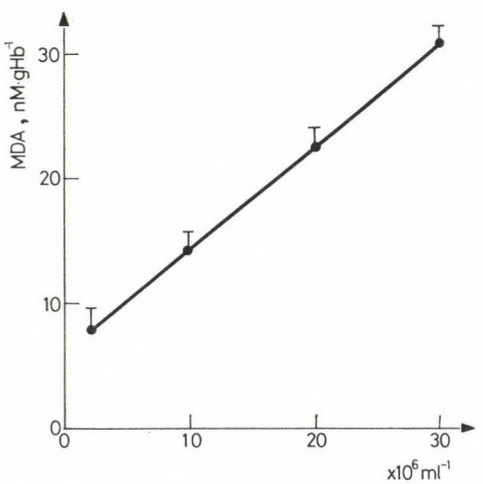

Fig. 1. Relationship between malondialdehyde (MDA) production in red blood cells and number of zymosan-activated granulocytes of healthy calves

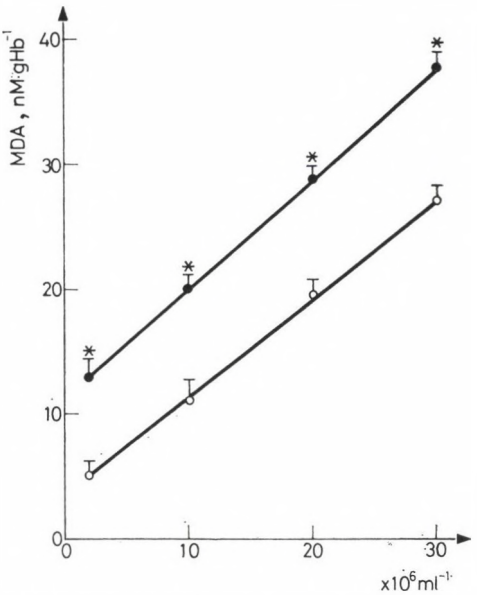

Fig. 2. Relationship between malondialdehyde (MDA) production in red blood cells and number of nonactivated (○) and zymosan-activated (●) granulocytes of diseased calves. *$p < 0.001$

Fig. 2 shows the correlation between MDA production in erythrocytes and the number of unstimulated and zymosan-activated granulocytes from bronchopneumonic calves. In the case of nonactivated phagocytic cells, there was an increase in MDA production from 5.0 ± 1.0 to 27.1 ± 1.1 nM × g Hb^{-1}, as the granulocyte number increased from 2×10^6 to 30×10^6 cells × ml^{-1}. In the case of zymosan-activated granulocytes, MDA concentration increased from 13.1 ± 1.1 to 37.9 ± 1.0 nM × g Hb^{-1}.

The causal relationship between the production of reactive oxygen species by unstimulated and zymosan-stimulated neutrophils and lipid peroxidation (MDA production) in red blood cells was examined with free radical scavengers (Table 1). Addition of catalase (10 μg × ml^{-1}) which scavenges H_2O_2, resulted in slightly less inhibition of peroxidation than was seen with superoxide dismutase, which scavenges O_2^-. Combination of these two scavengers resulted in a slight increase in inhibition, compared to values obtained with either superoxide dismutase or catalase alone.

Fig. 3 shows the influence of MDA on PS—MDA—PS and PE—MDA—PE adduct product concentration in red blood cells. The incubation of erythrocytes with increased concentrations of MDA shows a time-dependent increase in this product quantity.

Table 2 shows molar ratios of amines and glycerol to phosphorus in the phospholipid adduct. This composition confirms the hypothesis that this ad-

Table 1

The influence of superoxide dismutase (SOD) and catalase (CAT) on erythrocyte lipid peroxidation induced by free radicals generated in unstimulated and zymosan-stimulated neutrophils from calves (mean ± SD)

	Inhibition of MDA production (%)			
	Granulocytes of healthy calves		Granulocytes of diseased calves	
	Stimulated (A)	Unstimulated (B)	Stimulated (A)	Unstimulated (B)
SOD 20 $\mu g \times ml^{-1}$	13.2 ± 0.2	12.1 ± 0.2	31.4 ± 0.8*	27.6 ± 0.7*
CAT 20 $\mu g \times ml^{-1}$	10.4 ± 0.2	11.6 ± 0.2	27.6 ± 0.8*	22.1 ± 0.6*
SOD 20 $\mu g \times ml^{-1}$ + CAT 20 $\mu g \times ml^{-1}$	21.2 ± 0.3	18.6 ± 0.3	39.6 ± 0.7*	34.4 ± 0.7*

Student's *t* test for unpaired data: * $p < 0.001$ C vs. A and D vs. B; MDA = malondialdehyde

Table 2

Molar ratio of amines and glycerol to phosphorus in adduct moiety

	Molar ratio
Serine : phosphorus	0.47
Ethanolamine : phosphorus	0.42
Glycerol : phosphorus	0.95

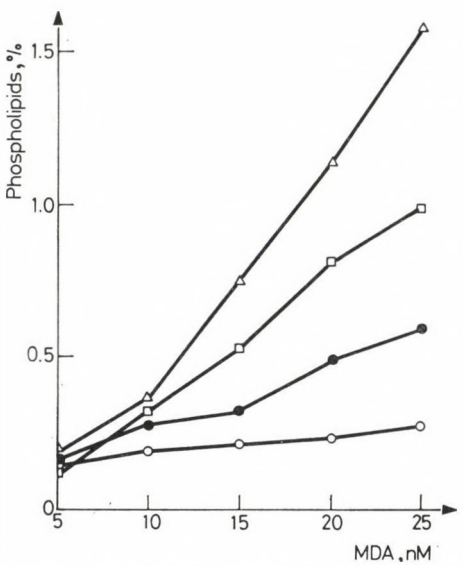

Fig. 3. The effect of malondialdehyde on adduct formation. ○ — 1 h incubation; ● — 6 h incubation; □ — 12 h incubation; △ — 24 h incubation

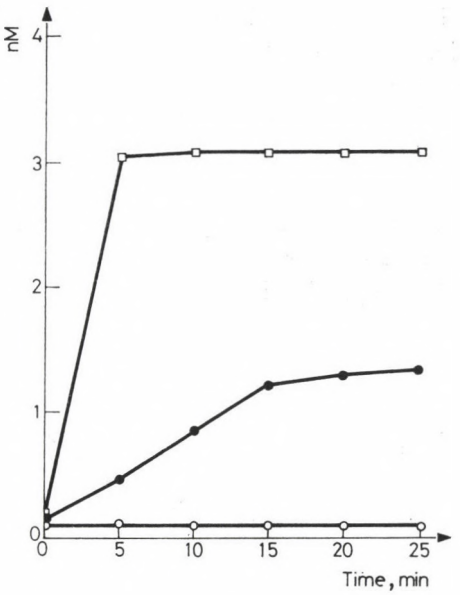

Fig. 4. Reduction of cytochrome c by the xanthine — xanthine oxidase system. ○ — superoxide dismutase (7.5 μM); ● — no additions; □ — Triton X-100 (0.2%)

duct is formed by crosslinking amino groups of PE and PS with MDA, according to the following reaction scheme:

$$PS{-}NH_2 + OHC{-}CH_2{-}CHO + H_2N{-}PS \rightarrow PS{-}N = CH{-}CH_2{-}CH = \\ = N{-}PS + 2\ H_2O$$

Tables 3 shows the quantities of phospholipid–MDA adduct, produced in red blood cells incubated with unstimulated and zymosan-stimulated polymorphonuclear leukocytes of calves. In unincubated red blood cells and those

Table 3

Adduct moiety concentration (% of total phospholipids) in erythrocytes incubated with stimulated and unstimulated granulocytes of calves (mean ± SD)

	Adduct moiety
Erythrocytes	0
Erythrocytes + unstimulated granulocytes of healthy calves	0
Erythrocytes + stimulated granulocytes of healthy calves	0.3 ± 0.1
Erythrocytes + unstimulated granulocytes of diseased calves	1.2 ± 0.2
Erythrocytes + stimulated granulocytes of diseased calves	2.2 ± 0.2

incubated with unstimulated granulocytes of healthy calves, this product was not observed. Incubation of erythrocytes with zymosan-stimulated granulocytes of healthy calves caused the appearance of negligible amounts of this adduct. Large amounts of this compound were observed in red blood cells incubated with unstimulated and zymosan-stimulated granulocytes of bronchopneumonic calves.

Fig. 4 shows incubation time dependent reduction of cytochrome c by the xanthine–xanthine oxidase system in erythrocyte ghosts. The initial rate of cytochrome c reduction depended both on xanthine oxidase concentration and

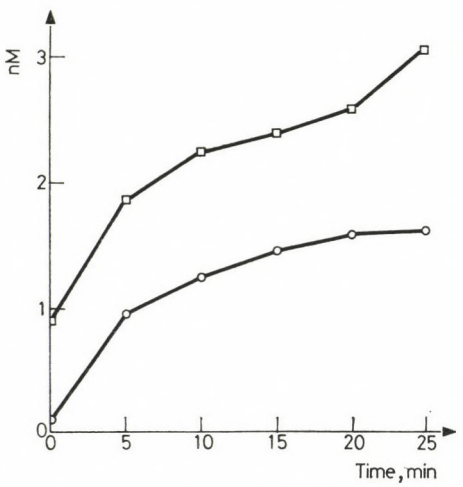

Fig. 5. Reduction of cytochrome c by zymosan-activated (□) and non-activated (○) neutrophils of diseased calves

on the number of erythrocyte ghosts. The reduction was completely inhibited by the addition of superoxide dismutase. The addition of Triton X-100 (nonionic detergent) increases the rate of cytochrome c reduction.

Fig. 5 shows cytochrome c reduction in erythrocyte ghosts incubated with polymorphonuclear leukocytes from healthy calves. The incubation of ghosts with unstimulated granulocytes causes a cytochrome c reduction comparable to that induced by the xanthine–xanthine oxidase system. Incubation of ghosts with zymosan-stimulated granulocytes causes a strong increase in cytochrome c reduction.

Fig. 6 shows the influence of granulocyte number on cytochrome c reduction in erythrocyte ghosts. A linear correlation between granulocyte number and reduced cytochrome c concentration was observed.

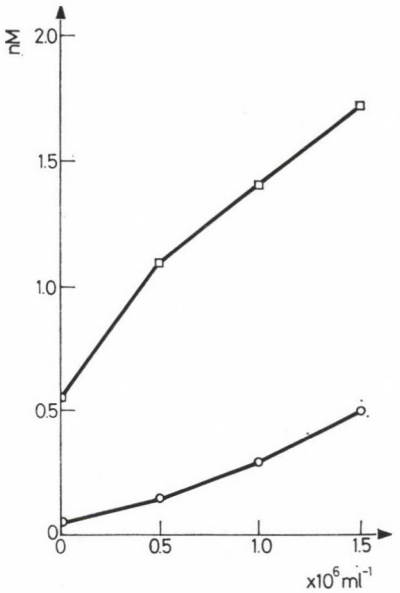

Fig. 6. Relationship between non-stimulated (○) and zymosan-stimulated (□) neutrophil number of diseased calves and cytochrome c reduction in erythrocyte ghosts

Discussion

We have shown in this work that zymosan-activated polymorphonuclear leukocytes from healthy calves and unstimulated neutrophils from diseased animals release reactive oxygen species in amounts sufficient to initiate and propagate lipid peroxidation in red blood cells.

The ratio of granulocytes to red blood cells (1 : 100) that we used is ten times as high as the values found *in vivo* (1 : 1000). However, in some cases e.g. in anaemia with concomitant neutrophilia, the *in vivo* value of this ratio may be similar to those used in this work.

We have shown that catalase and superoxide dismutase partially inhibit lipid peroxidation in red blood cells. This may explain the role of the superoxide radical (O_2^-) and hydrogen peroxide in this process. Since we inhibited lipid peroxidation with oxy radical scavengers only partially, these studies suggest an involvement of other radicals, such as the hydroxyl radical. In addition to oxygen species, activation of neutrophils results in the release of hydrolytic enzymes that may cause damage to the surrounding tissues and cells (Fleer et al., 1979; Voetman et al., 1981).

The increase in lipid peroxidation and MDA production in red blood cells incubated with granulocytes from bronchopneumonic calves might be caused by auto-oxidation of haemoglobin as a results of H_2O_2 overproduction (Hebbel

et al., 1982), or by the presence of large amounts of calcium ions (Jain and Shohet, 1981).

The presence of phospholipid–MDA adduct suggests the existence of peroxidative damage in red blood cells. More than half of peroxidation-susceptible polyunsaturated fatty acids in erythrocyte phospholipids is localized mostly in the PS and PE fractions. These phospholipids, in turn, are localized on the inner half of the lipid bilayer and, hence, close to the putative initial peroxidation source, the relatively redox-unstable haemoglobin.

The results of this work suggest that active granulocytes generate reactive oxygen species or similar products. These compounds can migrate to red blood cells and cause peroxidative damage in them. These phenomena occur *in vivo* in calves affected with bronchopneumonia.

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THE INVOLVEMENT OF POLYMORPHONUCLEAR LEUKOCYTES IN THE PATHOGENESIS OF BRONCHOPNEUMONIA IN CALVES

II. GRANULOCYTE-INDUCED CHANGES IN ERYTHROCYTE MEMBRANE PHOSPHOLIPID TOPOLOGY

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Phospholipid topology in erythrocyte membranes of bronchopneumonic calves and changes in phospholipid asymmetry induced by incubation of erythrocytes with neutrophils of diseased calves were examined.

Using aminophospholipid tracing by non-penetrating probe, trinitrobenzenesulfonic acid, and phospholipid hydrolysis by specific phospholipase A_2 from *Naja naja* venom and sphingomyelinase from *Staphylococcus aureus* it was stated that in erythrocytes from diseased calves phosphatidylserine and, to some extent, phosphatidylethanolamine are externalized on the outer leaflet of the membrane. Similar results were obtained for erythrocytes of healthy calves after incubation with granulocytes of bronchopneumonic animals.

It is suggested that granulocytes of bronchopneumonic calves cause perturbations in erythrocyte membrane phospholipid organization.

Key words: Calf, bronchopneumonia, erythrocytes, phospholipid asymmetry

The phospholipids of normal erythrocytes are arranged asymmetrically across the plasma membrane, i.e. specific classes of lipids are largely restricted to one or the other of the two leaflets of the lipid bilayer (van Deenen, 1981; Op den Kamp, 1979). Since the time required for flopping of lipids from one side of the membrane to the other lasts only a few hours (van Meer et al., 1980; Mohandas et al., 1982), and the life-span of the cell is several months, the erythrocyte must have some mechanism for maintaining this asymmetry. One important component of this maintenance system appears to be the spectrin network that underlies the bilayer (Haest and Deuticke, 1976; Mohandas et al., 1982).

The fact that this thermodynamically unstable lipid configuration is so scrupulously maintained argues for its functional significance.

Lubin and Chiu (1982) have shown abnormal phospholipid organization in membranes of pathologic erythrocytes (kryohydrocytes, stomatocytes). This abnormity is based on phosphatidylethanolamine (PE) and phosphatidylserine (PS) translocation from the inner to the outer leaflet of the bilayer, with con-

comitant translocation of phosphatidylcholine (PC) in the opposite direction. These erythrocytes adhere to endothelial cells in significantly higher numbers than do normal erythrocytes (Hebbel et al., 1980a; Hoover et al., 1979). Moreover, these abnormal adherence properties can be correlated with the clinical severity of the disease as measured by the frequency of microvascular occlusions and clinical status of these patients (Hebbel et al., 1980b).

The aim of this study was to define the phospholipid topology in erythrocyte membranes from bronchopneumonic calves. The influence of incubation of polymorphonuclear leukocytes from diseased calves with erythrocytes of healthy animals on phospholipid asymmetry in these cells was also investigated.

Materials and methods

Samples

The blood of 40 bronchopneumonic calves (4 weeks old) was used as the source of polymorphonuclear leukocytes. Red blood cells were obtained from the blood of 20 healthy animals of the same age.

The acquisition of granulocytes and erythrocytes and the conditions of incubation of neutrophils with red blood cells are described in the accompanying paper (Ledwożyw and Stolarczyk, 1991; see p. 175. of this issue).

PE and PS localization on the outer surface of bilayer, using trinitrobenzenesulfonic acid (TNBS)

TNBS forms covalent linkages with free amino groups, including those of PE and PS molecules, and does not penetrate the membrane of the intact erythrocyte (Chiu et al., 1979).

Washed erythrocytes (0.2 ml, haematocrit 0.5) were preincubated for 30 min at ambient temperature in 5 ml of bicarbonate buffer (50 mM, pH 8.5) containing 5.5 mM glucose and 0.5% bovine serum albumin. Then, 5 mM of TNBS was added and the mixture was incubated for 60 min at 37 °C. After incubation the mixture was centrifuged at 2500 *g* for 10 min and red blood cells were washed with the same buffer without TNBS. Lipids were extracted according to Folch et al. (1957). Phospholipids were analysed by thin-layer chromatography on plates precoated with silica gel G (E. Merck, Darmstadt, Germany) as described by Schick et al. (1972). The percentage of PE and PS labelled with TNBS was calculated from the ratio of lipid phosphorus remaining in the PE and PS bands to the PE and PS band of the corresponding non-TNBS-reacted samples.

Determination of phospholipid organization by phospholipase A_2 and sphingomyelinase

Phospholipase A_2 from *Naja naja* venom and sphingomyelinase from *Staphylococcus aureus* (Sigma, St. Louis, USA) were used in these experiments.

For phospholipase experiments, 0.25 ml of packed erythrocytes was suspended in 5 ml of 10 mM glycylglycine buffer containing 100 mM KCl, 50 mM NaCl, 0.25 mM Mg^{2+}, 0.25 mM Ca^{2+} and 44 mM sucrose (pH 7.4) and preincubated at 37 °C for 60 min. Fifteen international units (IU) of phospholipase A_2, or 10 IU of sphingomyelinase, were then added to the samples, and the incubations were continued for 3 h. Since calcium is required for phospholipase activity, the degradation of phospholipids by these enzymes was terminated by washing the red blood cells with 0.154 M NaCl containing 5 mM EDTA.

Phospholipids were extracted according to Rose and Oklander (1965) and analysed by thin-layer chromatography as described by Roelofsen and Zwaal (1976). Phosphorus in particular phospholipid spots was estimated according to Bartlett (1959). Malondialdehyde (MDA) concentrations were estimated as described previously (Ledwożyw et al., 1986).

Statistical significance of differences was analysed by Student's *t* test for unpaired data.

Results

Figure 1 shows the dependence between MDA accumulation and PS externalization on the outer surface of lipid bilayer in red blood cells of bronchopneumonic calves. A high correlation ($r = 0.92$; $p < 0.001$) was observed between these two events.

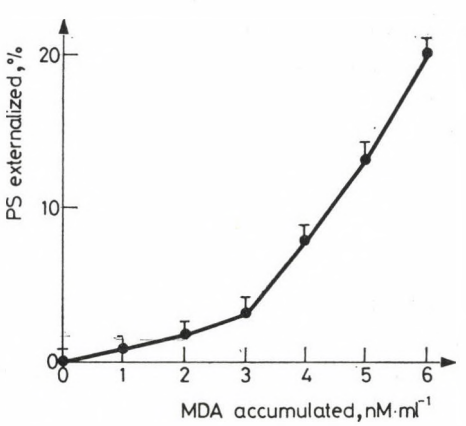

Fig. 1. Relationship between malondialdehyde accumulation and phosphatidylserine externalization in calf erythrocytes (PS = phosphatidylserine; MDA = malondialdehyde)

Table 1 shows phospholipid composition and MDA levels in erythrocytes of calves. Lysophosphatidylcholine and sphingomyelin content increases and phosphatidylserine and phosphatidylinositol content decreases in red blood cells of bronchopneumonic animals. MDA level was significantly higher in erythrocytes of diseased animals than in the control group.

Table 2 shows MDA production in healthy calves' red blood cells incubated with zymosan-stimulated and nonstimulated granulocytes of diseased

Table 1

Phospholipid composition (mole %) and malondialdehyde level ($nM \times ml^{-1}$) in red blood cells of calves (mean $\pm$ SD)

	Healthy	Diseased
LpC	2.2 ± 0.1	2.8 ± 0.2
PE	24.8 ± 0.8	25.0 ± 0.6
PS + PI	15.2 ± 0.4	12.5 ± 0.5*
PC	47.8 ± 1.1	48.2 ± 1.0
Sph	10.0 ± 0.3	11.5 ± 0.4*
MDA	2.2 ± 0.2	5.2 ± 0.4*

LpC = lysophosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PC = phosphatidylcholine; Sph = sphingomyelin; MDA = = malondialdehyde Student's t test for unpaired data: * $p < 0.001$

Table 2

Malondialdehyde production ($nM \times ml^{-1}$) in erythrocytes after incubation with zymosan-stimulated and unstimulated granulocytes from diseased calves (mean $\pm$ SD)

	Granulocytes of healthy calves		Granulocytes of diseased calves	
	Unstimulated A	Stimulated B	Unstimulated C	Stimulated D
MDA	2.2 ± 0.1	2.5 ± 0.1*	2.9 ± 0.2**	3.4 ± 0.3*,**

MDA = malondialdehyde

Student's t test for unpaired data: * $p < 0.001$ B vs. A and D vs. C; ** $p < 0.001$ C vs. A and D vs. B

and healthy calves. The level of this product in red blood cells which were incubated with unstimulated granulocytes of healthy calves was comparable to that observed in erythrocytes of the control group. However, the MDA level was significantly higher in erythrocytes incubated with zymosan-stimulated polymorphonuclear leukocytes of healthy calves. The level of this product was also higher in red blood cells incubated with unstimulated granulocytes from diseased calves.

Table 3 shows PE and PS reactivity with TNBS. In red blood cells of healthy animals, no reaction was observed between PS and chemical tracer.

Table 3

Phosphatidylserine and phosphatidylethanolamine reactivity with trinitrobenzenesulfonic acid in erythrocyte plasma membranes (mean ± SD)

	% of aminophospholipid reacted with TNBS	
	PE	PS
Healthy	12.2 ± 0.4	0
Diseased	25.3 ± 0.5	15.9 ± 0.2

PE = phosphatidylethanolamine; PS = phosphatidylserine

However, in red blood cells of diseased calves 15–16% of this phospholipid reacted with TNBS. PE reactivity was also higher in erythrocytes of diseased animals.

Incubation of red blood cells from healthy animals with unstimulated polymorphonuclear leukocytes of the same group caused no change in aminophospholipid tracing by TNBS, as compared with red blood cells which had not been incubated with granulocytes. Incubation of red blood cells with zymosan-stimulated granulocytes resulted in augmentation of PE tracing and reaction of 18% of the PS fraction with TNBS (Table 4).

Table 5 shows phospholipid degradation in red blood cell membrane by phospholipase A_2 and sphingomyelinase. The amount of phosphatidylcholine

Table 4

Phosphatidylethanolamine and phosphatidylserine reactivity with trinitrobenzenesulfonic acid in erythrocyte plasma membranes after its incubation with stimulated and non-stimulated granulocytes of healthy calves (mean ± SD)

	% of aminophospholipid reacted with TNBS	
	Stimulated granulocytes	Unstimulated granulocytes
Phosphatidylethanolamine	24.9 ± 0.5	12.0 ± 0.3
Phosphatidylserine	18.1 ± 0.4	0

hydrolysed by phospholipase A_2 was lower in erythrocytes of the control group than in those of diseased animals. The amount of hydrolysable PE was two times higher in red blood cells of bronchopneumonic calves. The PS fraction was not hydrolysed in erythrocytes of healthy animals; however, 12 to 16% of this fraction was hydrolysed by phospholipase A_2 in red blood cells of

Table 5

Phospholipid degradation (%) in calf erythrocytes by phospholipase A_2 and sphingomyelinase (mean ± SD)

	% of phospholipid degraded			
	by phospholipase A_2			by sphingomyelinase
	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine	Sphingomyelin
Healthy	59 ± 3	10 ± 1	—	84 ± 4
Diseased	40 ± 2	23 ± 2	16 ± 1	83 ± 3

diseased calves. The amount of sphingomyelin hydrolysed by sphingomyelinase was similar in both groups of animals.

In red blood cells of healthy animals which had been incubated with unstimulated granulocytes of healthy calves the percentages of PE, PS and phosphatidylcholice degradation by phospholipase A_2 and the percentage of sphingomyelin degradation by sphingomyelinase were comparable to those observed in red blood cells of the control group. The amount of phospholipase A_2-degradable phosphatidylcholine was significantly lower in red blood cells incubated with zymosan-stimulated granulocytes of healthy calves and with

Table 6

Phospholipid degradation (%) in erythrocytes after incubation with zymosan-stimulated and unstimulated granulocytes of healthy and diseased calves, by phospholipase A_2 and sphingomyelinase (mean ± SD)

	% of phospholipid degraded			
	by phospholipase A_2			by sphingomyelinase
	PC	PE	PS	Sph
Erythrocytes + unstimulated granulocytes of healthy calves	61 ± 2	10 ± 1	—	84 ± 4
Erythrocytes + stimulated granulocytes of healthy calves	55 ± 1	14 ± 1	8 ± 1	83 ± 4
Erythrocytes + unstimulated granulocytes of diseased calves	52 ± 1	14 ± 1	11 ± 1	83 ± 3
Erythrocytes + stimulated granulocytes of diseased calves	44 ± 2	19 ± 1	20 ± 1	82 ± 2

PC = phosphatidylcholine; PE = phosphatidylethamolamine; PS = phosphatidylserine; Sph = = sphingomyelin

unstimulated and zymosan-stimulated granulocytes of bronchopneumonic animals. In these cases, the amounts of phospholipase-hydrolysable PE and PS were higher than in the control group (Table 6).

Discussion

Membrane lipid peroxidation and its role in red blood cell pathology have been investigated by many authors (Claster et al., 1984; Jacob and Lux, 1968; Jain, 1984; Pfafferott et al., 1982). Lipid peroxidation can increase membrane permeability (Chiu et al., 1982; Markert et al., 1984), haemoglobin binding to the membrane (Sauberman et al., 1981; Weiss, 1982), and can decrease the deformability of red blood cells (Pfafferott et al., 1982) and their survival (Jain et al., 1983).

The results of this study suggest that membrane lipid peroxidation in red blood cells induced by free radicals released from polymorphonuclear leukocytes of bronchopneumonic calves can cause PS and PE externalization in the red blood cell membrane. This might be the cause of its ability to form microthrombi, as was observed by Jain (1985) in rats after phenylhydrazine administration. This compound initiates lipid peroxidation (Jain and Hochstein, 1980; Jain and Subrahmanyan, 1978), and generates oxygen-free radicals (Goldberg et al., 1975; Jain and Hochstein, 1979).

In red blood cells of healthy calves, both PS and PE occupied the outer side of the membrane only in very small amounts. Zwaal et al. (1977) have shown that the asymmetric distribution of phospholipids in red blood cell membrane plays an essential role in haemostasis. Wilschut and Papahadjopoulos (1979) have shown that negatively charged phospholipids (PS) and calcium ions are essential in cell-to-cell contact. This suggests that erythrocytes with abnormal PS externalization have a greater tendency to adhere to endothelial cells and may contribute to the formation of microvascular occlusions.

More convincing evidence of the role of phospholipid arrangement in erythrocyte behaviour has been provided by Schlegel et al. (1985), who showed that erythrocyte ghosts with abnormal symmetrical distribution of phospholipids adhered to endothelial cell monolayers to a greater extent than did the ghosts of normal red blood cells. Tanaka and Schroit (1983) showed that introduction of PS analogues into red blood cell plasma membrane increases their binding to macrophages.

The decrease in PS content in red blood cells of diseased calves may be due to the fact that this phospholipid is especially rich in peroxidation-susceptible polyunsaturated fatty acids (Dodge and Phillips, 1967). Contrary to other phospholipids, PS in red blood cells does not contribute to exchange processes with serum phospholipids (van Deenen and de Gier, 1974).

The mechanism responsible for PS and PE externalization is not known. Oxidation of spectrin was shown to cause alterations in membrane phospholipid asymmetry with increased amounts of PE and PS exposed on the outer leaflet of the lipid bilayer of erythrocytes (Haest and Deuticke, 1976; Haest et al.,

1978). Chiu et al. (1983) have shown that overloading of red blood cells with calcium ions results in the externalization of PS. Lubin et al. (1983) observed a similar situation in erythrocytes with congenital spectrin deficiency. Moreover, MDA generated during lipid peroxidation can induce cross-linkages between phospholipid molecules (Bidlack and Tappel, 1973; Jain and Shohet, 1984). This may be the cause of additional perturbations in lipid microenvironment of the membrane.

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THE INVOLVEMENT OF POLYMORPHONUCLEAR LEUKOCYTES IN THE PATHOGENESIS OF BRONCHOPNEUMONIA IN CALVES III. GRANULOCYTE ACTIVATION BY PLATELETS

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The influence of platelets and platelet lysate on superoxide anion production and β-glucuronidase release from neutrophils was investigated. Neither the suspension of unstimulated platelets from healthy calves nor its lysate had any influence on O_2^- production and enzyme release On the other hand, a great rise in superoxide anion production and β-glucuronidase release was observed in the case of platelets from bronchopneumonic calves.

It is suggested that platelets of bronchopneumonic calves can activate polymorphonuclear leukocytes, causing their degranulation and aggravating superoxide anion production.

Key words: Calf, bronchopneumonia, platelet, granulocyte activation

Polymorphonuclear leukocytes are often found adherent to, or closely associated with, blood platelets in experimentally induced thrombi (Mitchell and Sharp, 1969), a fact suggesting that they may participate in haemostatic and thrombotic processes (Nachman and Weksler, 1972; Ratcliff et al., 1971; Silbergleit, 1970). Evidence points to a possible role of both platelets and granulocytes in clinical conditions such as myocardial infarction (Hammerschmidt et al., 1982; Hill and Ward, 1971; Takur et al., 1979), shock lung (Hammerschmidt et al., 1980) and microvessel endothelial damage (Boogaerts et al., 1982). Products of the platelet-released material may promote migration of leukocytes into thrombi (Nachman and Weksler, 1972), and may increase phagocytic activity (Laghi-Pasini et al., 1983) and cellular aggregation (Redl et al., 1983). However, the factor released from platelets is not known.

The aim of this study was to investigate the effect of platelet release product(s) on granulocyte activation (β-glucuronidase release and superoxide anion production) in bronchopneumonic calves.

Materials and methods

The blood of 40 four-week-old calves affected with bronchopneumonia was used as the source of blood platelets. Polymorphonuclear leukocytes were obtained from the blood of 20 healthy animals of the same age.

Neutrophils were separated by dextran gradient sedimentation and differential centrifugation (Laghi-Pasini et al., 1983). Platelet-rich plasma and platelet-poor plasma were obtained as described by Laghi-Pasini et al. (1985). The platelet count in platelet-rich plasma was adjusted to 4×10^8 cells $\times$ ml^{-1}. The influence of platelet release products was investigated in Boyden chambers. When the maximal aggregation of platelets was reached (1–8 μM ADP), 0.5 ml platelet-rich plasma was removed and placed in the upper compartment of the Boyden chamber, whereas 0.125 ml of granulocyte suspension from healthy calves (2×10^6 cells $\times$ ml^{-1}) was placed in the lower compartment separated by a dialysis membrane (exclusion index: 20,000) to retain high molecular weight products and to avoid direct contact of platelets with neutrophils.

The effect of platelet lysate, prepared by three cycles of freezing and thawing, was also investigated.

After 20-min incubation at 37 °C, β-glucuronidase release from polymorphonuclear leukocytes was assayed according to Ward et al. (1983) and Weiss and Ward (1982). Superoxide anion generation was estimated as described by Johnston et al. (1975) and Yonetani (1965).

The statistical significance of differences was analysed by Student's t test for unpaired data.

Results

Fig. 1 shows the influence of platelet number and its lysate concentration on superoxide anion production in neutrophils. Unstimulated platelet suspension and its lysate have minimal influence on O_2^- production. However, production of superoxide anion was about 14 times higher in the case of unstimulated platelets from bronchopneumonic calves.

Similar changes were observed in neutrophil degranulation measured by β-glucuronidase release (Fig. 2).

Discussion

The present findings demonstrate that stimulated platelets from healthy calves and unstimulated platelets from bronchopneumonic animals can induce granulocyte activation expressed as specific degranulation and superoxide anion production. In these events, the immediate contact between platelets and granulocytes is not necessary. Separation of platelets from granulocytes by semipermeable membrane suggests that this phenomenon is caused by a factor with a molecular weight below 20,000. Sakamoto and Firkin (1984) have shown that activated platelets release a leukocyte phagocytosis stimulating

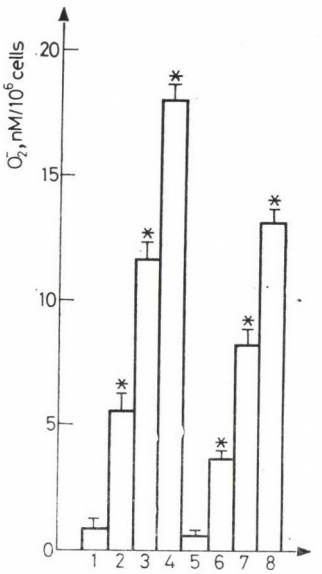

Fig. 1. The influence of platelets and platelet lysate on O_2^- production by neutrophils. 1 — non-activated from healthy calves; 2 — activated platelets from healthy calves; 3 — non-activated platelets from diseased calves; 4 — activated platelets from diseased calves; 5 — lysate of non-activated platelets from healthy calves; 6 — lysate of activated platelets from healthy calves; 7 — lysate of non-activated platelets from diseased calves; 8 — lysate of activated platelets from diseased calves. *$p < 0.001$

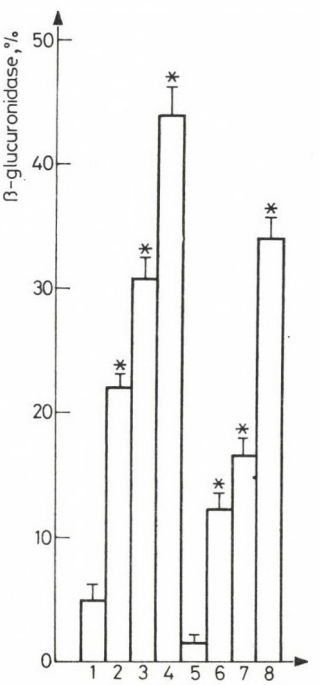

Fig. 2. The influence of platelets and platelet lysate on neutrophil degranulation (β-glucuronidase release). Other designations as in Fig. 1

agent with a molecular weight below 500. Redl et al. (1983) found that platelet lysate augmented chemotoxin-induced granulocyte aggregation. The lysates of aspirin- or indomethacin-pretreated platelets also retained the ability to produce this augmentative substance. This fact suggests that the production of this factor is independent of thromboxane synthesis.

Several authors (Hammerschmidt et al., 1982; Weksler and Coupal, 1973) have shown that platelets release proteinaceous chemotactic factors. Both platelets and thromboxane A_2 augment granulocyte adherence *in vitro* (Rasp et al., 1981; Spagnuolo et al., 1980).

In our experiments, activated platelets stimulated neutrophil degranulation and superoxide anion production. These events may play a role in the pathogenesis of bronchopneumonia in calves.

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THE INVOLVEMENT OF POLYMORPHONUCLEAR LEUKOCYTES IN THE PATHOGENESIS OF BRONCHOPNEUMONIA IN CALVES

IV. MYELOPEROXIDASE ACTIVITY

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Stable oxidant and hypochlorous acid production in neutrophils of bronchopneumonic calves was investigated. The production of these compounds was much more intense in neutrophils of diseased calves than in the control cells. The production of stable oxidants and hypochlorous acid was restrained by free radical scavengers. Functional response of neutrophils was inhibited by cyclooxygenase products and stimulated by lipoxygenase products of the arachidonic acid cascade. Superoxide anion production is not connected with the degranulation process (β-glucuronidase release), and the two events are under different control mechanisms.

Key words: Calf, bronchopneumonia, polymorphonuclear leukocyte, myeloperoxidase activity

Activated neutrophils release a variety of reactive oxygen species, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and, possibly, the hydroxyl radical (Babior, 1978; Klebanoff, 1980). By the myeloperoxidase enzymatic system, neutrophils also produce hypochlorous acid (HOCl) and a variety of chlorinated amines (stable oxidants) (Grisham et al., 1984a, b; Sagone et al., 1984; Thomas et al., 1983; Weiss et al., 1983). All these products play an important role in host defense against microorganisms, and in tissue injury.

Cell surface activation of neutrophils initiates, either by soluble or by particulate stimuli, a series of reactions ultimately leading to a functional response of the cell. Receptor–ligand interaction leads to a series of cell responses, including activation of phospholipases and protein kinases, production of 1,2-diacylglycerols, release of arachidonic acid metabolites, a rise in calcium level within the cytosol, an increase in oxygen consumption and, ultimately, a series of functional responses of the cell, including generation of oxygen-derived free radicals, secretion of lysosomal enzymes and changes in cell motility (Aswanikumar et al., 1977; Marasco et al., 1983; Schiffman et al., 1975; Williams et al., 1977).

There is now increasing evidence that lipoxygenase products, such as leukotrienes (LTs) and hydroxyeicosatetraenoic acids, generated by neutrophils, play a key role in these processes (Borgeat and Samuelsson, 1979; Honuna et al., 1982; Naccache et al., 1982; Sklar et al., 1982).

In the present work, we evaluated stable oxidant production in neutrophils of bronchopneumonic calves and studied the influence of lipoxygenase and cyclooxygenase inhibitors on granulocyte activation in such animals.

Materials and methods

The blood of 40 four-week-old bronchopneumonic calves was used as the source of polymorphonuclear leukocytes, and that of 20 healthy animals of the same age served as control material.

Granulocytes were obtained by dextran sedimentation and Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation as described by Boyum (1968).

Neutrophils (4×10^6 cells $\times$ ml^{-1}) were stimulated with zymosan (1 mg $\times$ $\times$ ml^{-1}, Sigma, St. Louis, USA) in autologous plasma as described in part I of this series (Ledwożyw and Stolarczyk, 1991). Some experiments were conducted in the presence of superoxide dismutase (10 μg $\times$ ml^{-1}, specific activity: 3000 units/mg protein, Sigma), sodium benzoate (10 mM), mannitol (40 mM) or dimethylsulfoxide (20 mM). Granulocytes were incubated also with lipoxygenase (nordihydroguaiaretic acid, nafazatrom) or cyclooxygenase inhibitors (indomethacin, piroxicam, ibuprofen, BW755C). Reactions were terminated at 2 h by icing and by adding 25 μg $\times$ ml^{-1} of catalase (specific activity: 5000 units/mg protein, Sigma). After centrifugation (30,000 *g*, 5 min), the cell-free supernatants were assayed for stable oxidants by spectrophotometric methods.

The first method depends on the ability of stable oxidants to oxidize I to I_2 (Sagone et al., 1984; Weiss et al., 1982). Supernatants (0.6 ml) were added to chilled tubes containing 2.4 ml of 1.25% KI in 100 mM phosphate buffer (pH 7.0) and changes in extinction at 352 nm were read immediately, assuming a molar extinction coefficient of 2.64×10^4 M$^{-1} \times$ cm^{-1} (Tsan and Chen, 1980).

The second method depends on the ability of stable oxidants to oxidize 5-thio-2-nitrobenzoic acid (TNB) to 5,5'-dithiobis (2-nitrobenzoic) acid (DTNB) (Aune and Thomas, 1977). A stock solution of TNB (0.6 mM) was prepared by adding 0.004 ml of 14.3 M solution of 2-mercaptoethanol to 100 ml of 1 mM DTNB solution in phosphate buffer (100 mM, pH 7.2). Supernatants (0.6 ml) were added to chilled tubes containing 2.3 ml of phosphate buffer and 0.1 ml of stock TNB solution. Extinction at 412 nm was read at exactly 3 min, assuming a molar extinction coefficient of. 1.36×10^4 M$^{-1} \times$ cm^{-1}.

HOCl production was quantitated by the taurine chlorination method (Weiss et al., 1982), myeloperoxidase activity was determined by oxidation of o-dianisidine (Henson, 1971), β-glucuronidase activity was measured by in-

crease of extinction at 540 nm with phenolphthalein β-glucuronide as substrate (Ward et al., 1983), and lactate dehydrogenase activity was assayed according to Showell et al. (1976). Superoxide anion generation was measured as cytochrome c reduction rate in the presence of superoxide dismutase (Weiss and Ward, 1982).

Statistical analysis was performed by Student's *t* test for unpaired data.

Results

Figure 1 showss table oxidant production in unstimulated and opsonized zymosan-stimulated neutrophils of calves as dependent on the stimulation time. The quantity of these compounds was higher in neutrophils from diseased calves.

Similar results were obtained in the case of HOCl production in granulocytes of calves thus investigated (Fig. 2).

Figure 3 illustrates the influence of free radical scavengers on stable oxidant production in zymosan-stimulated granulocytes. All scavengers thus investigated decrease the production of these compounds.

Figure 4 shows zymosan-dependent activation of neutrophils from healthy calves as measured by β-glucuronidase, myeloperoxidase and lactate dehydrogenase activities. This activation is specific, e.g. β-glucuronidase and myeloperoxidase are released in the degranulation process, but not by cell lysis (no changes in lactate dehydrogenase activity were observed).

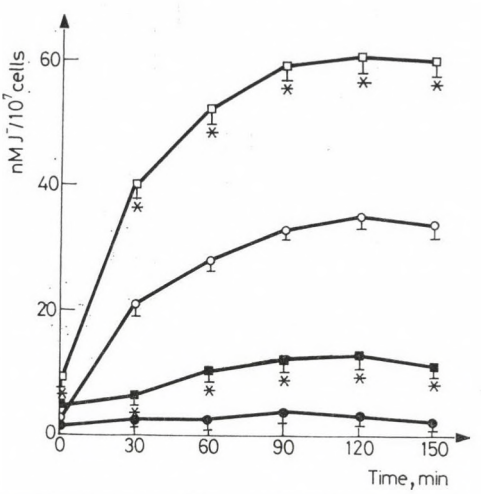

Fig. 1. Stable oxidant production in calf granulocytes, as dependent on stimulation time. ● — unstimulated granulocytes from healthy calves, ■ — stimulated granulocytes from healthy calves, ○ — unstimulated granulocytes from diseased calves, □ — stimulated granulocytes from diseased calves. × — $p < 0.01$

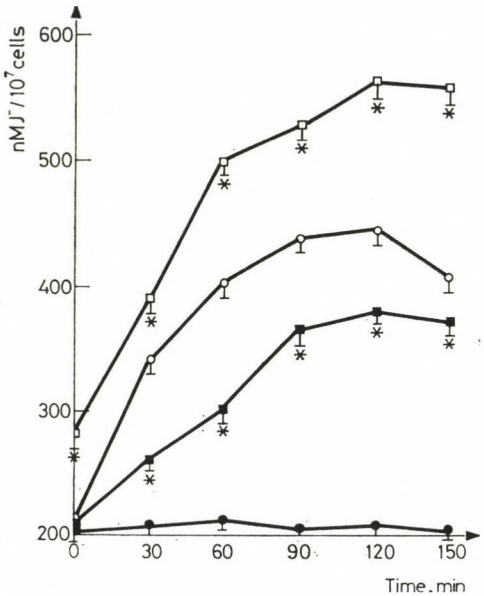

Fig. 2. Hypochlorous acid production in calf granulocytes, as dependent on incubation time. ● — unstimulated granulocytes from healthy calves, ■ — stimulated granulocytes from healthy calves, ○ — unstimulated granulocytes from diseased calves, □ — stimulated granulocytes from diseased calves. × — $p < 0.01$

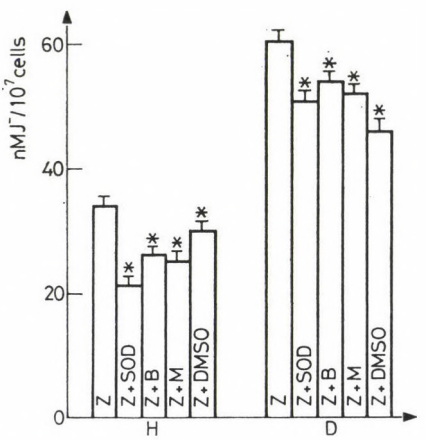

Fig. 3. The influence of free radical scavengers on stable oxidant production in stimulated granulocytes of calves. Z = zymosan, Z + SOD = zymosan + superoxide dismutase, Z + B = zymosan + sodium benzoate, Z + M = zymosan + mannitol, Z + DMSO = zymosan + dimethylsulfoxide, H = healthy animals, D = diseased animals, × — $p < 0.01$

Figure 5 indicates activation of granulocytes from bronchopneumonic calves. In this case, activation is also a specific process; however, a stimulator concentration of 0.5 mg per 2×10^6 cells causes a 3 times greater β-glucuronid-

ase and myeloperoxidase release as compared to granulocytes of healthy calves.

Figure 6 shows the effect of lipoxygenase inhibitors (nordihydroguaiaretic acid, nafazatrom) on β-glucuronidase release from zymosan-stimulated granulocytes of healthy calves. At a concentration of 100 μM nordihydroguaiaretic acid causes a nearly complete inhibition of enzyme release. In the case of

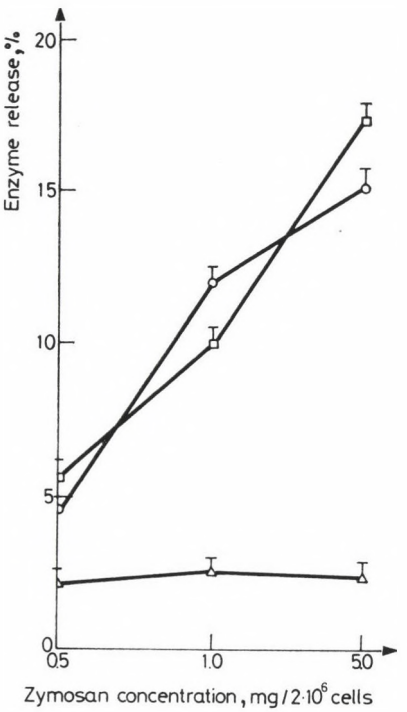

Fig. 4. Granulocyte activation in healthy calves as measured by β-glucuronidase (○), myeloperoxidase (□) and lactate dehydrogenase (△) release

nafazatrom, a comparable effect was observed at 10 μM inhibitor concentration.

Figure 7 shows the influence of cyclooxygenase inhibitors (indomethacin, piroxicam, ibuprofen, BW755C) on β-glucuronidase release from zymosan-stimulated neutrophils of healthy calves. Piroxicam, ibuprofen and BW755C all have an inhibitory dose-dependent effect on enzyme release. Indomethacin had no effect on β-glucuronidase release in this experiment.

In Fig. 8 the effect of lipoxygenase inhibitors on superoxide anion generation by neutrophils is presented. At concentrations above 0.1 μM nordi-

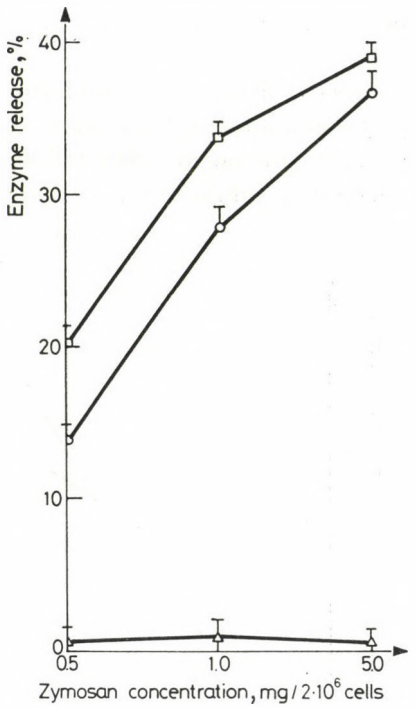

Fig. 5. Granulocyte activation in bronchopneumonic calves as measured by β-glucuronidase (○), myeloperoxidase (□) and lactate dehydrogenase (△) release

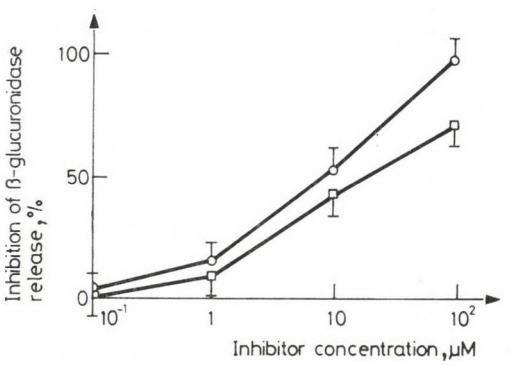

Fig. 6. The influence of lipoxygenase inhibitors on β-glucuronidase release from stimulated granulocytes of healthy calves. ○ — nordihydroguaiaretic acid, □ — nafazatrom

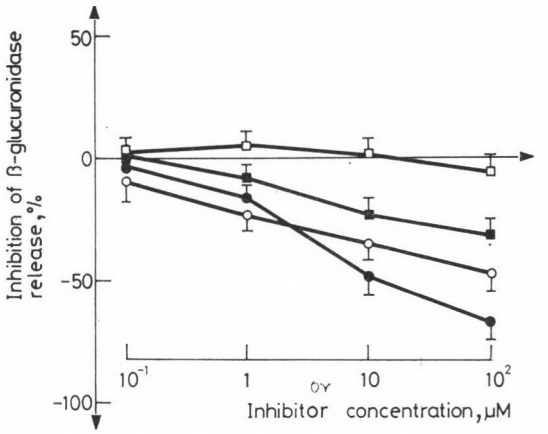

Fig. 7. The influence of lipoxygenase inhibitors on β-glucuronidase release from stimulated granulocytes of healthy calves. □ — indomethacin, ■ — ibuprofen, ○ — piroxicam, ● — BW755C

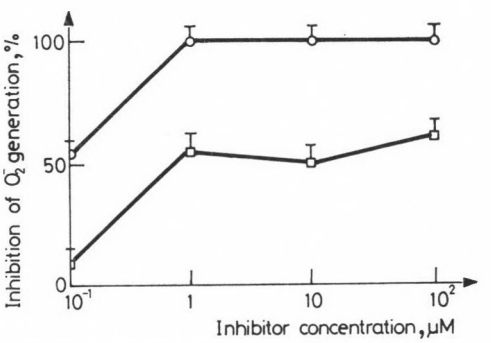

Fig. 8. The influence of lipoxygenase inhibitors on O_2^- generation in stimulated granulocytes of healthy calves. ○ — nordihydroguaiaretic acid, □ — nafazatrom

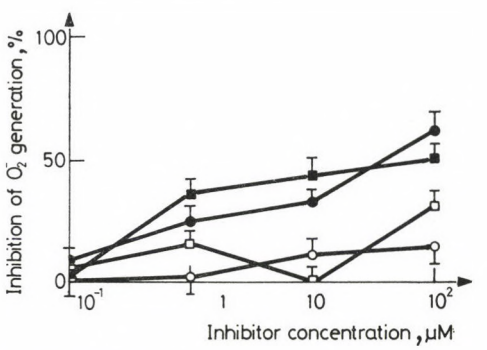

Fig. 9. The influence of cyclooxygenase inhibitors on O_2^- generation in stimulated granulocytes of healthy calves. □ — indomethacin, ■ — ibuprofen, ○ — piroxicam, ● — BW755C

hydroguaiaretic acid caused a complete inhibition of superoxide anion production. At this concentration nafazatrom caused a 50% inhibition of O_2^- generation.

Figure 9 shows the influence of cyclooxygenase inhibitors on O_2^- productionby zymosan-stimulated granulocytes of healthy calves. All compounds thus investigated cause a decrease in superoxide anion production.

Discussion

The present study provides evidence that unstimulated and zymosan-stimulated neutrophils of bronchopneumonic calves produce more HOCl and stable oxidants than do neutrophils of healthy animals. The fall in stable oxidants may be due to increased destruction of these compounds over time. Grisham et al. (1984a) found a significant decrease in stable oxidants between 30 and 60 min stimulation time. The time-dependent decrease in stable oxidants may be caused by interference with other radicals generated in granulocytes. Although increased dismutation of O_2^- to H_2O_2 could theoretically explain why superoxide dismutase yielded more stable oxidants, this effect in neutrophil oxidant production is usually of little importance (Baehner et al., 1975; Sagone et al., 1976). Sodium benzoate, a specific hydroxyl scavenger (Bors et al., 1982), also increases stable oxidant production. Thus, the experiments conducted with superoxide dismutase and benzoate support the concept that O_2 and OH can degrade the stable oxidants. It is also possible that the fall in stable oxidants over time is due to their ability to react with other cellular products including proteins, amino acids, ammonia and glutathione (Thomas et al., 1983). Since H_2O_2 can reduce HOCl, it is possible that the high levels of H_2O_2 destroy some portion of the HOCl before it can react with taurine (Weiss et al., 1982).

Oxygen radical production was found to be limited by the availability of oxygen at the site of inflammation (Edwards et al., 1983, 1984). However, our results suggest that production of stable oxidants exists for a long time after stimulator is added. Our data extend those obtained with rabbit and human granulocytes (Backer et al., 1979; Baxter et al., 1983; Fletcher et al., 1982; Goldstein et al., 1974).

The role of arachidonate metabolites in neutrophil activation is of much interest. There is no doubt that granulocyte activation by soluble and particulate stimuli results in the generation of cyclooxygenase and lipoxygenase products (Bass et al., 1981; Lindgren et al., 1981; Rubin et al., 1979; Stenson and Parker, 1979; Walsh et al., 1981). The latter category of products, especially, LTB_4, causes increased calcium flux into the cytosol (Naccache et al., 1982), a process that appears to be a requirement for the cell to respond to

chemotactic signals. It is not clear whether or not LTB_4 upon its generation in the cell acts entirely intracellularly or whether it is secreted and acts on other neutrophils (Ward et al., 1984). Generation of LTB_4 can be considered to be an important step in the response of neutrophils to chemotactic factors. Cyclooxygenase products appear to have an inhibitory effect on phagocytic function of the cells, perhaps related to their ability to elevate intracellular levels of cAMP (Marone et al., 1980). Accordingly, it is not surprising that cyclooxygenase inhibitors increase β-glucuronidase release through reduced cell generation of PGE_2 and PGI_2.

The results of our work suggest that superoxide anion generation in neutrophils is not connected with enzyme release, and that the two events are under different control mechanisms. The general conclusion that cyclooxygenase products inhibit functional responses of neutrophils, whereas lipoxygenase products facilitate the same cell responses is similar to that of another work using an entirely different cell system (Metz et al., 1983).

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PROTECTIVE EFFECT OF LIPOSOME-ENTRAPPED SUPEROXIDE DISMUTASE AND CATALASE ON BLEOMYCIN-INDUCED LUNG INJURY IN RATS

I. ANTIOXIDANT ENZYME ACTIVITIES AND LIPID PEROXIDATION

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The influence of liposome-entrapped catalase and/or superoxide dismutase on bleomycin-induced rat lung injury was studied. Liposome-entrapped catalase and/or superoxide dismutase increase antioxidant enzyme activities in the lung tissue of bleomycin-treated rats. The level of lipid peroxidation products (malondialdehyde, conjugated dienes, lipid hydroperoxides) was significantly lower in liposome-entrapped catalase and/or superoxide dismutase supplemented rats with bleomycin-injured lungs. It is suggested that liposomes are good vectors for drugs in the treatment of bleomycin-induced lung fibrosis.

Key words: Liposomes, antioxidant enzymes, bleomycin, lung fibrosis

Bleomycin, a mixture of glycopeptides from *Streptomyces vesticillus*, is used in the therapy of selected human tumours with high efficacy and low bone marrow toxicity (Umezawa et al., 1966). It is widely used in the treatment of lymphomas, squamous cell carcinomas and testicular tumours (Crooke and Bradner, 1976; Friedman, 1978; Garnick, 1985).

One of the serious adverse side effects limiting the clinical use of this drug is that it produces interstitial pulmonary fibrosis (Delena et al., 1972; Krous and Hamlin, 1973). The pulmonary toxicity of bleomycin can take several forms. A dose-related fall in diffusing capacity (gas transfer coefficient) is the commonest (Van Barneveld et al., 1985), but a 5–20% incidence of pneumonitis has also been reported (Dalgleish et al., 1984; White and Stouer, 1984) which may develop into an injury showing the characteristics of adult respiratory distress syndrome (Jones, 1978).

The lung effects of bleomycin are unpredictable, although several conditions may predispose to injury. These include concomitant exposure to radiation (Catane et al., 1979), use in combination chemotherapy programmes, total drug dose above 450 mg, and impairment of renal function. Exposure

to high inspired oxygen concentrations has also been suggested as a stimulus to produce acute lung injury (Douglas and Coppin, 1980; Goldiner et al., 1978), although there is some controversy in this respect (Goldiner and Rooney, 1984; LaMantia et al., 1984).

Intratracheal administration of bleomycin is employed to produce an animal model of interstitial pulmonary fibrosis (Raisfeld, 1980; Laurent et al., 1981).

Some of the morphological and biochemical events associated with the fibrotic phase of the injury have been examined (Laurent, 1985, 1986). There is an early inflammatory phase with a marked influx of neutrophils. Fibrosis develops rapidly with maximal collagen deposition 2–4 weeks after intratracheal administration. The rate of collagen synthesis also increases, and there may be a reduced rate of collagen degradation (Laurent and McAnulty, 1983). There is preliminary evidence for an increased burden of fibroblast growth-promoting activity (Oliver et al., 1985).

The biochemical mechanisms responsible for bleomycin-induced lung fibrosis are poorly understood. It is known, however, that bleomycin is able to generate reactive oxygen species such as superoxide and hydroxyl radicals in the presence of molecular oxygen (Sugiura and Kikuchi, 1978; Sausville et al., 1978; Oberley and Buettner, 1979). The availability of excess reactive oxygen species was shown to cause DNA strand scission (Ekimoto et al., 1980; Sugiura et al., 1982) and lipid peroxidation of the cell membranes (Tom and Montgomery, 1980; Giri et al., 1983).

Phospholipid molecules exhibit ampiphilic properties and therefore they aggregate either in their crystalline state or, in polar solvents, into ordered structures with typical lyotropic liquid crystalline symmetries. In aqueous solutions phospholipid molecules normally form self-closed spherical or oval structures where one or several phospholipid bilayer(s) entrap(s) part of the solvent in its interior (Lasic, 1988). Vesicles are very important in many different areas of science and technology. In basic research they serve as models for cell mambranes and their fusion, transport studies and as delivery vehicles for drugs, genetic material, enzymes and other molecules into living cells and through other hydrophobic barriers in pharmacology and medicine (Tyrrell et al., 1976; Szoka and Papahadjopoulos, 1980). Nucleic acids (Schaefer-Ridder et al., 1982; Lavelle et al., 1982), proteins (Takada et al., 1982) and antitumour drugs (Kimelberg et al., 1976; Patel and Baldeschwieler, 1984) can become cellassociated and biologically active, using liposomes as transmembrane vectors. The intracellular content of these macromolecules can be multiplied.

In this report evidence is presented that rats can be protected against bleomycin-induced lung fibrosis by intraperitoneal administration of liposomes containing catalase and/or superoxide dismutase.

Materials and methods

Preparation of liposomes

Liposomes were prepared by reverse-phase evaporation as described by Szoka and Papahadjopoulos (1978) and consisted of L-α-dipalmitoylphosphatidylcholine/cholesterol/stearylamine (molar ratio: 14:7:4). Lipids dissolved in chloroform (25 ml; 7 μm dipalmitoylphosphatidylcholine per ml) were mixed with an aqueous solution of enzyme (8 ml) in 4 mM potassium phosphate (pH 7.4) and sonicated until a homogeneous emulsion was formed. Chloroform was then removed *in vacuo* by rotary evaporation.

Bovine liver catalase (50,000 U/mg protein, Sigma, St. Louis, USA) and bovine liver Cu, Zn-superoxide dismutase (3000 U/mg protein, Sigma) were dissolved in 4 mM phosphate buffer (pH 7.4). As found by Turrens et al. (1984), aqueous solutions of these enzymes are stable to emulsification with chloroform and heating to 46 °C, and no loss of enzymatic activity is observed under these conditions. Liposome concentrations were expressed per μmole phospholipid, with 100 μM phospholipid corresponding to 105 mg of total liposome lipid. Phospholipid phosphorus was estimated according to Bartlett (1959).

Approximately 35% of enzyme in aqueous solution was liposome associated after reverse-phase evaporation, centrifugation and washing. Enzyme-to-phospholipid ratios before reverse-phase evaporation were 1.5×10^4 U catalase/μM phospholipid and 2.5×10^2 U superoxide dismutase/μM phospholipid. Liposomes were prepared the day before injection and assayed for enzyme content. Following reverse-phase evaporation, liposomes were separated from non-entrapped protein by centrifugation at 105,000 *g* for 30 min and resuspended in 0.154 M NaCl buffered with 10 mM potassium phosphate (pH 7.4), yielding a liposome phospholipid concentration of about 20 μM/ml of buffer.

Control or "empty" liposomes contained enzymes in the external milieu. "Empty" liposomes were prepared by reverse-phase evaporation in the presence of 4 mM potassium phosphate (pH 7.4). After centrifugation, liposomes were resuspended in 0.154 M NaCl and 10 mM potassium phosphate (pH 7.4), containing catalase and superoxide dismutase in concentrations equivalent to those of enzyme-containing liposomes (per μM liposome phospholipid).

Treatment of rats with bleomycin and liposomes

Studies were carried out on male Sprague Dawley rats weighing 320–350 g and certified free from specific bacterial, viral and parasitic pathogens. The rats were provided with food and water *ad libitum*, housed in laminar

flow cages with bedding, and maintained on a 12-h light/dark cycle. Bleomycin hydrochloride (Bleocina, Nippon Kayaku Co. Ltd., Tokyo, Japan) was used throughout the study. After an adaptive phase of 4 weeks, intratracheal instillation of bleomycin was accomplished via a stainless steel cannula inserted into the trachea under general anaesthesia (Ketamine 100 mg/kg + Diazepam 5 mg/kg).

One group of rats (A, n = 10) received no treatment at all. A second group (B, n = 10) was instilled intratracheally with saline (0.2 ml), whereas group C (n = 50) received a single dose of bleomycin (10 mg/kg) in a volume of 0.2 ml. One week after beleomycin administration, rats of group C were divided into 5 subgroups of 10 animals each. One subgroup (C-1) was left without treatment. A second subgroup (C-2) received "empty" liposomes as intraperitoneal injection of about 40 μM liposome phospholipid in 2.0 ml of NaCl-potassium phosphate. Animals of the third subgroup (C-3) were injected with liposome-entrapped catalase, while those of the fourth subgroup (C-4) with liposome-entrapped superoxide dismutase. The remaining 10 rats (C-5) were injected intraperitoneally with liposomes containing both catalase and superoxide dismutase.

Before injection, liposomes were passed through a 27-gauge needle to disperse aggregates. Liposome injections were repeated every 48 h thereafter over the 8-day period of the experiment. Two h after the last liposome injection (day 16 after bleomycin instillation), all rats were killed by decapitation, the lungs were perfused, through the pulmonary artery, with 0.154 M NaCl to remove blood elements for the measurement of lung superoxide dismutase and catalase activity.

Table 1

Antioxidant enzyme activities

	A	B	C-1
Cu, Zn-superoxide dismutase	62.5 ± 5.1	60.3 ± 4.6	201.3 ± 8.1[a]
Mn-superoxide dismutase	21.3 ± 3.0	20.5 ± 2.8	107.5 ± 4.1[a]
Glutathione peroxidase	1.14 ± 0.21	1.10 ± 0.14	4.40 ± 0.14[a]
Glutathione reductase	0.34 ± 0.02	0.35 ± 0.03	1.02 ± 0.04[a]
Catalase	180 ± 17	192 ± 18	840 ± 25[a]

Mean values ± SD. Statistical significance $p < 0.05$: a = C-1 vs. A; b = C-2 vs. A; c = C-3 vs. A; d = C-4 vs. A; e = C-5 vs. A; f = C-3 vs. C-1; g = C-4 vs. C-1; h = C-5 vs. C-1. Each group consisted of 10 animals. Group designation as in Materials and methods. Definition of units: one unit of Cu, Zn-superoxide dismutase activity = the amount of enzyme which produces 50% inhibition of epinephrine autoxidation. One unit of Mn-superoxide dismutase activity = the

Other assays

Lungs were homogenized with a Waring Blendor homogenizer in 10 ml of 5 mM Tris-HCl buffer containing 0.2% sodium dodecyl sulfate (pH 7.4). Homogenates were centrifuged at 1500 *g* for 10 min to remove cell debris.

Catalase activity was assayed to Bergmeyer (1955). Superoxide dismutase activity was measured as described by Crapo et al. (1978). Glutathione peroxidase and glutathione reductase were assayed as described previously (Ledwożyw and Kądziołka, 1989). Conjugated dienes and hydroperoxides were estimated according to Buege and Aust (1978). Malondialdehyde levels were assayed as described previously (Ledwożyw et al., 1986). Protein was measured according to Lowry et al. (1951). DNA content was assayed by the diphenylamine method (Richards, 1974).

All data were evaluated by one-way analysis of variance followed by Duncan's multiple-range test (Duncan, 1955) with statistical significance defined as $p < 0.05$.

Results

Table 1 summarizes the influence of bleomycin and enzyme-containing liposomes on five antioxidant enzyme activities in rat lung. Bleomycin alone (group C-1) causes a significant increase in all enzyme activities thus investigated. Injection of "empty" liposomes (group C-2) causes a further small rise in enzyme activities. Intraperitoneal administration of catalase- and/or superoxide dismutase-containing liposomes (groups C-3, C-4 and C-5) causes a further significant increase in antioxidant enzyme activities in rat lung.

units × mg DNA^{-1}) in rat lung

C-2	C-3	C-4	C-5
210.3 ± 9.6^{b}	231.4 ± 8.4^{cf}	335.4 ± 9.2^{dg}	306.2 ± 9.5^{eh}
112.4 ± 5.2^{b}	132.3 ± 6.1^{cf}	169.3 ± 6.8^{dg}	151.2 ± 5.5^{eh}
4.54 ± 0.17^{b}	5.47 ± 0.20^{cf}	5.56 ± 0.22^{dg}	5.62 ± 0.25^{eh}
1.10 ± 0.06^{b}	1.20 ± 0.05^{cf}	1.24 ± 0.08^{dg}	1.24 ± 0.07^{eh}
985 ± 30^{b}	1136 ± 42^{cf}	920 ± 35^{dg}	1060 ± 40^{eh}

amount of enzyme which produces 50% inhibition of epinephrine autoxidation in the presence of 2 mM KCN. One unit of glutathione peroxidase activity = 1 nM of NADPH oxidized/min. One unit of glutathione reductase activity = 1 nM of NADPH reduced/min. One unit of catalase activity = 1 μM H_2O_2 metabolized/min

Table 2

Malondialdehyde (nM × mg protein^{-1}), conjugated dienes (optical density at 233 nm × g tissue^{-1})

	A	B	C-1
Malondialdehyde	0.74 ± 0.05	0.76 ± 0.06	2.10 ± 0.08[a]
Conjugated dienes	0.77 ± 0.06	0.80 ± 0.07	2.23 ± 0.08[a]
Hydroperoxides	0.021 ± 0.003	0.023 ± 0.003	0.205 ± 0.010[a]

Mean values ± SD. Statistical significances as in Table 1. Each group consisted of 10

Table 2 summarizes the influence of bleomycin and antioxidant enzyme-containing liposomes on lipid peroxidation products in rat lung. Intratracheal bleomycin instillation causes a significant rise in malondialdehyde, conjugated dienes and lipid hydroperoxides in the lung tissue. Administration of "empty" liposomes causes a severe fall in lipid peroxidation products. Supplementation with catalase- and/or superoxide dismutase-containing liposomes causes a dramatic fall in peroxidation product levels in rat lung tissue.

Discussion

Bleomycin has two functionally different domains: pyrimidine and imidazole moieties, which are responsible for iron binding, and two thiazole rings and a positively charged sulfur group, which binds to DNA. *In vitro* studies indicate that bleomycin is converted into an active species complexing with iron(II) and oxygen (Burger et al., 1981). This complex generates hydroxyl radicals (Oberley and Buettner, 1979).

Cells with high oxygen tension and readily available ferrous ions are the most sensitive to the toxic effects of bleomycin. A pathway of hydrogen peroxide generation has also been described in rat lung (Turrens et al., 1982). This may add to the vulnerability of lung tissue to bleomycin injury. Normal lung has considerable reducing capacity (Slade et al., 1985; Castranova et al., 1983) compared with other tissues.

In our previous work (Ledwożyw et al., 1991) we showed that intratracheal administration of bleomycin causes a significant rise in antioxidant enzyme activities and a parallel rise in lipid peroxidation product levels. We have hypothesized that these phenomena are signs of the induction of adaptive mechanisms in the lungs, mechanisms which protect the tissue from the dangerous effects of bleomycingenerated free radicals. Antioxidant enzymes might therefore be expected to have a protective function against bleomycin-

and lipid hydroperoxides (optical density at 353 nm × g tissue^{-1}) levels in rat lung tissue

C-2	C-3	C-4	C-5
1.74 ± 0.07^{b}	1.24 ± 0.07^{cf}	1.10 ± 0.08^{dg}	$0.92 \pm 0.0v^{eh}$
1.84 ± 0.10^{b}	1.50 ± 0.10^{cf}	1.62 ± 0.11^{dg}	1.12 ± 0.07^{eh}
0.185 ± 0.011^{b}	0.125 ± 0.008^{cf}	0.082 ± 0.006^{dg}	0.055 ± 0.005^{eh}

animals. Group designation as in Materials and methods

induced lung injury by decreasing the availability of hydrogen peroxide and other active oxygen species.

Several investigators have taken therapeutic advantage of the rapid phygocytosis of liposomes by cells of the liver and spleen in the treatment of diseases such as leishmaniasis (Alving et al., 1980; New et al., 1981), malaria (Prison et al., 1980), fungal infections (Lopez-Bernstein et al., 1983) and cancer (Kataoka and Kobayashi, 1978; Kobayashi et al., 1977; Mayhew et al., 1978; Patel et al., 1982).

Aerosolized superoxide dismutase has been found ineffective against oxygen-induced injury of the lung tissue (Crapo and Tierney, 1974), although polyethylene glycol-conjugated catalase and superoxide dismutase appeared to be effective when given intravenously (Walther et al., 1986).

Premature human infants treated with unmodified subcutaneous antioxidant enzymes (Rosenfield et al., 1984) showed some apparent radiological improvement of their bronchopulmonary dysplasia. In contrast, attempts to protect the endothelium against oxygen toxicity by supplementing endogenous intracellular antioxidant activity using liposomes as transmembrane vectors have been successful both *in vitro* (Freeman et al., 1983) and *in vivo* (Turrens et al., 1984).

A more recent *in vivo* study (Padmanabhan et al., 1985), in which liposomes were delivered to the pulmonary epithelium by intratracheal administration, has confirmed the protective effect of antioxidant enzymes delivered in liposome carriers against oxygen-induced lung damage. Similar protection has been observed by Tanswell and Freeman (1987) in newborn rat pups exposed to 95% oxygen.

Our observation that liposomes alone ("empty" liposomes) may also be protective clearly indicates the need to use control liposomes in such studies. The mechanism by which this effect is achieved is uncertain. It may reflect a general effect, with the liposomes acting as a lipid supplement and improving energy input, which has been reported to influence oxygen toxicity (Frank

and Groseclose, 1982), or a specific effect on the lipid composition of cell membranes, which has also been reported to modify pulmonary oxygen toxicity (Schatte and Mathias, 1982).

The experiments described in this report show that augmentation of lung defence against superoxide and hydrogen peroxide using catalase- and/or superoxide dismutase-containing liposomes can decrease bleomycin-induced lung injury. These observations offer promise of a potential therapeutic tool for use in humans with lung injury induced by bleomycin therapy. The use of vehicles to carry antioxidant enzymes, which allows an increased uptake by lung cells, may improve the results obtained with the injection of native drugs.

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FATTY ACID COMPOSITION OF NEAR-TERM AND BEYOND-TERM PIG PLACENTA

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The fatty acid composition of four major phospholipid classes of near-term and beyond-term pig placenta was investigated. In beyond-term pig placentas much lower levels of linoleic, dihomo-γ-linolenic and arachidonic acids were observed, as compared to near-term organs. It is suggested that beyond-term pregnancy in the pig may be caused by a deficiency of prostaglandin precursors in the placenta.

Key words: Pig, placenta, prostaglandin precursors

Although the events surrounding parturition have been studied for many years, the factors responsible for its initiation and propagation are still poorly understood. Numerous studies suggest that enhanced prostaglandin biosynthesis within the uterus appears to be a prerequisite for the sustained increase in myometrial activity which is associated with labour (Mitchell, 1984; Noden et al., 1981). Endogenous prostaglandins increase in the peripheral circulation during natural labour in women (Green et al., 1974), in laboratory (Moussard et al., 1986) and domestic animals (Sharma, 1975). Exogenous prostaglandins will induce uterine contractions (Liggins et al., 1973; Mitchell et al., 1977), while suppression of prostaglandin biosynthesis delays the onset of dexamethasone- or ACTH-induced pre-term delivery (Mitchell and Flint, 1978*a*, *b;* Evans et al., 1982) and delay natural fetal expulsion (Lewis and Shulman, 1973).

Prostaglandins have a number of actions on the myometrium, including stimulation of phospholipid metabolism, mobilization of intracellular calcium stores, increased transmembrane calcium flux and gap junctions formation (Casey and MacDonald, 1984; Thornburn, 1985).

It is well known that $PGF_{2\alpha}$ and PGE_2 are produced by fetal membranes (Bloch et al., 1985; Olson et al., 1984). $PGF_{2\alpha}$ increased in amniotic fluid before and during human labour (Hillier et al., 1974), as did the prostaglandin precursor, arachidonic acid (Keirse et al., 1977; Ramwell et al., 1977).

The precise role of prostaglandins in placental function is far from being clear. It has been suggested that PGE_2 and $PGF_{2\alpha}$ produced by the placenta, with opposite vascular effects, regulate maternal and fetal blood flow within the placenta (Rankin, 1976).

The placenta is known to have a large capacity for metabolizing prostaglandins, comparable to that of the lung (Hansen, 1976). Moussard et al. (1986) have shown that the placenta may contribute to the augmented intrauterine availability of prostanoids near parturition.

The primary source of intrauterine prostaglandins in pregnant gilts is not clear, as the secretion of $PGF_{2\alpha}$ into the uterine venous blood is less in pregnant than in non-pregnant animals (Moeljono et al., 1977; Rampacek et al., 1979).

Since the availability of free arachidonic acid is a limiting step in prostanoid synthesis (Weissmann, 1983), it may be a matter of interest to determine if the levels of prostaglandin precursors, e.g. linoleic, dihomo-γ-linolenic, arachidonic and eicosapentaenoic acid, are different in near-term and beyond-term gilt chorion and placenta.

Materials and methods

Tissues were recovered from a total of 12 gilts of Polish Large White breed slaughtered in the 15th week of pregnancy. Beyond-term placentas were obtained from the Clinic of Gynaecology and Obstetrics for our Faculty at the Caesarean section opportunities ($n = 10$).

The uterus was clamped between fetal bulges, and the bulge excised and placed on a plastic tray. The uterus was cut open at the antimesometrial border exposing the fetus enclosed in the fetal membranes. The outer vascular membrane enclosing the fetus was dissected away from the margin of the placenta nearest the uterine wall. This tissue was termed the chorion. Amnion and allantois were snipped free from the placenta and its fetal attachment, and discarded. Next, the red vascular placenta and its underlying layer, termed the decidua, were gently pulled away from the uterine wall. Individual tissues were weighed and then frozen in the refrigerator until processed.

Lipids were extracted twice with 10 v/w of 2 : 1 chloroform : methanol mixture. The crude extracts were pooled and mixed with 0.2 vol of a 0.73% aqueous KCl solution. After gentle shaking and centrifugation, the upper phase was discarded and the interphase was carefully washed with a "synthetic" upper phase containing the same solvents and salts as the original upper phase (Folch et al., 1957). The chloroform layer was evaporated to dryness, the dry residue was dissolved again in a small volume of the above-mentioned chloroform : methanol mixture and analysed by thin-layer chromatography on precoated silica gel G plates (E. Merck, Darmstadt, Germany) as described previously (Wawrzkiewicz et al., 1987). Individual phospholipids were identified by comparing their R_f values with those of standards (Sigma, St. Louis, USA). Fatty acids in individual phospholipid classes were analysed

by gas-liquid chromatography on Perkin Elmer F-30 gas chromatograph (Perkin Elmer Ltd., Beaconsfield, Bucks., England) as described earlier (Michalak et al., 1988). Statistical analyses were performed by Student's *t* test for unpaired data.

Results

The prostaglandin precursor levels of particular phospholipid classes are shown in Tables 1 and 2. It is of interest that in all four lipid classes in placenta

Table 1

Prostaglandin precursors in phosphatidylcholine and phosphatidylethanolamine fractions in placenta, chorion and decidua of gilts (% of total fatty acids; mean values ± SD)

	Placenta		Chorion		Decidua
	15 weeks	beyond term	15 weeks	beyond term	15 weeks
Phosphatidylcholine					
18 : 2(n–6)	2.83 ± 0.52	1.32 ± 0.44*	1.43 ± 0.12	0.72 ± 0.06*	2.41 ± 0.19
20 : 3(n–6)	3.66 ± 0.39	1.15 ± 0.12*	2.25 ± 0.20	1.30 ± 0.12*	2.98 ± 0.26
20 : 4(n–6)	6.62 ± 0.73	2.21 ± 0.20*	4.42 ± 0.40	3.25 ± 0.30*	6.22 ± 0.55
20 : 5(n–3)	0.14 ± 0.03	0.08 ± 0.01*	0.07 ± 0.01	0.07 ± 0.02	0.07 ± 0.02
Phosphatidylethanolamine					
18 : 2(n–6)	1.86 ± 0.21	0.65 ± 0.07*	2.45 ± 0.20	1.76 ± 0.15*	3.76 ± 0.32
20 : 3(n–6)	3.21 ± 0.35	1.46 ± 0.16*	1.94 ± 0.16	1.42 ± 0.12*	2.62 ± 0.22
20 : 4(n–6)	4.47 ± 0.50	2.11 ± 0.24*	4.81 ± 0.40	3.50 ± 0.30*	5.94 ± 0.52
20 : 5(n–3)	0.17 ± 0.02	0.10 ± 0.01*	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01

Student's *t* test for unpaired data: * $p < 0.05$ beyond term vs. 15 weeks of pregnancy.
Short-hand notation of fatty acids: 18 : 2(n–6) = linoleic acid; 20 : 3(n–6) = dihomo-γ-linolenic acid; 20 : 4(n–6) = arachidonic acid; 20 : 5(n–3) = eicosapentaenoic acid

Table 2

Prostaglandin precursors in phosphatidylserine and phosphatidylinositol fractions in placenta, chorion and decidua of gilts (% of total fatty acids; mean ± SD)

	Placenta		Chorion		Decidua
	15 weeks	beyond term	15 weeks	beyond term	15 weeks
Phosphatidylserine					
18 : 2(n–6)	4.22 ± 0.50	2.31 ± 0.26*	2.03 ± 0.18	1.10 ± 0.12*	2.31 ± 0.20
20 : 3(n–6)	3.30 ± 0.35	1.62 ± 0.18*	2.51 ± 0.22	1.60 ± 0.15*	2.77 ± 0.23
20 : 4(n–6)	7.36 ± 0.85	3.21 ± 0.35*	5.90 ± 0.53	2.04 ± 0.18*	7.31 ± 0.60
20 : 5(n–3)	0.10 ± 0.02	0.07 ± 0.01*	0.07 ± 0.02	0.05 ± 0.01	0.06 ± 0.01
Phosphatidylinositol					
18 : 2(n–6)	2.61 ± 0.29	1.05 ± 0.10*	3.81 ± 0.33	2.62 ± 0.20*	4.60 ± 0.37
20 : 3(n–6)	3.64 ± 0.40	2.20 ± 0.25*	2.44 ± 0.20	1.54 ± 0.12*	3.06 ± 0.35
20 : 4(n–6)	6.41 ± 0.68	3.41 ± 0.40*	6.42 ± 0.56	3.83 ± 0.35	7.65 ± 0.82
20 : 5(n–3)	0.15 ± 0.02	0.08 ± 0.01*	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.02

Student's *t* test for unpaired data: * $p < 0.05$ beyond term vs. 15 weeks of pregnancy.
For short-hand notation of fatty acids see footnote to Table 1

and chorion from beyond-term pregnancies the level of prostaglandin precursor was much lower than in near-term tissues.

Discussion

Cycloxygenase activity increased in the uterine tissues of ewes near the time of luteolysis (Huslig et al., 1979) when increased $PGF_{2\alpha}$ also occurred (Baird et al., 1976). An increase in cycloxygenase activity probably reflects an increased ability of the various tissues to synthesize one or more prostaglandins.

It is tempting to speculate that the increased capacity for prostaglandin production by the gestational compartment precipitates, or participates in, delivery. The increase in $PGF_{2\alpha}$ found in the plasma of near-term rabbits supports this idea (Challis et al., 1975). A similar observation was reported in ewes (Mitchell and Flint, 1978*b*). The data of Noden et al. (1981) support the idea that prostaglandin production in intrauterine tissues changes both quantitatively and qualitatively during gestation. Conversion of radioactive arachidonate to $PGF_{2\alpha}$ and PGE_2 was increased in human amnion (Okazaki et al., 1980) and in rhesus decidua parietalis and myometrium (Mitchell et al., 1978) near term. More PGE_2 was produced in the maternal and fetal cotyledons of ewes than in the myometrium, and this production increased at delivery (Mitchell and Flint, 1978*a*). In a previous publication (Ledwożyw and Kądziołka, 1989) we have shown that with the advancement of gestation a permanent rise in linoleic, dihomo-γ-linolenic and arachidonic acid levels occurs in the pig amnion and allantois.

On the other hand, it is well established that more precursor is available near term (Keirse et al., 1977), probably due to an increased liberation from phospholipids (Grieves and Liggins, 1976). The increased availability of arachidnoic acid at parturition may be due to an increased release or accelerated activity of phospholipase A_2 from fetal membrane lysosomes (Schwarz et al., 1976).

Wilson et al. (1982) have defined the relationship of individual prostaglandins to one another and have compared their alterations with gestational age in rats. These authors have shown that a significant enhancement in uterine 6-keto-$PGF_{1\alpha}$, TXB_2, $PGF_{2\alpha}$ and PGE_2 concentrations occurs on day 20 compared to day 15 of pregnancy, and further dramatic increases were observed on day 21 and at delivery. Placental PGE_2 and $PGF_{2\alpha}$ concentrations increased only slightly a delivery, but the augmentation in 6-keto-PGF_1 and TXB_2 levels were of greater magnitude.

Experiments with blastocystic tissue cultured from ewes (Marcus, 1981), cows (Lewis et al., 1982), and pigs (Lewis and Waterman, 1983) showed that

conceptus tissues were more active in converting arachidonic acid to prostaglandins than was endometrium from the same animals. The results presented in this paper are consonant with the hypothesis that uterine prostaglandins have an important function in parturition and uterine-placental physiology, and suggest that an adequate level of arachidonic acid is a decisive factor in the proper term of delivery in swine.

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EFFECT OF NICKEL DEFICIENCY ON BIOCHEMICAL VARIABLES IN SERUM, LIVER, HEART AND KIDNEYS OF GOATS*

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Nickel deficiency was induced in 2- to 4-year-old goats by feeding 0.1 mg Ni/kg dry matter with a semisynthetic diet. The control group consumed 5.0 mg Ni/kg d.m. Activity of several enzymes (SDH, LDH, HBDH, AST, ALT, ALD, CK, CHE) was determined in the serum, liver, heart and kidneys. Serum urea-N level was also measured and transmission electron microscopic (TEM) examinations were performed.

Signs characteristic of nickel deficiency (retarded growth, increased mortality of dam and offspring, parakeratosis of the skin) appeared in the low-nickel group. The activity of SDH and ALD, as well as the level of urea-N was significantly lower in the serum of Ni-deficient animals than in the control. Ni-deficient animals also had significantly lower enzyme activities in the heart (SDH, HBDH, AST, ALT, ALD and CK), liver (SDH and CHE) and kidneys (HBDH and CK). Electron micrographs showed degeneration of cardiac and skeletal muscle in the Ni-deficient animals.

Ni deficiency elicited changes primarily in the heart and these resulted in depressed activity of several enzymes.

Key words: Nickel deficiency, biochemical variables, enzyme activities, myodegeneration, heart, electron microscopy, goat

The clinical signs and pathologic lesions of nickel toxicity have been described by several authors (Sunderman et al., 1978; Sunderman et al., 1989; Hildebrand et al., 1986; Knight et al., 1987). Because of the chemical and physical relationships between iron, cobalt and nickel, in the 1950s and 1960s a number of research teams tried to demonstrate the biological essentiality of nickel. After a long series of unsuccessful experiments the first indications of the biological essentiality of Ni for plants were reported by Dixon et al. (1975), who showed that plant urease contained the element.

Nickel was also found to be essential for the miniature pig (Anke et al., 1974, 1976, 1978), chicken (Nielsen et al., 1975*a*), rat (Nielsen, 1971; Nielsen et al., 1975*b*; Schnegg and Kirchgessner, 1975*b*; Kirchgessner and Schnegg, 1976), goat (Anke et al., 1977, 1980*d*, 1983*a*, *b*; Hennig et al., 1978) and sheep (Spears and Hatfield, 1977, 1978).

Nickel deficiency manifested itself in decreased gain, increased mortality of mother and offspring, decreased haemoglobin concentration and haema-

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tocrit, lowered activity of several enzymes (Schnegg and Kirchgessner, 1975*a;* Anke et al., 1980*d;* Szilágyi et al., 1981, 1982) and parakeratosis of the skin (Anke et al., 1974, 1976, 1980*d;* Nielsen et al., 1975*b).* Mineral metabolism was also affected, lowered zinc levels were observed in the ribs and liver, less calcium and iron was absorbed and more excreted by the gut (Schnegg and Kirchgessner, 1976; Anke et al., 1980*a, b, c;* Kirchgessner and Schnegg, 1980).

Elevated serum nickel concentrations were found following cardiac infarction (Leach et al., 1985), and nickel was found to inhibit cardiac contractions and oxidative metabolism (Rubányi and Kovách, 1980).

In spite of numerous studies the biological role of nickel has remained largely unknown.

In the experiment reported here we made comparative studies on nickel-deficient and control goats. Activity of several enzymes taking part in the intermediary metabolic processes of the most important compounds was determined in the serum, liver, heart and kidneys, and serum urea-N level was measured. Ni concentration in the liver, heart and kidney was also determined, and transmission electron microscopic (TEM) examinations were performed.

Materials and methods

Hornless, white, 2- to 4-year-old female goats (offspring of Ni-deficient dams) were kept in polystyrol sties and were fed a semisynthetic diet (Anke et al., 1977) for two years. This diet contained 0.1 mg or less Ni/kg dry matter of feed ("Ni-deficient" group, 8 animals). Control goats (12 animals) were fed on the same diet supplemented with $NiCl_2$, resulting in 5 mg Ni/kg d.m. Distilled water was offered to drink.

Blood samples were taken from the jugular vein one week before slaughter. The serum samples were stored at —20 °C until used. Tissue samples were collected from the liver, heart and kidneys immediately after slaughter. The samples were kept frozen in liquid nitrogen until used. After thawing, the samples were homogenized and diluted with saline.

Activity of sorbitol dehydrogenase (SDH; EC 1.1.1.14), lactate dehydrogenase (LDH; EC 1.1.1.27), hydroxybutyrate dehydrogenase (HBDH; EC 1.1.1.27), aspartate aminotransferase (AST; EC 2.6.1.1), alanine aminotransferase (ALT; 2.6.1.2), aldolase (ALD; EC 4.1.2.13), creatine kinase (CK; EC 2.7.3.2), cholinesterase (CHE; EC 3.1.1.8), and concentration of serum urea nitrogen (UN) were determined.

Boehringer test collections and an Eppendorf ACP 5040 equipment were used. The methods used were as recommended by the International Society for Animal Clinical Biochemistry and Die Deutsche Gesellschaft für Klinische

Chemie: "Optimized Standard Methods" for LDH, HBDH, AST and ALT, "NAC active monotest" for CK; the substrate for CHE was acetylthiocholine. After dry ashing at 450 °C, Ni was determined colorimetrically with dimethylglioxime (Ölschläger, 1955).

Inter-group significance levels were calculated by Student's *t* test.

For transmission electron microscopy, specimens were fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide. After embedding in Durcupan, examinations were performed in a JEOL 100B electron microscope.

Results

The Ni concentration of kidney, liver and heart samples was found significantly decreased in Ni-deficient animals as compared to the control (Table 1). The activities of ALD and SDH and the level of urea-N were signi-

Table 1

Ni concentration in different organs of goats (μg/kg d. m.) fed on Ni-deficient (0.1 mg Ni/kg d.m.) and control (5 mg Ni/kg d.m.) diet ($\bar{x} \pm$ SE)

Organs	Ni-deficient (n = 8)	Control (n = 12)	P	%*
kidney	432 ± 262	1205 ± 642	< 0.01	36
liver	542 ± 326	1128 ± 450	< 0.01	48
heart	281 ± 163	602 ± 408	< 0.05	47

* Control: 100%

Table 2

Serum enzyme activities (U/l) and the concentration of urea-nitrogen (UN mg/l) in Ni-deficient and control goats ($\bar{x} \pm$ SE)

Enzyme	Ni-deficient (n = 8)	Control (n = 12)	P	%*
ALD	2.23 ± 0.25	4.11 ± 1.71	< 0.01	54
SDH	3.31 ± 3.01	10.03 ± 3.89	< 0.001	33
AST	21.2 ± 5.74	30.5 ± 9.72	n.s.	69
ALT	11.2 ± 5.30	13.0 ± 4.31	n.s.	86
CK	18.1 ± 8.3	22.3 ± 9.4	n.s.	81
LDH	305 ± 70	298 ± 63	n.s.	102
HBDH	230 ± 47	196 ± 65	n.s.	117
$\frac{\text{HBDH}}{\text{LDH}}$	0.75	0.66	—	114
UN	112 ± 25.7	203 ± 45.6	< 0.001	55

n.s. = non-significant
*control: 100%

Table 3

Kidney enzyme activities (U/g wet weight) in Ni-deficient and control goats ($\bar{x} \pm$ SE)

Enzyme	Ni-deficient (n = 6)	Control (n = 8)	P	%*
ALD	0.44 ± 0.08	0.39 ± 0.02	n.s.	113
LDH	104 ± 17	121 ± 14	n.s.	86
HBDH	69.7 ± 12.2	89.7 ± 4.0	< 0.05	78
SDH	1.19 ± 0.16	1.02 ± 0.11	n.s.	117
AST	8.37 ± 3.83	12.0 ± 1.9	n.s.	70
ALT	1.81 ± 0.48	2.35 ± 0.12	n.s.	77
CK	7.82 ± 4.75	15.6 ± 3.09	< 0.05	50
CHE	247 ± 54	259 ± 41	n.s.	95

n.s. = non-significant
*Control: 100%

Table 4

Heart enzyme activities (U/g wet weight) in Ni-deficient and control goats ($\bar{x} \pm$ SE)

Enzyme	Ni-deficient (n = 6)	Control (n = 8)	P	%*
ALD	0.88 ± 0.37	1.98 ± 0.54	< 0.05	44
LDH	198 ± 24	243 ± 35	n.s.	81
HBDH	152 ± 21	181 ± 14	< 0.05	83
SDH	0.15 ± 0.03	0.20 ± 0.05	< 0.05	75
AST	63.7 ± 10.3	94.4 ± 12.3	< 0.01	67
ALT	4.78 ± 0.68	6.53 ± 1.07	< 0.05	73
CK	135 ± 8.1	195 ± 31.9	< 0.01	69
CHE	47 ± 15	51 ± 24	n.s.	92

n.s. = non-significant
* Control: 100%

ficantly lower in the sera of Ni-deficent animals (Table 2). The activity of renal HBDH and CK was significantly lower in the Ni-deficient goats than in the control animals (Table 3). Decreased hepatic SDH and CHE activity was measured in the Ni-deficient animals (Table 4). Six of the investigated enzymes (SDH, ALD, HBDH, AST, ALT, CK) were found to be significantly lowered in the heart of Ni-deficient goats (Table 5).

By electron miscroscopy of the heart muscle, decreasing density of mitochondrial matrix, occasionally ruptures of cristae, widening of sarcoplasmic and T-tubules, hypercontracted Z-band and widened Z-band material

Table 5

Liver enzyme activities (U/g wet weight) in Ni-deficient and control goats ($\bar{x} \pm$ SE)

Enzyme	Ni-deficient (n = 6)	Control (n = 8)	P	%*
ALD	0.30 ± 0.05	0.31 ± 0.10	n.s.	97
LDH	48.4 ± 8.0	46.3 ± 6.0	n.s.	104
HBDH	38.3 ± 4.25	30.1 ± 5.82	n.s.	127
SDH	7.04 ± 3.91	11.2 ± 2.81	< 0.05	63
AST	19.1 ± 1.31	20.3 ± 0.94	n.s.	95
ALT	3.19 ± 0.68	3.25 ± 0.44	n.s.	98
CK	3.78 ± 0.63	4.89 ± 0.77	n.s.	77
CHE	340 ± 138	817 ± 143	< 0.01	42

n.s. = non-significant
* Control: 100%

were observed (Figs 1, 2). Changes of the capillary endothelial cells included oedema of endothelial cells, swelling of endothelial mitochondria and pericapillary oedema.

Discussion

According to Anke et al. (1980*c*, 1983*a*) the bone seems to be the most suitable tissue for indicating the nickel status of animals, followed by the kidneys and the liver. In the present experiment, the Ni concentrations found in the kidneys, liver and heart of control goats showed that the control ration covered their nickel requirement. Significantly lower Ni concentrations were measured in the same organs of the goats of the deficient group (Table 1), and the signs of deficiency were the same as described previously (Anke et al., 1980*a*).

Urea, a product of protein degradation in mammals, significantly decreased in the sera of Ni-deficient goats as compared to the control. Similar changes were observed by Schnegg and Kirchgessner (1978) in sera of Ni-deficient rats. Supplementation of cows' ration with 5 mg Ni/kg dry matter of feed increased serum urea-N concentrations (Spears et al., 1986). The activity of bacterial urease in the rumen contents decreased in goats fed a low-Ni diet (Hennig et al., 1978), while 5 mg Ni/kg d.m. supplementation of steer rations increased the urease activity (Starnes et al., 1982).

Practically, the N requirement of ruminants is partially covered both by *in vivo* recirculating and by dietary urea fermented by Ni-containing microbial urease. It has been suggested that the urease activity of bacteria attached to

the surface of the rumen mucosa can enhance the excretion of urea via the rumen wall.

Serum, liver and heart SDH activities were significantly lowered in Ni-deficient animals than in the controls. Recently, Jeffrey et al. (1984) have found SDH to be a Zn-dependent enzyme. Anke et al. (1984) reported that Ni-deficiency markedly decreased Zn concentration in the heart, liver and kidneys of goats. Secondary Zn deficiency accompanied by a Ni-malnourished status of goats could cause lowered SDH activity.

Both AST and ALT were less active in Ni-deficient animals than in the controls. However, the activity differed significantly only in the heart muscle. Decreases were also observed in several other organs of rats (Schnegg and Kirchgessner, 1977) and sheep (Spears and Hatfield, 1977, 1978) fed on low-Ni rations. On the other hand, a 5 mg/kg dietary Ni supplementation significantly increased ALT activity in lambs (Spears et al., 1986).

We found significantly depressed ALD and CK activities in the Ni-deficient goats, particularly in the heart tissues.

Schnegg and Kirchgessner (1977) found depressed LDH and HBDH activity in the liver of Ni-deficient rats (0.015 mg Ni/kg diet). In this experiment we found significantly lowered activity of HBDH only in the kidneys of Ni-deficient animals.

Like other trace elements, Ni can affect the activity of numerous enzymes *in vitro*. Some enzymes have been reported to be activated or inhibited by Ni (Nielsen, 1971). However, no enzymes of animal tissues have been found to require Ni as an intrinsic component.

The ultrastructural changes seen in the Ni-deficient goats are not specific and are localized mainly in the mitochondria and in the wall of blood vessels. Similar histological changes were found in Ni-deficient chickens as well (Nielsen et al., 1975*a*).

Both the present and earlier morphological observations (Balogh et al., 1985; Balogh and Szilágyi, 1988) indicated that in Ni deficiency the sarcotubular system of the striated muscle was damaged; also, mitochondria and muscle fibres were swollen in the heart.

It is interesting to note that both deficiency and overfeeding of Ni resulted in similar morphologic changes. Both acute CO intoxication (Balogh et al., 1983) and myocardial infarction (Leach et al., 1985; Balogh et al., 1983) were reported to affect cardiac Ni concentration. All these findings show that the heart is involved in the metabolism of Ni as a target organ.

Acknowledgement

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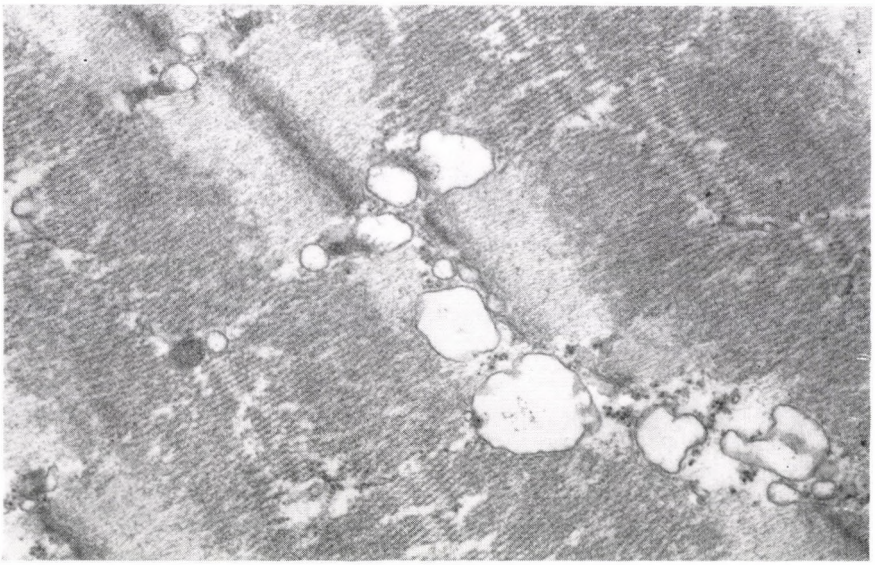

Fig. 1. Widening of sarcoplasmic and T tubules of the heart muscle in Ni-deficient goats. Transmission electron micrograph (TEM), × 28,800

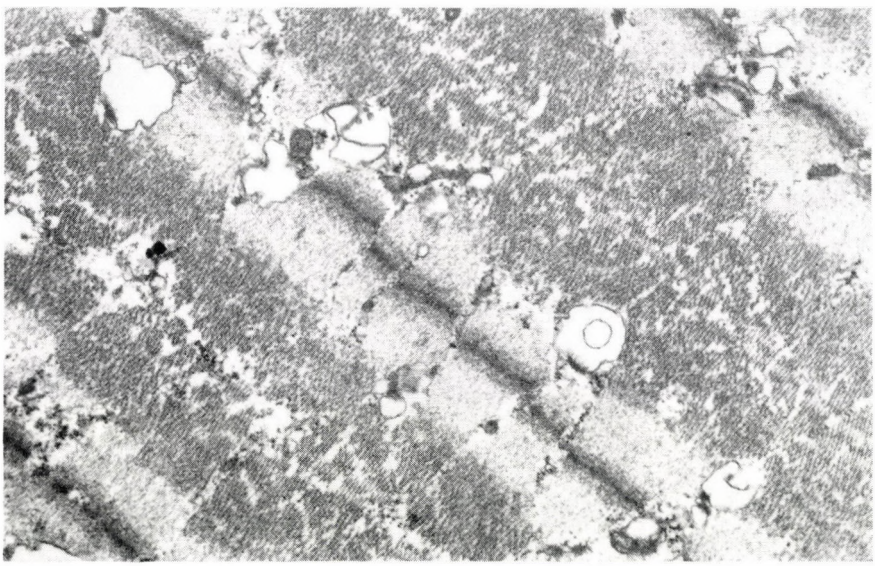

Fig. 2. Hypercontracted Z-band, widened Z-band material of the heart muscle in Ni-deficient goats. TEM, × 21,600

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AGE-RELATED VARIATIONS IN SOME BLOOD PARAMETERS OF GEESE

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Blood samples from a total of 44 geese of the Hungarian breed, representing three age groups (3 months old, 6 months old, and adult) and originating from the Babat Goose Farm, were examined for packed cell volume (PCV), differential count, haemoglobin content, and serum levels of glucose, total protein, total lipid, calcium and phosphorus. The results revealed significant age-related variations in PCV, proportions of lymphocytes and eosinophils, total protein, total lipid, calcium and phosphorus. The changes were comparable to those obtained for 3 months old and adult Landes geese.

Key words: Blood parameters, goose, age-related variations, breed

As compared to other poultry species, blood parameters of the goose have been little studied. Biochemical variables and packed cell volume (PCV) are mainly available in the literature (Hunt et al., 1964; Hunsaker et al., 1964; Bernhardt and Kaleta, 1968; Kaleta and Bernhardt, 1968; Bokori and Karsai, 1969; Szép et al., 1972), but values of the cellular components are still lacking.

This paper presents the major clinico-biochemical parameters of blood from 44 geese representing three age groups.

Materials and methods

Birds and blood samples. A total of 44 geese of the Hungarian breed originating from the Babat Goose Farm were examined. The geese represented three age groups: 3 months old, 6 months old, and adult. The birds were housed in groups of 5–6 in an animal house supplied with a fowl run. They provided a pelleted goose rearing, a sustenance and a layer diet, respectively, as well as drinking water *ad libitum*.

Blood was taken from the wing vein between 9 and 11 a.m. Heparinized blood was used for packed cell volume (PCV) and haemoglobin determination, while native blood and serum served for blood films and determination of the biochemical variables.

Clinical values. PCV was determined by the micromethod described by Frankel et al. (1970). Differential count was appraised from 100 cells of May-Grünwald and Giemsa stained blood films.

Biochemical data. Biochemical variables were determined with the aid of a SPEKTROMOM 401 spectrophotometer. Haemoglobin concentration was determined by the cyanhaemoglobin method (Richterich, 1969). Serum levels of glucose, total protein, total lipid, calcium and phosphorus were obtained using commercial REANAL and CHEMAPOL Test Combinations.

Biometry. Means and standard deviations were computed for all three age groups of geese and compared by Student's *t* test (Sváb, 1973).

Results

Body mass and major blood parameters of the three age groups of geese belonging to the Hungarian breed are presented in Table 1.

Table 1

Body mass and major blood parameters obtained for the three age groups of geese belonging to the Hungarian breed (mean ± SD)

Parameter	Age group		
	3 months old (n = 16)	6 months old (n = 18)	adult (n = 10)
Body mass (kg)	4.0 ± 0.7	5.5 ± 0.7	5.2 ± 0.7
PCV (1/1)	0.37 ± 0.02	0.42 ± 0.02	0.41 ± 0.03
Lymphocyte (%)	0.37 ± 0.15	0.64 ± 0.15	0.569 ± 0.152
Monocyte (%)	0.02 ± 0.02	0.01 ± 0.01	0.018 ± 0.016
Heterophil granulocyte (%)	0.40 ± 0.19	0.31 ± 0.15	0.314 ± 0.169
Eosinophil granulocyte (%)	0.19 ± 0.08	0.04 ± 0.03	0.099 ± 0.052
Basophil granulocyte (%)	0.02 ± 0.02	0.00 ± 0.00	0.000 ± 0.000
Hemoglobin (mval/1)	6.4 ± 0.6	7.1 ± 0.8	7.2 ± 1.0
Glucose (mmol/1)	—	11.4 ± 1.7	10.4 ± 1.3
Total protein (g/l)	42.0 ± 5.8	62.5 ± 3.7	67.5 ± 11.9
Total lipid (g/l)	4.2 ± 1.0	—	6.6 ± 1.5
Ca (mmol/l)	2.7 ± 0.4	2.4 ± 0.4	1.9 ± 0.7
P (mmol/l)	2.8 ± 0.7	1.9 ± 0.5	2.1 ± 0.8

Blood samplings were performed on 3 months old geese in July, then at the age of 6 months in November and on adults in September

Parallel with aging, PCV, total protein, total lipid and haemoglobin tended to increase while serum calcium and phosphorus tended to decrease. The lymphocyte: heterophil ratio of ca. 1:1 also showed a right shift to ca. 2:1. This was accompanied by a decrease in the proportion of eosinophils.

Statistically significant differences between the 6 months old group and the adult geese were found only in the proportion of eosinophils ($p < 0.01$)

and in serum calcium ($p < 0.05$). A comparison of 3 months old geese with 6 months old and adult birds, respectively, revealed significant differences in PCV ($p < 0.001$), proportions of lymphocytes ($p < 0.001$ and $p < 0.01$, respectively) and eosinophil granulocytes ($p < 0.001$ and $p < 0.01$, respectively), total protein ($p < 0.001$), total lipid ($p < 0.001$), serum calcium ($p < 0.01$) and phosphorus ($p < 0.001$ and $p < 0.05$, respectively). No significant differences were found among the three groups in the other variables studied.

Discussion

Blood parameters are known to vary by age, breed, sex, season and other factors. Of the variables presented here, PCV, haemoglobin, total protein, glucose, calcium and phosphorus were reported for 4 to 18 weeks old geese of the Hungarian, Rhine, Landes, Italian and Tiszasüly (a Hungarian farm breed) breeds (Szép et al., 1976). Variations in these parameters between 6 and 12 weeks and 12 and 18 weeks of age followed no definite breed-, sex- and age-related pattern of increase or decrease.

In our study with the Hungarian (white) goose breed, the 3 months old geese were sampled in July and the two older groups in autumn. Yet the differences found in the blood parameters among the three groups may reflect age-related changes rather than seasonal ones. Except eosinophils and serum calcium, the blood values obtained for the 6 months old and the adult geese showed good agreement. The differences may be attributed to the fact that adult geese were sampled during the autumn production season.

With the Landes (gray) breed we observed similar age-related variations in blood parameters between the 3 months old ($n = 10$) and adult ($n = 7$) geese sampled both in July and in October. Namely, increases occurred in PCV (from 0.33 to 0.40 l/l; $p < 0.001$), total protein (from 47.5 to 58.7 g/l; $p < 0.05$), and in total lipid (from 2.8 to 5.7 g/l; $p < 0.001$). Again, a similar right shift was found in the lymphocyte:heterophil ratio of ca. 1:1 to ca. 2.6:1. Basophils occurred only in blood samples of the 3 months old geese in both breeds.

Statistical comparison of values obtained for the two breeds showed a significantly ($p < 0.001$) higher serum calcium and a slightly lower ($p < 0.1$) total protein level in adult Landes geese. (It should be mentioned, however, that these geese were sampled outside the production season). In the 3 months old age group, significant differences ($p < 0.001$) were found between the two breeds in PCV, total lipid, serum phosphorus and in the proportion of eosinophils and again a slight decrease ($p < 0.1$) was noted in total protein. Sample size was, however, relatively small.

We hope that the blood parameters presented here may serve as useful information for studying the clinicochemical changes of goose blood.

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BOOK REVIEWS

Gustav ROSENBERGER: *Die klinische Untersuchung des Rindes (Clinical Examination of Cattle)*. Third revised edition. Edited by Prof. Dr. h. c. *Gerrit Dirksen*, Prof. *Hans-Dieter Gründer* and Prof. Dr. h. c. *Matthaeus Stöber*. Written in collaboration with Prof. Dr. G. *Dirksen* (Munich), Prof. Dr. *H.-D. Gründer* (Giessen), Prof. Dr. *E. Grunert* (Hanover), Prof. Dr. *D. Krause* (Hanover) and Prof. Dr. *M. Stöber* (Hanover). Verlag Paul Parey, Berlin und Hamburg, 1990. 744 pages with 676 illustrations in the text, 21 colour plates and 76 tables. Bound DM 198.—

The book first brought out by Professor *Rosenberger* in 1964, as well as its second edition published in 1977, was received favourably by all, and has become an indispensable university text-book and manual of bovine practitioners. The success of the book is illustrated by the fact that it has been translated into seven languages (Italian, French, English, Spanish, Japanese, Portuguese and Polish). As a result, it has met with a worldwide recognition almost unprecedented for books on veterinary science.

Because of Professor Rosenberger's death, the now published third edition was edited by Professors *Dirksen*, *Gründer* and *Stöber*, renowned buiatricians and Rosenberger's co-authors in the earlier editions, and also written by them in collaboration with Professors *Grunert* and *Krause*.

As compared to the second edition, the third edition of the book has been substantially revised and enlarged: its size and number of illustrations are more than 1.5 times those of the second one. The editors of the book have, however, retained the structure and arrangement of the earlier editions.

The third edition consists of *15 chapters* and a *Subject index*. When dealing with the individual chapters, I shall try to point out all important changes made with respect to the earlier editions.

Handling cattle (Stöber, pp. 1–74). The first chapter specifies the conditions which are indispensable for a successful clinical examination. These conditions range from the veterinarian's protective clothing to the restraining of cattle by physical means, their sedation, local and block anaesthesia as well as narcosis (general anaesthesia).

The abundance of illustrations and the well-arranged table summarizing the diagnostic conclusions that can be drawn from the sensation of pain are worthy of special mention. The chapter has been enlarged with the treatment of temper (temperament), sensation of pain, issues of animal protection, and electric immobilization.

Identification of cattle, history (anamnesis), procedure for clinical examination, general examination (Stöber, pp. 75–141). This chapter describes the methods and steps of expert clinical examination. It also lists the examinations which should be carried out before the detailed examination of organs and organ systems.

The description of herd history (anamnesis), up-to-date methods serving for the identification of animals, and of the general clinical examination of herds has been added to the chapter.

Hair, skin, subcutaneous tissue, visible mucous membranes, horns (Gründer, pp. 142–162).

Lymphatic system (Stöber, pp. 163–170).

Circulation (Stöber and *Gründer*, pp. 171–241). The first part of the chapter is devoted to examination of the output of the *heart*, *blood vessels* and *blood circulation*. This has been completed with the description of echocardiography.

The larger part of the chapter offers a detailed description of *haematological examination*. The physiological values of almost all blood parameters that may be of interest to specialists are presented in excellent and well-arranged tables.

The chapter is completed by a description of sampling and examination of the *bone marrow*.

Respiratory system (Stöber, pp. 242–287). This chapter has been enlarged with the description of the aetiopathogenesis of respiratory diseases and the unique respiratory defence mechanism. Several up-to-date instrumental techniques, such as respiratory endoscopy (thoracoscopy), tracheobronchoscopy and thoracic X-ray examination are also described. The chapter also deals with the effect exerted by respiration on acid-base balance, and with other extrarespiratory functions of the lungs.

The clearly arranged table summarizing the differential diagnosis and symptoms of respiratory diseases is worthy of special mention.

Digestive system (*Dirksen*, pp. 288–400). The longest chapter of the book. Owing to its subject, it is one of the chapters which command the greatest interest. It deals with *food intake*, *digestive processes*, the examination of *organs preparing the food for digestion*, the *forestomachs*, the *abomasum* and the *intestines*.

The chapter involves a range of subjects wider than its title implies, as it supplies the information which should be known about the examination of the *liver*, the *abdominal wall* and the *abdominal cavity*.

Besides the physical and instrumental examination, the chapter deals with the clinical-laboratory methods of examination of the *saliva*, *rumen liquor*, *faeces* and *ascitic fluid* and gives, with an up-to-date and practical approach, a sufficiently detailed description of the clinical laboratory *tests for liver function*. This chapter is illustrated with well-arranged tables.

Of the new material added to the chapter, the description of the defence mechanism of the digestive system, the functional examination of the omasum, intra-abdominal (peritoneal) endoscopy, intra-abdominal X-ray examination, cholecystography and the detailing of several other instrumental methods are worth mentioning.

Urinary system (Gründer, pp. 401–421). The large part of the chapter is constituted by the description of *renal function test* and *urine analysis* (urinalysis). Instrumental examination of the urinary organs is also dealt with in sufficient detail.

Male genital system (Krause, pp. 422–471). Besides the physical and instrumental examination of the male genital system, this chapter deals with sexual behaviour, semen collection techniques and examination of the ejaculate.

Female genital system (Grunert, pp. 472–548). This chapter contains a description of the *gynaecological* and *obstetrical diagnostic* examinations and describes the handling and care of *newborn calves*. The methods serving for the diagnosis of *udder diseases* are also discussed here.

Locomotor system (Dirksen, pp. 549–591). This chapter deals with the diagnostics of locomotor diseases and discusses the examination of individual animals and herds separately. The judgement of motion-associated *behaviour* and *recumben. animals* is also described. The reader gets a picture of the methods of examination of hooves, feet, joints, synovia, muscles, bones and tendons, and of ways of differentiating between physiological and pathological conditions.

Central nervous system (Stöber, pp. 592–625). The chapter describes the general nervous, cerebral and spinal symptoms and the examination of reflexes, the technique of taking samples from the cerebrospinal liquor, and the clinical-laboratory examination of the liquor.

Perhaps one of the most valuable parts of the entire book is the 9-page table in which the author summarizes the symptoms and differential diagnostic characteristics of the major nervous diseases with great professional virtuosity and practicability.

Organs of sense (Rosenberger and *Stöber*, pp. 626–646).

Evaluating the results of examinations (Stöber, pp. 647–662).

Application of medicaments (Stöber, pp. 663–700).

Index (pp. 701–708).

All chapters of the book are abundantly illustrated with perfect *photographs*, beautiful *drawings* and highly informative *tables*. The illustrations are in perfect harmony with the text, complement it and make the book very attractive and highly readable.

As it were to call attention to the high instrument and material demand of up-to-date clinical examination, at the end of each chapter there is a *catalogue of the instruments and diagnos tica* necessary for the methods described in the given chapter. Thist list also specifies the names of the manufacturers and the sources of supply.

The chapters are completed with an up-to-date, detailed *reference list* containing references which were not included in the earlier editions.

Perusing the book, we can state that *Rosenberger's* original work has been substantially improved and expanded by his former co-workers who are authors of this third edition. The book gives a very favourable impression in every respect. We can only render full homage to

the authors whose work displays a high command of up-to-date theoretical and practical knowledge. They have succeeded in excellently synthesizing and summarizing this body of knowledge and bring it out in an accurate wording and clear style. We can state without any exaggeration that their work is unparalleled on this subject and in future years it will certainly serve as the basic reference book on the high-level clinical examination of cattle.

The editing and typographic make-up of the book are equal to its contents and reflect the high standard which has been customary for veterinary publications of *Verlag Paul Parey* for many decades.

Prof. Dr. Ferenc KARSAI

W. JAKSCH and E. GLAWISCHNIG: *Klinische Propädeutik der inneren Krankheiten und Hautkrankheiten der Haus- und Heimtiere* (Clinical Propedeutics of the Inner and Skin Diseases of Domestic Animals and Pets). Third revised and enlarged edition. Verlag Paul Parey, Berlin und Hamburg, 1990. Edited by Prof. Dr. *Erich Glawischnig*. Written by *E. Glawischnig*, *W. Baumgartner*, *G. Schlerka*, *Lore Vasicek*, *G. Schusser* and *A. Tipold*. 293 pages, 102 figures and 25 tables.

The success of the clinical propedeutics book first published in 1975 is reflected by the fact that in a rather short time it now comes out in the third edition. This is a piece of news that all veterinarians should be delighted to hear.

Although the title and authors of the third edition are different, it has maintained the mentality, structure and form of the earlier editions.

The authors have included the word "Heimtiere" (pet animals) in the title, meeting the claim constituted by the increased interest taken in pets and describing the veterinary duties to be performed with them.

The present third edition of the book, once written by the recently deceased Professor *Jaksch*, has now been brought out by Professor *Glawischnig* who was co-editor of the second edition. The third edition was written by several authors including, besides the Editor, five specialists from the two clinics of medicine of the University of Veterinary Science of Vienna, who had not taken part in writing the earlier editions.

The book, which is of not too large extent, is divided into not less than 46 chapters. The authors of the individual chapters are not indicated.

Looking over the chapters we can get a good picture of the contents and structure of the book. In the following these chapters are listed, indicating their page numbers and, here and there, important features.

1. Introduction (pages 13–16).

2. Course of the clinical examination (pages 16–21). Preparations for, and steps of, the individual examination of patients, herd examination, case history.

3. General knowledge concerning clinical examination. Restraining procedures (pages 22–30). This chapter describes the preparation and restraint of horses, cattle, pigs, dogs, cats, rabbits, fur-bearing animals and pets for the examination.

4. Identity, marks and signs of the patient (pages 30–40). Colour, size, age, recognition marks.

5. History (pages 40–50). The anamnesis for individual animals and the herd (group) history.

6. Environmental conditions (pages 50–56). Management, keeping and feeding of the animals.

7. Behaviour and posture (pages 57–59). Depression, uninterestedness, excitation, colic.

8. Body condition, state of nourishment (pages 59–61). Judgement of emaciation and obesity.

9. The skin and its appendages. General part (page 61).

10. The haircoat and the horny structures (pages 61–64).

11. The surface of the skin (pages 67–72). Skin symptoms, pruritus, ectoparasites.

12. The flexibility of the skin (page 72).

13. The temperature of the skin (pages 73–74).

14. Internal temperature (pages 75–78). Taking the patient's temperature (thermometry), fever.

15. Examination of the mucous membranes. General part (pages 75–81).

16. The conjunctiva and the eyelids (pages 81–82).

17. The nasal sinuses and the accessory sinuses of the head (pages 83–87).

18. The oral cavity and the pharynx (pages 87–90), including examination of the tongue and the teeth.

19. Food and drinking water intake (pages 90–94).

20. The upper cervical region (pages 94–97).

21. The larynx (pages 98–101), including the examination of coughing and the bronchial secretion.

22. The neck and the jugular groove (pages 101–105). Examination and probing of the oesophagus.

23. The lymph nodes (pages 105–109).

24. The pulse and peripheral circulation (pages 109–116).

25. Respiration (pages 116–122). Dyspnea, pathological types of respiration.

26. Inspection and palpation of the cardiac region (pages 122–123).

27. Percussion. General part (pages 123–127). Percussion techniques. Resonances and their pathological alterations.

28. Examination of the lungs by percussion (pages 128–130). Borders of the lungs and their abnormities.

29. Examination of the cardiac region by percussion (pages 131–132).

30. Auscultation over the lungs (pages 133–137). Methodology, physiological and pathological respiratory sounds.

31. Auscultation over the heart (pages 137–145). Cardiac sounds, cardiac murmurs, phonocardiography.

32. Electrocardiography (pages 145–153). Methodology of electrocardiography and evaluation of the electrocardiogram (ECG).

33. Examination of the abdomen (pages 153–166). This chapter describes the examination of the abdomen, forestomachs, intestinal peristalsis, tests for the presence of foreign bodies, examination ol the rumen liquor, laparoscopy, and physical as well as laboratory examination of the liver.

34. Rectal examination (pages 166–176).

35. Examination of defecation and the faeces (pages 176–185). Most of the chapter is devoted to parasitological examination.

36. Genital organs (pages 185–188).

37. Examination of the mammary glands (pages 189–194).

38. Urinary organs (pages 194–198). Urination disturbances, urethral catheterization.

39. Urine analysis (pages 199–210). Physical, chemical, microscopic and bacteriological examination of the urine, and renal function tests.

40. Examination of the blood (pages 211–228). The methodology of blood sampling and examination, the commonly used haematological examinations.

41. Nervous system (pages 228–250): Techniques for examination of the nervous system, nervous symptoms and changes, examination of the sensory nervous system, cranial nerve pairs and reflexes, the sampling and examination of the cerebrospinal liquor, complementary instrumental examinations. This well-written chapter ends with a summary of conclusions that can be drawn from the nervous symptoms.

42. Exploratory puncture (pages 250–252).

43. Special diagnostic procedures in infectious diseases (pages 253–257). Allergy and serologic tests, demonstration of pathogens.

44. Sending in test materials (pages 258).

45. Application of medicaments and diagnostica (pages 259–268).

46. The examination of birds (pages 268–285). All that which should be known about the examination of domestic and ornamental birds.

Similarly to its earlier editions, the third edition of Jaksch and Glawischnig's "Clinical Propedeutics" produces a very favourable impression. The authors have updated and completed the material and have, to a varying extent, revised the different chapters. The outer get-up as well as the internal characteristics and subject-matter of the book (the treatment and handling of animals, the course and methods of clinical examination, and the therapeutic procedures used in the practice) have remained the same as were in the first two editions. The methods of examination, which range from the classic physical examination to the laboratory and instrumental methods, are described in the fullest detail. As the book is not a textbook of diagnostics, it hardly deals with the diagnosis and differential diagnosis of the different diseases, and offers little information on the usability of the individual diagnostic procedures.

Considering its size, the book embraces an almost uniquely vast body of knowledge on the examination of diverse groups of animals ranging from farm animals through dogs and cats to ornamental birds and exotic pets, namely all species of animals that vets might encounter in their practice. To compile all this diverse knowledge often requiring dissimilar approaches must have been a very difficult task and in a single book it could be done only in a short, compendium-like form, often only sketchily. It should also be noted that though the main

emphasis of the books has shifted towards the examination of pets, its strong point, apart from the chapter dealing with the examination of birds, is still the part devoted to the examination of cattle and pigs.

In summary: it can be stated that the authors have accomplished their difficult task successfully, on high professional level. Their work shows them to be the representatives and followers of the old, famous Viennese (and Budapest) veterinary school and clinical attitude. The text is clearly and concisely written and well arranged. The chapters dealing with the nervous system and the examination of birds are especially well written. Although it does not affect the professional merits and usability of the book, the reviewer cannot help ointing out some disproportions: e.g. why is the examination of the skin dealt with in five separate chapters when that of the nervous system, which is at least as complicated as the former, hss been successfully incorporated in a single chapter; or why is the examination of the abdomen described so compendiously: the examination of the forestomachs and liver would have deserved a separate and much more detailed treatment when much less important topics are dealt with in more detail than would have been necessary.

Similarly to the earlier editions, this work does not contain photographs either, although the subject-matter would call for illustration of the text with nice pictures. The book is illustrated with — mostly simple — drawings.

The book contains a useful and practical body of knowledge. It can be recommended mainly for veterinarians who wish to get an overall picture of the first stage of clinical work, the treatment and handling of animals and the diagnostic procedures. In addition, it serves as a university text-book.

The book was published in the "Studien Texte" series of *Verlag Paul Parey* in a simpler but unobjectionable get-up.

Prof. Dr. Ferenc KARSAI

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