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RENAL FUNCTION IN NORMAL AND KIDNEY TRAUMATIZED GERMFREE RATS*

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A comparative study was made between kidney-traumatized (unilateral nephrectomy, contralateral ligation of renal artery, and release after 120 min) germfree (GF) and conventional (CONV) rats, and normal control GF and CONV rats. In the kidney-traumatized animals the following trends were indicated. (i) Deaths in 3 to 5 days occurred approximately 25% of the time in GF and 60% in the CONV groups. (ii) Two to 4 days post-operation the renal blood flow was essentially unchanged in GF in comparison to normal controls, while it was 30% of the normal values in CONV rats. (iii) Blood urea nitrogen was approximately 4 times the normal values in GF and 28 times in CONV animals. (iv) The ratio of urine and blood plasma osmolality indicated that the concentrating ability of the kidneys was well maintained in GF while it was virtually lost in CONV traumatized rats. (v) The clearance of intravenously administered, labelled thiourea into the urine and caecal lumen of GF rats was markedly elevated in comparison to the low clearance of this label in CONV rats. No essential differences in kidney function were noted among normal (unoperated or sham-operated) GF and CONV groups. The results indicate that the absence of the intestinal flora is advantageous to the host. This effect might be caused by relief from microbial burden and by the enlarged caecum of GF rats acting as an auxiliary "dialysis membrane". Based on these data the role of oral antibiotic treatment in the management of human uraemia is worth investigating.

It is widely known that as a result of the gastrointestinal microflora altering the metabolism, numerous compounds may be observed in the urine or faeces of experimental animals [1]. Few observations have been made on the renal function of germfree (GF) animals. Early work conducted in a colony of GF rats fed a special autoclaved diet [2] indicated high urinary calcium, citrate and oxalate, as well as low phosphate excretion. These conditions were associated with high incidence of urinary bladder stone formation, particularly in males. Conventional (CONV) control rats, fed the same diet, were free from this anomaly. These findings suggested that among conditions which pre-

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dispose animals to urinary calculus formation, the absence of the intestinal microbial flora is a contributing factor.

Reports dealing with GF animals fed different diets (including the works listed below), generally failed to observe the tendency of urinary calculus formation. In one study, concentrating on cardiovascular parameters of GF rats, renal blood flow was found to be essentially comparable to that of CONV controls [3].

Other experiments carried out on the renal function of GF animals were undertaken to test theories on the role of the microbial flora in the uraemic host. After bilateral nephrectomy in food and water deprived rats, it was shown that the survival time of the GF group was significantly longer than that of the CONV control animals [4]. In an extension of this work [5] it was found that in nephrectomized GF rats blood urea nitrogen remained lower and the concentration of urea in caecal contents was higher in comparison to CONV counterparts. In uraemic rats, intestinal lesions (e.g. inflammation, ulceration) were found only in CONV and not in GF groups. On the other hand, cardiovascular lesions associated with uraemia (myocardial and arterial necrosis and calcification) had an earlier onset and higher occurrence in the GF group. In general, these observations supported the theory that the presence of the microbial flora in the animal during uraemia is detrimental to the animal. It appeared that in this context the GF state may result in two benefits for an animal. (a) When defenses are impaired by uraemia, GF animals escape microbial invasion. (b) The intestinal elimination of nitrogenous waste is enhanced in GF animals, possibly by the marked caecal enlargement and the chronic mild diarrhoea which are displayed by GF rodents [6]. The cause of the more severe cardiovascular lesions observed in uraemic GF rats was not clear.

The purpose of the present work was to study the effect of the microbial flora of renal function and on related parameters in rats that were either intact or exposed to a defined but less drastic uraemic episode than in the previously mentioned experiments. Death rate, blood urea nitrogen, concentrating ability and blood flow of the kidney were observed on unilateral nephrectomy and on temporary ligation and successive release of the vessels of the other kidney. In addition, the renal and intestinal clearance of intravenously administered thiourea was studied. This work was conducted as a comparative study between GF and CONV rats.

Materials and methods

Animals. The GF and CONV animals included in these experiments were CDF rats (Charles River Breeding Laboratories, Wilmington, MA, USA). They were male, 9–10 months old, fed autoclaved L–462 diet [7] ad libitum. The GF rats were maintained in flexible plastic isolators [8] using routine procedures for rearing [9] and of sterility testing [10]. The CONV

controls were kept in the open environment in the same air-conditioned room. In this work a total of 62 GF and 53 CONV rats were used.

Surgical procedures. Surgical procedures were carried out under pentobarbital sodium anaesthesis (35 mg/kg body weight, intramuscularly). The GF animals were operated within the sterile isolators where they remained until the term of the experiment. The surgical procedures in CONV rats were carried out in the open environment, maintaining conditions of surgical asepsis. Kidney and sham-control trauma to the rats were inflicted by an adaptation of a previously described method [11]. The operative procedures were as follows.

(a) In kidney-traumatized (KTR) rats, after medial laparotomy, the right renal artery, vein and ureter were ligated and the kidney removed. On the left side the renal artery was ligated, occluding blood flow completely. After 120 min the blood flow in the left kidney was restored by removing the ligature without damaging the involved tissue. Then the abdominal incision was closed and the animal was allowed to recover from the anaesthesis. Via previous experience in CONV rats, this type of renal trauma precipitated signs of uraemia within 2–4 days, resulting in 50–60% mortality within 3–5 days after the operation.

(b) Sham-operated control rats were exposed to anaesthesis, laparotomy and comparable manipulation of the abdominal organs without additional surgical intervention.

(c) In both KTR and control rats (except those that participated in the death rate study), prior to term, in the anaesthetized and laparotomized animals, a polyethylene cannula (ID 1.19, OD 1.70, length 75 mm, respectively) was inserted and tied into urinary bladder, and urine was collected for a period of 2–3 h, following the ligation of the urethra.

Parameters studied. (a) Death rate. KTR rats were set aside for this purpose and the number of animals spontaneously lying were recorded daily.

(b) Blood urea nitrogen was determined terminally in blood serum samples obtained by cardiac puncture, using the diacetyl method [12].

(c) Osmolality was determined in heparinized blood plasma (500 U/ml) and in urine, using a freezing point depression-type osmometer (Advanced Instruments, Inc., Newton Highlands, MA).

(d) Thiourea clearance. A standard dose of labelled thiourea (5 pc, 14 C THU) was injected into the tail vein of anaesthetized rats. After one hour, blood samples were obtained and the rats were killed by decapitation. The contents of the caecum were collected and the caecal lumen was repeatedly rinsed with saline. Contents and rinsings were combined and centrifuged at 15 000 *g* for 10 min and the supernatant decanted. The sediment was repeatedly washed with saline and recentrifuged, permitting thus the transfer of the cleared label to the combined supernatants. The activities of various samples were determined (Liquid Scintillation Spectrometer, Packard Instruments, Downers Grove, IL).

(e) Cardiac output was determined by constructing haemoconcentration curves of a label (400 000 cpm) 100 g body weight of ^{86}RbCl, intravenously, in anaesthetized rats by an established method [13].

(f) Regional blood flow was assessed by an extension of the same method [13], on determining the distribution of the label in various organs.

In reference to all parameters studied, separate groups of rats were used in case of the determinations listed under (a), (b–c), (d), (e) and (f), respectively. For details on the number of rats participating in various groups, see Tables I–III.

Results

The observations made on death rate/survival of these animals are shown in Fig. 1. Among the GF rats 2 from 7 died after renal trauma whereas among CONV rats 6 were lost from 10 following that episode. In the GF group death occurred on the fifth day, while in the CONV group the rats died within 1–3 days after the operation. All sham-operated control rats survived and recovered promptly from the operation (not shown in Fig. 1).

The data on blood urea nitrogen and on the kidney's concentrating ability are given in Table I. In CONV KTR rats, blood urea nitrogen rose to 27 times the control value. In GF KTR rats this increment was approximately

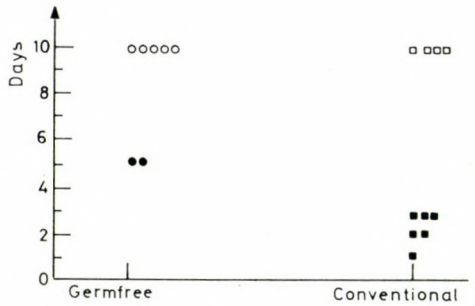

Fig. 1. Death rate/survival of germfree and conventional rats during 10 days following kidney trauma. Full circles and squares = dead animals; open circles and squares = survivors

4-fold. In CONV KTR rats blood plasma osmolality was found a significant 27% higher than in controls, whereas this elevation was 13% in GF KTR rats. The concentrating ability of the kidney during post-traumatic period (i.e. the urine/plasma osmolality ratio) was found reduced in both KTR groups, but to a somewhat smaller extent among GF rats than in CONV controls.

The results of the thiourea clearance are given in Table II. In reference to sham-operated rats, the urinary elimination of this marker was generally higher in the GF group than in CONV controls. After kidney trauma the

Table I

Blood urea nitrogen, blood plasma and urine osmolality

		Conventional	Germfree
Blood urea nitrogen, mg/100 ml blood	KTR	336±27(6)	60±26(10)
	control	12±4(7)	14±2(4)
	% diff.	+2700	+328
Blood plasma, mOsm	KTR	389±30(6)	332±13(12)
	control	306±9(7)	295±8(4)
	% diff.	+27±	+13
Urine, mOsm	KTR	389±30(5)	474±138(11)
	control	1818±297(7)	1932±257(4)
	% diff.	−79	−75
Urine/plasma osmolality	KTR	1.03±0.05(5)	1.43±0.61(11)
	control	5.93±0.94(7)	6.73±0.93(4)
	% diff.	−87	−79

Arithmetic means and standard deviations given; in parenthesis, number of rats. KTR = kidney traumatized rats; control = sham operated normal controls; % diff. = percent difference between KTR and control values. All differences between KTR and controls, numerically listed, are statistically significant ($p < 0.02$); non-significant differences ($p > 0.02$) are so indicated (NS).

Table II

Distribution of intravenously administered ^{14}C thiourea (dose: 5 μC) in urine, caecal contents and blood serum, observed at one hour post-injection

		Conventional	Germfree
Clearance into (cpm $\times 10^3$) urine	KTR	11.2 $\pm$ 8.4(4)	121.1 $\pm$ 132.6(8)
	control	191.2 $\pm$ 44.3(5)	341.6 $\pm$ 55.9(5)
	% diff.	−96	−65
ceacal contents	KTR	60.2 $\pm$ 26.5(5)	346.8 $\pm$ 197.6(8)
	control	31.3 $\pm$ 3.7(4)	195.6 $\pm$ 31.6(5)
	% diff.	+92	+77
Concentration in (cpm $\times 10^3$/ml) blood serum	KTR	35.3 $\pm$ 4.2(5)	45.4 $\pm$ 13.1(8)
	control	25.4 $\pm$ 4.6(4)	29.7 $\pm$ 5.5(5)
	% diff.	NS	NS

Same legend as in Table I

Table III

Cardiac output and regional blood flow

		Conventional	Germfree
Cardiac output, ml/min/kg body weight	KTR	224 $\pm$ 5(3)	209 $\pm$ 14(4)
	control	180 $\pm$ 28(6)	130 $\pm$ 23(6)
	% diff.	+25	+61
Regional blood flow, μl/min/g tissue kidney	KTR	1490 $\pm$ 170(3)	2530 $\pm$ 710(7)
	control	4750 $\pm$ 1330(4)	2900 $\pm$ 380(6)
	% diff.	−69	NS
heart (coronary)	KTR	1860 $\pm$ 200(4)	1690 $\pm$ 110(6)
	control	1750 $\pm$ 250(7)	1250 $\pm$ 370(10)*
	% diff.	NS	NS
caecum	KTR	660 $\pm$ 140(4)	460 $\pm$ 170(10)
	control	630 $\pm$ 150(3)	409 $\pm$ 65(10)*
	% diff.	NS	NS

* Data obtained from 3 months old wister rats, comparable to the CDF rats presently used
Other legends as in Table I

decrease of thiourea clearance into the urine of GF rats amounted to approximately −65% of the sham-control rats' value, whereas in CONV KTR rats this drop corresponded to about −96% of the level observed in CONV sham-controls. In terms of thiourea clearance into the caecal sack, both GF values were substantially higher than in the CONV counterparts. After kidney trauma the percentage increase of caecal clearance of the marker was approximately

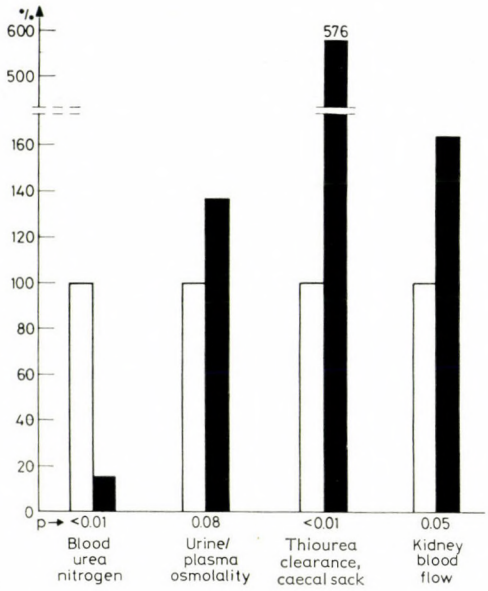

Fig. 2. Selected characteristics in kidney traumatized germfree rats (closed columns) and conventional rats (open columns). Conventional: 100%

the same in both GF and CONV rats (about 92–97%). However, considering total amounts, after kidney trauma the GF caecum was capable of clearing five times more thiourea than its CONV counterpart. The concentration of the label in blood showed essentially similar values both in GF and CONV sham-operated rats. After kidney trauma the marker averaged somewhat higher levels in both groups of rats.

The observations on cardiac output and regional blood flow are given in Table III. Among sham-operated rats, cardiac output was substantially lower in the GF group in comparison to CONV controls. On kidney trauma, cardiac output increased both GF and CONV rats, this was particularly conspicuous in the GF group. Regional blood flow in the kidney tissue of CONV KTR rats was found reduced by over 60% in comparison to CONV sham-controls, whereas no significant drop was found in this context between GF KTR and sham-control rats. Blood flow tested in other areas (coronary and caecal wall) did not indicate significant differences in any of these organs between KTR sham-control rats (Fig. 2).

Discussion

This work confirms previous findings which indicated that in GF hosts uraemic episode took a milder course than in CONV control animals. It appears that the relief experienced by GF rats as illustrated by thiourea clearance, is

in part due to the increased penetration of nitrogenous waste into the gut-lumen, notably into the enlarged caecum of these animals (which is generally characteristic of rodents maintained in the GF state). In GF rats the surface area of the caecal sack is approximately 3 times greater than in CONV controls [14], its contents weigh 5–10 times more, and because of water absorption inhibition and marked water secretion into the lowel bowel, GF rodents display chronic mild diarrhoea [15]. In addition, it is possible that the better maintained blood circulation in the remaining kidney of the traumatized GF rat also contributes to the milder course of the uraemic episode (in spite of the reduced cardiac output which is typical of GF rats vs CONV controls [16]). This was indicated by the essentially unchanged renal blood flow in GF KTR rats in comparison to GF sham-controls. In contrast to this, in CONV KTR rats the renal blood flow fell to approximately 1/3 of the CONV sham-control value. The relatively high renal blood flow in GF KTR rats may well have been the reason for a better urinary clearance of thiourea (which is known to essentially follow the pathway of urinary urea elimination [17]), in comparison to CONV controls. It might be speculated that the better maintained renal blood flow in GF animals under these circumstances results from the refractoriness of their vascular smooth muscle to vasoconstrictive agonists (norepinephrine, vasopressin, angiotensin [18]). This could have a protective effect on the vascular shut-down which is known to occur after temporary ischemia of the kidney (19–21). This speculation is supported by another observation. It was shown that severing the splanchnic nerve, preceding the temporary ligation of the renal artery, improved the renal blood flow [22]. It is tempting to consider similar experiments in caecectomized GF rats, first because the large "caecal dialysis membrane" in these animals is permanently removed [6], and secondly, because the removal of the large pool absorbable vasoconstrictive agonist-inhibitory substances in caecal contents [23] has been shown to result in virtually normal sensitivity of vascular smooth muscle in GF rats [24]. At this point it is assumed that the caecectomized GF rats, after comparable kidney trauma will lose to some extent the advantages, which were displayed by the non-caecectomized GF rats in this context. This experimental approach might help to pin-point microbial components among uraemic toxic substances [25].

In concluding, preliminary results obtained in our laboratory from comparable, CONV, KTR and sham-operated, orally antibiotic-treated rats should be mentioned. These animals, as previously shown [6, 26, 27], on partial neutralization of their intestinal flora, display qualitatively GF-like characteristics. Presently established routines of the implicated antibiotic treatment were used (for 4 days preoperatively and during the entire postoperative period the following were added to the drinking water which was taken ad libitum: bacitracin, streptomycin 4 mg/ml of each, nystatin 1 mg/ml [28]).

The results indicated that the KTR antibiotic-treated rats aligned clearly with the GF KTR and not with the CONV KTR group. Thus in the antibiotic-treated CONV rats the following trends were observed (the details of observation as well as the units are the same as given in Tables I–III). Blood urea nitrogen, KTR: 62 ± 40, sham-control: 14 ± 2. Blood plasma osmolality, KTR: 321 ± 27, sham-control: 297 ± 9. Thiourea clearance into the caecum, KTR: 126 ± 42, sham-control: 45 ± 10. Among six antibiotic treated CONV KTR rats, one died on the 5th day and five survived. Accordingly, the qualitative GF similarity of antibiotic treated CONV rats can be extended to characteristics displayed following the presently used kidney trauma. Based on these findings, oral antibiotic treatment gains an added justification in the clinical management of uraemia [28].

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STABILITY OF THE HUNGARIAN NATIONAL BCG REFERENCE PREPARATIONS TREND OF VIABILITY DURING 4–7 YEARS STORAGE AT +4 °C

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The stability of the viability of the Hungarian BCG Reference Preparations (Vaccine Bch. Nos: 265, 456, 505) was tested according to the recommendation of the World Health Organization. The BCG viable units of the vaccines were estimated from the colony counts determined on solid medium. The control data of the BCG viable units expressing the stability in 10^6/ml values were analysed up to 7 years in a three-step statistical model applying unbiased parameter estimates, valid hypothesis test and multiple comparison. The presented model verifying the stability of the reference preparations ensures the reliability of the assays to accept or reject the viability at controlling routine BCG vaccines in comparison with the reference preparations.

The stability and the inter-ampoule homogeneity of the active substance are the basic requirements for the international and for the national standard and reference biological preparations [1–3]. These two characteristics, the stability and uniformity ensure the reliability, i.e. the precision and the accuracy in the biological assays to control the test preparations.

The International Reference Preparation of BCG Vaccine (IRP-BCG) was established in 1966 and its stability has been controlled [4–10]. However, data on National BCG Reference Preparations from manufacturing laboratories and from National Control Authorities have not yet been published.

The aim of this paper is: (i) to report the data on stability of viability of three batches of the Hungarian National BCG Reference Preparations (HUN-BCG-RP's) controlled from 1970 in the BCG Laboratory of the National Institute of Hygiene, Budapest, and (ii) to present the three-step statistical model used in the quality control of the HUN-BCG-TP's.

Material and methods

BCG strain. The French 1173-P2 BCG strain of the Pasteur Institute Paris is used in Hungary for vaccine production since 1960 and as HUN-BCG-RP's since 1970 [11, 12].

BCG vaccine. The production of the Hungarian freeze-dried BCG vaccine was described and the HUN-BCG-RP's were prepared with the same method [13, 14].

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The batch numbers and the control period of the three batches of the HUN-BCG-RP's were as follows: (i) 265/220970-281174, (ii) 456/250973-230277, (iii) 505/020774-230781. The vials of the dried vaccines were stored at +4 °C to +10 °C.

Determination of the viability. BCG viability is expressed in viable units of 10^6/ml values (VU 10^6/ml) and is estimated from the colony counts. The method of the determination of

Table I

Stability of the viability expressed in VU×10^6/ml of the Hungarian BCG Reference Preparation (Bch. No. 265-220970) stored at 4 °C during 1527 days[a]

Control		Sampling	BCG VU	Control		Sampling	BCG VU	Control		Sampling	BCG VU
No.	date	day	10^6/ml	No.	date	day	10^6/ml	No.	date	day	10^6/ml
1	03 11 70	35	3.517	39	08 09 72	707	0.523	78	30 11 73	1162	0.668
				40	13 09 72	712	2.483	79	07 12 73	1169	2.491
2	19 06 71	263	3.763	41	15 09 72	715	2.991	80	14 12 73	1176	1.794
				42	20 09 72	720	2.458	81	21 12 73	1183	1.950
3	24 01 72	513	2.732	43	27 09 72	727	1.938	82	30 12 73	1192	0.608
4	30 03 72	547	2.666	44	04 10 72	734	2.975				
5	07 04 72	555	0.089	45	11 10 72	741	1.188	83	08 01 74	1201	1.990
6	13 04 72	561	2.490	46	18 10 72	748	2.700	84	11 01 74	1204	0.900
7	19 04 72	567	0.990	47	20 10 72	750	1.485	85	18 01 74	1211	2.883
8	22 04 72	570	4.750	48	25 10 72	755	1.710	86	23 01 74	1216	0.650
9	26 04 72	574	0.625	49	04 11 72	765	3.250	87	25 01 74	1218	0.941
10	04 05 72	582	0.225	50	15 11 72	776	2.183	88	05 02 74	1229	0.491
11	06 05 72	584	0.750	51	22 11 72	783	1.482	89	08 02 74	1232	0.625
12	11 05 72	589	2.050	52	29 11 72	790	2.650	90	15 02 74	1239	1.441
13	18 05 72	596	1.418	53	06 12 72	797	0.450	91	22 02 74	1246	0.483
14	24 05 72	602	2.016	54	14 12 72	805	1.266	92	01 03 74	1255	0.841
15	25 05 72	603	1.108					93	08 03 74	1262	3.000
16	26 05 72	604	0.450	55	06 01 73	834	3.058	94	15 03 74	1269	5.287
17	02 06 72	611	2.908	56	28 02 73	887	2.866	95	22 03 74	1276	1.233
18	08 06 72	615	1.483	57	14 03 73	901	2.258	96	29 03 74	1283	2.533
19	14 06 72	621	3.366	58	16 03 73	903	4.150	97	06 04 74	1291	2.858
20	15 06 72	622	1.108	59	23 03 73	910	1.866	98	12 04 74	1297	2.791
21	17 06 72	624	4.158	60	30 03 73	917	2.933	99	20 04 74	1305	2.250
22	21 06 72	628	3.708	61	06 04 73	924	1.670	100	26 04 74	1311	1.716
23	22 06 72	629	1.825	62	10 05 73	958	8.562	101	03 05 74	1318	3.016
24	28 06 72	635	0.383	63	17 05 73	965	1.750	102	10 05 74	1325	2.200
25	29 06 72	636	0.800	64	26 05 73	974	2.116	103	17 05 74	1332	2.150
26	06 07 72	643	2.375	65	31 05 73	979	3.283	104	06 06 74	1352	1.275
27	07 07 72	644	4.140	66	21 06 73	1000	1.191	105	13 06 74	1359	1.208
28	13 07 72	650	3.080	67	21 09 73	1092	2.900	106	20 06 74	1366	2.791
29	19 07 72	656	0.840	68	29 09 73	1100	2.900	107	26 06 74	1372	1.762
20	27 07 72	664	.0833	69	08 10 73	1109	0.966	108	10 07 74	1386	3.433
31	03 08 72	671	2.041	70	12 10 73	1113	2.397	109	24 07 74	1400	1.466
32	10 08 72	678	0.533	71	16 10 73	1117	6.400	110	12 09 74	1450	3.616
33	11 08 72	679	3.666	72	19 10 73	1120	1.915	111	19 09 74	1457	2.533
34	17 08 72	685	2.258	73	22 10 73	1123	1.375	112	21 11 74	1520	3.116
35	24 08 72	692	1.216	74	30 10 73	1131	1.112	113	28 11 74	1527	3.850
36	28 08 72	696	2.280	75	02 11 73	1134	1.625				
37	30 08 72	698	2.433	76	17 11 73	1149	1.625				
38	06 09 72	705	2.245	77	29 11 73	1161	1.467				

[a] Mean VU 10^6/ml values were estimated from colony counts determined on BOAA medium from reconstituted vials. Batch consists of 10 000 vials divided into 8 lots (1250 vials). Concentration per vial before lyophilization is 1.0 mg semi-dry weight of Pasteur BCG strain (Seed-Lot System) in 0.3 ml sodium glutamate, dextran and glucose medium. Reconstitution before vaccination in 1.0 ml Aqua dest. pro inj. Vial: 2.0 ml white neutral glass. Rubber stopper: Pharma, 1188 pH 182 grau, with aluminium seeling capsule

Table II

Stability of the viability expressed in VU×10⁶/ml of the Hungarian BCG Reference Preparation (Bch. No. 456-27 09 73) stored at 4 °C during 1270 days[a]

Control		Sampling day	BCG VU 10^6/ml	Control		Sampling day	BCG VU 10^6/ml	Control		Sampling day	BCG VU 10^6/ml
No.	date			No.	date			No.	date		
1	06 12 73	70	3.091	29	25 06 75	663	1.948	58	13 04 76	954	3.500
				30	24 07 75	691	1.990	59	23 04 76	964	2.316
2	29 03 74	183	2.381	31	31 07 75	698	1.820	60	28 04 76	969	2.335
3	06 04 74	192	2.633	32	06 08 75	704	2.730	61	05 05 76	976	5.614
4	12 04 74	222	2.408	33	21 08 75	719	3.083	62	26 05 76	997	2.114
5	20 04 74	230	0.380	34	03 09 75	732	3.591	63	01 06 76	1003	1.792
6	26 04 74	236	1.966	35	10 09 75	739	2.971	64	16 06 76	1018	2.214
7	03 05 74	243	3.416	36	17 09 75	746	2.371	65	25 06 76	1027	1.976
8	10 05 74	250	2.466	37	24 09 75	753	1.821	66	30 06 76	1032	2.457
9	17 05 74	257	1.150	38	01 10 75	760	6.557	67	06 07 76	1038	2.606
10	29 05 74	269	1.612	39	10 10 75	769	2.960	68	15 07 76	1047	1.716
11	06 06 74	277	1.066	40	23 10 75	782	3.343	69	20 07 76	1052	2.428
12	13 06 74	284	1.175	41	12 11 75	802	2.020	70	02 09 76	1096	2.001
13	20 06 74	291	2.291	42	18 11 75	808	3.740	71	20 10 76	1144	2.435
14	26 06 74	297	1.525	43	03 12 75	823	2.385	72	23 10 76	1147	0.793
15	10 07 74	311	0.850	44	19 12 75	839	3.550	73	27 10 76	1151	1.157
16	24 07 74	325	4.425					74	02 11 76	1157	2.750
17	29 08 74	361	3.783	45	02 01 76	853	2.571	75	10 11 76	1165	1.892
18	12 09 74	376	3.483	46	14 01 76	865	3.135	76	24 11 76	1179	1.942
19	19 09 74	383	2.327	47	21 01 76	872	2.942	77	01 12 76	1186	2.192
20	21 11 74	444	3.666	48	27 01 76	878	4.514	78	08 12 76	1193	1.135
21	28 11 74	453	3.850	49	04 02 76	886	2.642	79	15 12 76	1200	2.250
				50	11 02 76	893	3.964	80	21 12 76	1206	3.181
22	19 02 75	537	2.641	51	25 02 76	907	3.580				
23	26 02 75	544	3.370	52	05 03 76	915	3.057	81	12 01 77	1228	2.031
24	05 03 75	551	2.633	53	10 03 76	920	1.386	82	19 01 77	1235	1.772
25	12 03 75	558	5.433	54	17 03 76	927	4.592	83	25 01 77	1241	2.135
26	19 03 75	565	2.616	55	24 03 76	934	2.345	84	02 02 77	1249	1.079
27	16 05 75	623	4.550	56	31 03 76	941	3.730	85	23 02 77	1270	2.821
28	28 05 75	635	2.333	57	07 04 76	948	2.714				

[a] Mean VU 10^6/ml values were estimated from colony counts determined on BOAA medium from reconstituted vials. Batch consists of 10 000 vials divided into 8 lots (1250 vials). Concentration per vial before lyophilization is 1.0 mg semi-dry weight of Pasteur BCG strain (Seed-Lot System) in 0.3 ml sodium glutamate, dextran and glucose medium. Reconstitution before vaccination in 1.0 ml Aqua dest pro inj. Vial: 2.0 ml white neutral glass. Rubber stopper: Pharma, 1188 pH 182 grau, with aluminium seeling capsule

culturable particles i.e. the colony counts of the vaccine on Blood–Oleic Acid–Albumin Agar (BOAA) medium in Petri dishes during storage was described [15–17]. In order to test the stability of the viability of the three HUN-BCG-RP's, their VU 10^6/ml values were estimated from colony counts parallel with the routine batches manufactured for Hungary using the same control technique.

Statistical evaluation. The stability of viability of HUN-BCG-RP's was evaluated in a three-step statistical model: (i) from colony counts unbiased parameter point estimates of VO 10^6/ml values and 95% confidence limits were computed; (ii) with unbiased $\log_{10}$ transformed VU 10^6/ml values (dependent variable) linear regression analyses (Regranal) were performed in function of the storage time (days, independent variable) to test the trend of viability; (iii) the hypothesis of the equality of the regression coefficients (parallelism) and of the adjusted group means (AGM, identity) of the regression lines were tested by analysis of variance (Anova) applied for Regranal, then after rejected null hypothesis (non-parallelism), Gabriel's Simultaneous Test Procecure (STP) was performed for the multiple comparison of the ranked regression coefficients and of AGM values in order to diminish the risk of Type I error [18–22].

Table III

Stability of the viability expressed in VU · 10^6/ml of the Hungarian BCG Reference Preparation (Bch. No. 505-02 07 74) stored at 4 °C during 2533 days[a]

Control No.	Control date	Sampling day	BCG VU 10^6/ml	Control No.	Control date	Sampling day	BCG VU 10^6/ml	Control No.	Control date	Sampling day	BCG VU 10^6/ml
1	06 08 74	35	2.800	52	30 03 77	959	2.529	104	14 06 78	1390	1.757
				53	06 04 77	966	2.186	105	21 06 78	1397	1.871
2	05 02 75	218	7.300	53	06 04 77	966	2.186	106	02 08 78	1439	1.100
3	09 07 75	372	1.150	54	15 04 77	975	4.586	107	23 08 78	1460	1.943
4	19 12 75	535	2.625	55	20 04 77	980	2.443	108	12 10 78	1510	1.929
				56	28 04 77	988	2.652	109	25 10 78	1523	2.100
5	02 01 76	549	2.842	57	04 05 77	994	1.693	110	01 11 78	1530	3.500
6	14 01 76	561	2.228	58	10 05 77	1000	2.941	111	10 11 78	1539	2.857
7	21 01 76	568	2.514	59	20 05 77	1010	1.386	112	06 12 78	1565	1.686
8	27 01 76	574	2.400	60	01 06 77	1022	1.400	113	22 12 78	1581	3.900
9	04 02 76	582	2.500	61	08 06 77	1029	1.826	114	28 12 78	1587	1.600
10	11 02 76	589	2.800	62	15 06 77	1036	2.743				
11	20 02 76	598	3.592	63	22 06 77	1043	1.843				
12	25 02 76	603	3.059	64	13 07 77	1064	1.826	115	18 01 79	1609	1.392
13	05 03 76	611	2.685	65	19 07 77	1070	2.063	116	31 01 79	1622	2.000
14	10 03 76	616	2.828	66	20 07 77	1071	2.286	117	21 02 79	1643	2.300
15	17 03 76	623	2.457	67	27 07 77	1078	1.278	118	08 03 79	1658	1.829
16	24 03 76	630	2.266	68	03 08 77	1085	2.337	119	20 03 79	1670	1.500
17	31 03 76	637	3.070	69	10 08 77	1092	1.828	120	10 04 79	1691	0.400
18	07 04 76	644	2.342	70	17 08 77	1099	2.185	121	18 04 79	1699	2.543
19	13 04 76	650	2.091	71	31 08 77	1103	2.654	122	09 05 79	1720	1.710
20	17 04 76	654	3.700	72	07 09 77	1110	1.893	123	16 05 79	1727	2.200
21	23 04 76	660	2.800	73	14 09 77	1117	1.768	124	23 05 79	1734	2.329
22	28 04 76	665	3.214	74	22 09 77	1125	1.884	125	05 06 79	1747	1.074
23	05 05 76	671	3.428	75	27 09 77	1130	2.178	126	13 06 79	1755	1.074
24	13 05 76	679	2.075	76	06 10 77	1139	2.593	127	21 06 79	1763	3.186
25	26 05 76	692	1.594	77	12 10 77	1145	1.550	128	04 07 79	1776	1.655
26	01 06 76	698	1.871	78	19 10 77	1152	2.707	129	12 07 79	1784	1.871
27	16 06 76	703	1.357	79	26 10 77	1159	2.823	130	25 07 79	1797	1.458
28	25 06 76	712	1.765	80	02 11 77	1166	2.049	131	07 08 79	1810	2.571
39	30 06 76	717	1.903	81	10 11 77	1174	2.564	132	08 08 79	1811	1.072
30	06 07 76	723	2.985	82	16 11 77	1180	1.361	133	28 08 79	1839	1.614
31	15 07 76	732	2.085	83	25 11 77	1189	1.514	134	01 09 79	1843	2.457
32	20 07 76	737	2.628	84	30 11 77	1194	3.113	135	10 09 79	1852	1.500
33	02 09 76	750	1.090	85	07 12 77	1201	1.250	136	18 09 79	1860	0.828
34	30 09 76	778	3.071	86	10 12 77	1204	0.929	137	20 09 79	1862	1.350
35	20 10 76	798	2.008	87	21 12 77	1215	1.000	138	04 10 79	1876	1.228
36	23 10 76	801	1.550	88	28 12 77	1222	2.028	139	07 10 79	1879	1.350
37	27 10 76	805	2.142					140	21 11 79	1923	3.857
38	02 11 76	811	2.257	89	04 01 78	1229	1.571	141	12 12 79	1944	1.228
39	10 11 76	819	1.891	90	11 01 78	1236	1.704	142	19 12 79	1951	2.800
40	24 11 76	833	2.307	91	20 01 78	1245	0.300				
41	01 12 76	840	1.771	92	25 01 78	1250	1.757	143	24 01 80	1987	4.980
42	08 12 76	847	1.228	93	02 02 78	1258	1.448	144	25 03 80	2027	2.071
43	15 12 76	854	1.514	94	09 02 78	1265	1.628	145	12 03 80	2034	2.814
44	21 12 76	860	1.075	95	22 02 78	1278	1.450	146	26 03 80	2048	1.843
				96	01 03 78	1285	1.207	147	09 04 80	2062	0.733
45	12 01 77	882	1.061	97	08 03 78	1292	1.178	148	16 04 80	2069	1.029
46	19 01 77	889	1.800	98	19 04 78	1334	1.714	149	28 04 80	2081	2.443
47	25 01 77	895	1.542	99	17 05 78	1362	1.600	150	21 05 80	2104	1.786
48	02 02 77	903	2.757	100	21 05 78	1366	1.871	151	29 05 80	2112	3.114
49	23 02 77	924	4.171	101	24 05 78	1369	1.285	152	19 06 80	2133	0.515
50	26 03 77	945	2.700	102	31 05 78	1376	1.107	153	26 06 80	2140	2.200
51	22 03 77	951	2.657	103	07 06 78	1383	1.864	154	10 07 80	2154	1.586

Table III (continued)

Control No.	Control date	Sampling day	BCG VU 10^6/ml	Control No.	Control date	Sampling day	BCG VU 10^6/ml	Control No.	Control date	Sampling day	BCG VU 10^6/ml
155	17 07 80	2161	0.736	164	20 02 81	2380	1.250	174	07 05 81	2456	1.778
156	24 07 80	2168	0.743	165	26 02 81	2386	1.486	175	21 05 81	2470	1.421
157	06 08 80	2181	1.028	166	06 03 81	2394	0.821	176	04 06 81	2484	0.478
158	13 08 80	2188	1.043	167	12 03 81	2400	1.457	177	11 06 81	2491	0.471
159	28 08 80	2203	1.777	168	19 03 81	2407	0.357				
160	12 11 80	2279	0.483	169	26 03 81	2414	0.071	178	24 06 81	2504	0.514
161	19 11 80	2286	2.042	170	02 04 81	2421	2.243	179	02 07 81	2512	0.850
				171	09 04 81	2428	1.936	180	09 07 81	2519	1.164
162	29 01 81	2358	0.843	172	16 04 81	2435	0.557	181	17 07 81	2527	0.164
163	12 02 81	2372	0.957	173	24 04 81	2443	0.866	182	23 07 81	2533	1.490

[a] Mean VU 10^6/ml values were estimated from colony counts determined on BOAA medium from reconstituted vials. Batch consists of 10 000 vials divided into 8 lots (1250 vials). Concentration per vial before lyophilization is 1.0 mg semi-dry weight of Pasteur BCG strain (Seed-Lot System) in 0.3 ml sodium glutamate, dextran and glucose medium. Reconstitution before vaccination in 1.0 ml Aqua dest pro inj. Vial: 2.0 ml white neutral glass. Rubber stopper: Pharma, 1188 pH 182 grau, with aluminium seeling capsule

Computation. Commodore 64 computer was used for data processing and statistical analysis. Programmes and computer printed tables are available on request from the BCG Laboratory of Budapest.

Results

It is a prerequisite in control assays to know individual estimated VU 10^6/ml values and the stability of a BCG Reference Preparation determined during storage. Therefore, all BCG VU control data will be listed and also Regranal of each batch then Anova followed by STP will be presented.

Tables I, II and III list the BCG VU 10^6/ml estimates of the three HUN-BCG-RP's batches in a chronological order. It is seen that batch 265 was used during 1527 days (4.2 years), batch 456 during 1270 days (3.5 years) and batch 505 during 2533 days (6.9 years) as RP.

Table IV presents the Regranal of the BCG VU 10^6/ml values on storage time of the three batches. Footnotes explain the statistical and microbiological meaning of the estimated parameters. It is shown that the viability of batches 265 and 456 is stable. The trend of the BCG VU 10^6/ml values of batch 505 drops under the mean ($1\,690 \times 10^6$) after 2 000 days (5.5 years) i.e. the stability has begun to decrease.

Table V has two parts. The upper part presents the Anova applied for Regranal testing the equality of the regression coefficients of the three batches. Since the viability of batch 505 has a regression it is seen that the slopes are unequal, i.e. the null hypothesis on parallelism is rejected. The lower part presents the STP of the ranked parameters showing that the "B" values of batches 265 and 456 form a homogeneous subset while after analysis of covariance the "AGM" values of batches 456 and 505 are in the same subset.

Table IV

Regranal of VU 10^6/ml values on storage time of the three

Vacc. Bch. No.	Regranal					
	Intercept			Regr. coeff.		
	a	(95% conf. limit)		b	(95% conf. limit)	
265	1.496	(1.021	2.280)	0.000070	(−0.000118	0.000259)
456	2.371	(1.849	3.048)	0.000008	(−0.000122	0.000138)
505	3.304	(2.748	3.972)	−0.000209*	(−0.000261	−0.000156)

* Sign. at 5% level

a Intercepts express VU 10^6/ml values at the origins

b Regression coefficients express in log VU 10^6/ml values the daily decrease (increase) of the viability, i.e. the stability

Discussion and conclusions

The study had two aims: (i) to control the stability of the BCG VU 10^6/ml values of the HUN-BCG-RP's, and (ii) to present the three-step statistical model considered to be essential in the analysis of the control data of the

Table V

Anova of the equality of the regression lines and STP for multiple comparison of the estimated parameters of the three HUN-BCG-RP. Analysis of data from Tables I, II, III and IV

Anova applied for Regranal

Source	SS	DF	MS	F
Equality of slopes	0.99	2	0.49	8.14*
Error 1	22.61	373	0.06	
Total	23.60	375	0.06	
Equality of adjusted means	0.66	2	0.78	5.26*
Common slope	2.04	1	2.04	32.42*
Error 2	23.60	375	0.06	
Total	26.30	378		

STP of the estimated and ranked "b" and "AGM" values

Rank	Bch	"b"	HS	Bch	"AGM"	HS
1	265	0.000070	|	456	0.328	|
2	456	0.000008	|	505	0.271	|
3	505	−0.000209	|	265	0.211	|

* $p < 5\%$

HS Vertical bars indicate homogeneous subsets of Gabriel's STP

HUN-BCG-RP's Analysis of data from Table I, II and III

r^2	T 1/2 day	Y %/X	VU survivals at day 1825 10^6/ml	Anova		
				Regr. MS	Resid. MS	Resid. DF
0.0049	4 262	0.016	2.010	0.0477	0.097	110
0.0002	37 555	0.002	2.453	0.0004	0.04	83
0.2562	1 438	0.048	1.374	2.97*	0.05	180

r^2 Coefficients of determination express in % the variation of y (VU) due to the linear regression on x (day)
T1/2 Express in day the 50% reduction of VU 10^6/ml values
Y%/X Average daily decrease of VU expressed in %
VU Survivals at day 1825 (5 years) are estimated from the regression equations

biological products for having an objective interpretation from statistical inference.

The stability of the biological standard and/or reference preparations is a basic requirement to control reproducibility of the experimental designs, the sensitivity of the reagents, the precision of the technique, the sampling and the experimental error in the biological assays to determine the potency of the tested biological preparations [23].

The main epistemological and pragmatical problems of the statistical quality control is to accept or reject a test preparation in comparison with the standard. Therefore, the stability and homogeneity of the standard and/or reference preparations and the use of exact statistical methods, i.e. unbiased parameter and confidence interval estimates, valid hypothesis tests, multiple comparison to reduce Type I error in decision, have to be ensured.

The study has demonstrated that the HUN-BCG-RP's can be used generally up to 5 years as stable reference preparations. Further studies will analyse the factors decreasing the stability of the BCG VU 10^6/ml values after 5 years. Also more developed statistical designs will be applied to analyse interaction effects in experimental models in testing the stability.

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NOURSEOTHRICIN (STREPTOTHRICIN) INACTIVATED BY A PLASMID pIE636 ENCODED ACETYL TRANSFERASE: NATURE OF THE INACTIVATED NOURSEOTHRICIN

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Nourseothricin, a mixture of several streptothricins, is inactivated by an acetyl transferase produced by *Escherichia coli* containing the plasmid pIE636. Nourseothricin inactivated in the presence of ^{14}C-acetate was purified and submitted to partial hydrolysis. In the hydrolysate besides others a radioactive and ninhydrin-reactive substance moving only slightly towards the cathode was found. It proved to be [^{14}C]-acetyl beta-lysine.

Nourseothricin, an antibiotic isolated from *Streptomyces lavendulae* [1] represents a mixture of several streptothricins, mainly D and F [2]. Streptothricin consists of three moieties: gulosamine, streptolidine, and a β-lysine peptide chain differing in chain length from 1–6 amino acid residues (streptothricin F–A). As the antibiotic activity of streptothricins depends directly on the number of β-lysine residues it is supposed that the peptide moiety represents the biologically active centre of the molecule [2]. Recently we found that *Escherichia coli* strains containing the plasmids pIE636 or pIE638 encoding streptothricin resistance produce a streptothricin acetylating enzyme [3, 4]. The present paper describes the purification of the acetylated streptothricin and investigations to localize the acetyl group in the molecule.

Materials and methods

Preparation of the crude enzyme. E. coli strain IE956 [3] was disrupted by sonication (20 kHz, 250 W, 3×30 s, [5]) and the sonicate centrifuged 90 min at 105 000 *g*. To the supernatant an equal amount of a saturated solution of $(NH_4)_2SO_4$ in water was added, the precipitate collected by centrifugation, dissolved in the original volume of TMED buffer (10 mM tris(hydroxymethyl)aminomethane, 10 mM $MgCl_2$, 0.15 mM ethylenediaminetetraacetate, and 1 mM mercaptoethanol, [6]) and dialyzed overnight against the same buffer.

Inactivation of nourseothricin. The inactivation mixture contained 0.5 ml of the enzyme solution, 150 mmol acetyl coenzyme A (CoASAc) in 0.1 ml TMED, and 20 mg nourseothricin in 0.4 ml TMED. After incubation overnight at 37 °C the solution was heated 3 min at 100 °C.

Preparation of ^{14}C-labelled inactivated nourseothricin. Twenty μl coenzyme A (1.6 mg/ml), 20 μl 1[^{14}C]-CH_3COONa (37 MBq/ml; Zentralinstitut für Kernforschung, Dresden GDR),

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20 μl adenosine-5'-triphosphate (25 mg/ml), 100 μl of the enzyme solution, and 30 μl nourseothricin (3 mg/ml) were incubated and handled as described above.

Purification of the inactivated nourseothricin. The solution of the crude inactivated nourseothricin (20 mg) in water (containing about 0.5 MBq/ml ^{14}C-labelled nourseothricin as a tracer) was diluted to obtain an electrical conductivity comparable with that of a 0.05 M NaCl solution. This solution was filtered through a column (16 × 50 mm) of Amberlite CG50I (Na^+). Most of the solutes are not absorbed under these conditions. Some impurities were eluted from the column with 0.2 M NaCl, the inactivated nourseothricin with 0.3 M NaCl. The fraction obtained thus was concentrated to about 5 ml and applied to the head of a 26 × 900 mm column of Sephadex G-10 (v_0 = 145 ml, v_t = 480 ml). In the effluent (Fig. 1) two fractions were detectable, the first containing the practically pure inactivated nourseothricin.

Thin layer chromatography (TLC) and thin layer electrophoresis (TLE) were performed on 20 × 20 cm glass plates coated with a 0.5 mm layer of cellulose powder MN 300 (Macherey, Nagel & Co., Düren, FRG). Solvent for TLC was pyridine:n-butanol:acetic acid:water = = 10:15:3:12 (solvent A, [2]) buffers for TLE were formic acid:acetic acid:water = 25:75:900, pH 2.0 (buffer A, [7]) and pyridine:acetic acid:water = 10:4:86, pH 5.2 (buffer B, [8] slightly modified).

Assay methods. For the estimation of total acetyl the sample was hydrolyzed overnight with 4 N HCl at 100 °C, cooled, and extracted with ether [9]. To the extracts propionic acid as internal standard was added and the separation and quantification was performed on 5% DEGA on Chromosorb-G-HP (2 m × 3 mm column, 140 °C). For the estimation of O-acetyl the solutions of the inactivated nourseothricin were reacted with hydroxylamine and, after acidification with HCl to pH 1.2, with $FeCl_3$ in 0.1 N HCl. The optical density was read at 540 nm [10]. Amino groups were quantified by means of dinitrofluorobenzene in 1% $Na_2B_4O_7$ buffer [11], after 30 min at 60 °C the samples were acidified with 2 N HCl and the optical density was read at 420 nm.

Results

Characterization of the inactivated nourseothricin

1. Thin layer electrophoresis (buffer A, 45 min, ~20 V/cm). Purified inactivated nourseothricin moves towards the cathode, after staining with ninhydrin producing a similar pattern as unmodified nourseothricin, however, with reduced velocity (about 75% of that of the unmodified substance, Fig. 2). [^{14}C]-acetyl labelled inactivated nourseothricin in each spot contains radioactivity.

2. Thin layer chromatography (solvent A). After staining with ninhydrin only one strongly elliptic spot is visible, containing all the radioactivity (R_f ~0.4).

3. Thin layer electrophoresis of the hydrolyzed (4 M HCl, 16 h, 100 °C, ninhydrine-staining) inactivated nourseothricin (buffer A, 45 min, ~20 V/cm). The patterns of both the active and inactivated nourseothricins were indistinguishable (Fig. 2), indicating the identity and purity of the substance.

4. Estimation of the percentage of acetyl in inactivated nourseothricin. Inactivated nourseothricin contains about 4% acetyl. This value points to only one acetyl residue per molecule streptothricin.

Estimation of the kind of linkage of the incorporated acetyl residue. 1. Nourseothricin inactivated in the presence of CoAS-[^{14}C]Ac was dissolved in 0.01 N NaOH. After two hours at room temperature [10] an excess of acetic acid was added, the solution concentrated by evaporation in vacuo and subjected to

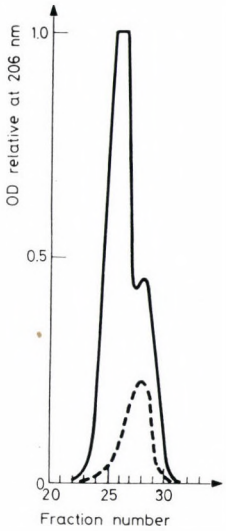

Fig. 1. Effluent diagram of chromatography on Sephadex G-10 of inactivated nourseothricin. —— optical density (OD) at 206 nm; – – – conductivity (no absolute data); fraction size 10 ml

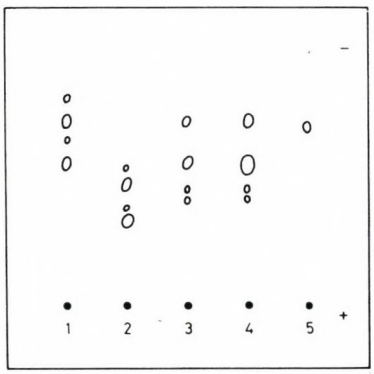

Fig. 2. Thin layer electropherogram (buffer A). Lane 1, nourseothricin; lane 2, inactivated nourseothricin; lane 3, hydrolyzed nourseothricin; lane 4, hydrolyzed inactivated nourseothricin; lane 5, — α-lysine

TLE (buffer A, 45 min, ~10 V/cm). The pherogram was stained with ninhydrin and the radioactivity localized and counted with the Dünnschichtscanner II (Fa. Berthold, Wildbad, BRD). No difference could be detected between the pherograms of nourseothricin either treated with NaOH or untreated, i.e., no splitting of the linkage between the streptothricin molecules and the acetyl residues had occurred. Similarly *N*-acetylglucosamine was stable under these conditions, while *O*-acetylserine and *O*-acetylcholine were completely deacetylated.

2. Treatment of a sample of inactivated nourseothricin with hydroxylamine and afterwards with $HCl/FeCl_3$ [10] yielded in an optical density at

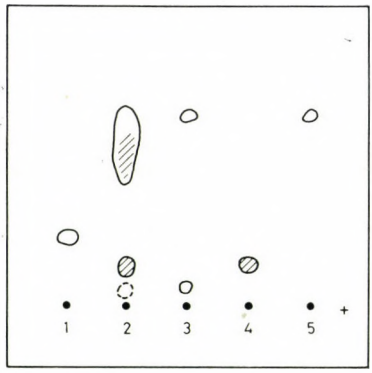

Fig. 3. Thin layer electropherogram (buffer B). Lane 1, ε-amino caproic acid; lane 2, partially hydrolyzed inactivated nourseothricin; lane 3, mixture of α-lysine (upper spot) and glycine (lower spot); lane 4, purified unknown compound; lane 5, purified unknown compound after complete hydrolysis (→ β-lysine); open area: ninhydrine reactive spot; shaded area: radioactivity

540 nm corresponding to about 10% of the acetyl content of the sample. Solutions of either *N*-acetylglucosamine and nourseothricin remained colourless under these conditions; while *O*-acetylserine produced the anticipated colour yield.

3. The results of the foregoing experiments point to an amide bound acetyl causing the inactivation of streptothricin. The precise localization of the acetyl group was determined by partial hydrolysis of the [^{14}C]-acetate labelled inactivated nourseothricin (1 N HCl, 3 h, 100 °C) and isolation and investigation of the labelled splitting products. In high-voltage TLE (buffer B, 1h, ~20 V/cm) besides unhydrolyzed inactivated nourseothricin only one labelled compound moving only slowly towards the cathode could be detected (Fig. 3). This compound was purified by preparative high voltage paper electrophoresis (buffer B) and subsequent TLC (solvent A; R_f ~0.5) and then submitted to complete hydrolysis (4 NHCl, 16 h, 100 °C). In TLE (buffer A and B, see Figs 2 and 3, resp.), and in TLC (solvent A) only β-lysine could be detected in this sample.

Discussion

One of the possible mechanisms of bacteria to become resistant against the harmful action of antibiotics is the enzymatic modification of the antibiotic molecule. The β-lactamases in the case of β-lactam antibiotics and several transferases (nucleotidyl, phosphoryl, acetyl) in the case of aminoglycoside antibiotics [7] are examples. Though nourseothricin (streptothricin) contains an aminoglycosidic linkage it cannot be regarded as an aminoglycoside antibiotic in the true sense. Likewise one cannot anticipate that nourseothricin

inactivating enzymes act in the same manner as aminoglycoside antibiotics inactivating enzymes [7]. Recently we found that *E. coli* strains containing the plasmids pIE636 or pIE638 inactivate nourseothricin by means of an acetylating enzyme [3] which in the case of *E. coli* J53 as recipient of the plasmid is localized at the inner side of the cytoplasmic membrane [12]. Very recently, however, we found wild type *E. coli* strains containing at least part of the acetyl transferase in the periplasmic space or at the outer side of the cytoplasmic membrane (Seltmann and Wolter, unpublished).

Purification of the inactivated nourseothricin yielded a preparation producing the same pattern in TLE as the unmodified substance, however, moving with reduced velocity. Analysis of purified streptothricin F under identical conditions resulted in only one spot (not shown in "Results"). The TLE patterns of the totally hydrolyzed active and inactivated nourseothricins were identical and that of the latter contained no additional spots, i.e., the purified inactivated nourseothricin did not contain detectable amounts of impurities. It must be mentioned, however, that acidic hydrolysis of nourseothricin does not cause complete splitting of the molecule into its three components, because (i) the linkage between gulosamine and streptolidine is rather acid-stable and therefore splitted only in part; (ii) besides gulosamine significant quantities of 1,6-anhydrogulosamine have been formed during hydrolysis [2]. Only β-lysine seems to be liberated more or less quantitatively. Therefore quantitative estimations of gulosamine, β-lysine and streptolidine in the hydrolysate does not justify conclusions on the ratio of these components in the unhydrolyzed molecule. However, qualitatively and quantatively identical TLE patterns of both the active and the inactivated nourseothricin should indicate that no variations in the ratio of the three components had occurred. Speculations that the enzyme could act by acetylating one of the hydroxy groups of gulosamine proved to be incorrect as we found practically all the acetyl groups to be amide-bound. The fact that mild acidic hydrolysis of the [^{14}-C]-acetate labelled inactivated nourseothricin liberates, besides others, [^{14}C]-acetyl β-lysine demonstrates that at least part of the acetyl residues incorporated into the nourseothricin molecules are bound to the β-lysine (peptide) chain. This result is in agreement with the finding [2] that the longer the β-lysine (peptide) chain in the streptothricin molecule the higher is its antibiotic activity.

From the facts that in the partial hydrolysate acetyl-β-lysine can be found and that one molecule of inactivated nourseothricin apparently contains only one acetyl residue it can be speculated that only the terminal ε-amino group of the β-lysine (peptide) chain is substituted by the plasmids pIE636 or pIE638 determined acetyl transferase. This possibility is under further investigation.

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VIRULENCE FACTORS OF *ESCHERICHIA COLI*

IV. ASSOCIATION IN *ESCHERICHIA COLI* OF LD_{50} WITH HAEMOLYSIN PRODUCTION, HAEMAGGLUTINATING CAPACITY, ANTIGENS K1, K5 COLICINOGENICITY AND PATHOGENICITY

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Haemolysin production (HLY), mannose resistant haemagglutinating activity (MRA), presence of antigens K1 and K5 and colicinogenicity (Col) were compared with LD_{50} for mice in 663 *Escherichia coli* strains, including 281 faecal, 129 urinary and 253 other extraintestinal isolates. Those isolates that LD_{50} value fell into $\leq 10^6$ LD_{50} category were arbitrarily termed highly virulent (HV) and those which belonged to $\geq 10^7$ LD_{50} category were considered avirulent (AV). HV isolates occurred significantly more frequently (58%) among strains from different extraintestinal samples than from faeces (14%) or urine (16%). The incidence of HV strains was significantly higher in patients with sepsis (43%) or meningitis (100%) than in patients with enteritis (20%), urinary tract infections (UTI, 16%) or in healthy subjects (28%). The incidence of HV strains in the most frequent (O1, O2, O4, O6, O7, O18, O75) serogroups was significantly higher (60%) than in others (10%). Strains with different virulence markers (HLY, MRA, K1, K5, Col) belonged significantly more frequently to the HV group than those which failed to have these markers (44 vs 27%, 51 vs 25%, 83 vs 17%, 78 vs 27%, 52 vs 16%, respectively). Important role of antigen K1 playing in pathomechanism of meningitis was confirmed by data of analysis according to which significant difference was revealed in the incidence of HV strains between groups of isolates with MRA^+K1^+ (71%) and MRA^+K1^- (44%, $p < 0.02$), or between groups of isolates with MRA^+K1^- and MRA^-K1^+ (91%, $p < 0.001$). Moreover there were significant differences in the incidences of HV strains in $K1^+Col^-$ (73%) and $K1^-Col^+$ (29%, $p < 0.001$), and in $K1^+Col^+$ (86%) and $K1^-Col^+$ (29%, $p < 0.001$) groups. Further evidence was given by those data that there were no significant differences between groups of HV strains with MRA^+K1^+ and MRA^-K1^+ ($p > 0.05$) or with $K1^+Col^+$ and $K1^+Col^-$ ($p > 0.1$) properties. Isolates that possessed simultaneously two of MRA, HLY, Col markers were more pathogenic in LD_{50} assay than those that had one or the other of these markers alone. Strains in serogroup O18 killed mice significantly more frequently than those of other serogroups independently of having any virulence factor, suggesting that bacteria in serogroups O18 must have some special virulence other than K1, Col, MRA or K5. MRA^+HLY^+ HV strains occurred frequently in extraintestinal diseases (42%) supporting the preconception that these properties play an important role also in the pathomechanism of extraintestinal infections other than UTI. HLY^+ strains isolated from septic patients belonged also in a high proportion to the HV group (63%). On the basis of special characteristics of the isolates, two distinct groups of extraintestinal diseases could be distinguished beside UTI: (i) "meningitis of newborn" where the presence of antigen K1, and lack of MRA and HLY, serogroups O7, O18; and (ii) "sepsis and inflammation" where MRA^+, HLY^+ serogroups or MRA^+, HLY^+, O18:K5 strains are common. Strains causing meningitis in elder children or adults and UTI belong also to this category.

In systemic infection with *Escherichia coli* it is well known that various bacterial factors may be involved. There are *E. coli* with certain K antigens which possess a particular pathogenicity and are the cause of peritonitis, cystitis, pyelitis, meningitis etc [1–3]. The great significance of haemolysin

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production for the pathogenicity was confirmed by investigations of many workers during the last 30 years [4–6]. The general importance of microbial adhesion has been recognized in recent years [3, 7, 8]. Colicin V production appears also to be associated with ability to cause septicaemia [3, 9–12]. There are several possibilities to approach the question of virulence of *E. coli* [13, 14], however, determination of the median lethal dose (LD_{50}) in mice by intraperitoneal injection of bacteria is one of the commonly accepted method for proving the pathogenicity of an isolate. The aim of this study was to examine the correlation of LD_{50} with different virulence factors (haemolysin production, haemagglutinating capacity, O antigen, K1 and K5 antigens, colicinogenicity) of *E. coli* implicated in extraintestinal infections, in isolates originating from faecal, urinary and other extraintestinal sources.

Materials and methods

E. coli strains were isolated from faecal samples of healthy subjects (119), or patients suffering from enteritis (162), urine of patients with pyelonephritis (46), cystitis (62), asymptomatic bacteriuria (19), and 253 from other extraintestinal sources (blood 38, cerebrospinal fluid 35, autopsy material 23, wound swab 57, umbilical cord 8, vagina 37, upper respiratory tract excretions 35, placenta, lochia 20).

Classification according to diagnostic criteria of patients, serological examination of O antigens, mannose resistant haemagglutination (MRA), haemolysin production (HLY), detection of K1 and K5 capsular antigens, determination of colicinogenicity (Col) were carried out as described previously [15–17].

LD_{50} assay. Four groups of 10 CFLP mice weighing 16–16 g were used. Each mouse was injected intraperitoneally with 0.5 ml of bacteria grown for 4–6 h in broth and serially diluted 10 fold in physiological saline [9, 18]. The mortality rate was recorded after 7 days (no additional deaths occurred later on). The results were expressed as the viable number of bacteria corresponding to LD_{50}. Statistical analysis of LD_{50} was carried out by the formula of Reed and Muench [19]. Those isolates that LD_{50} value fell $\leq 10^6$ category were arbitrarily termed highly virulent (HV), oppositely, those strains that LD_{50} were $\geq 10^7$ were regarded as avirulent [AV]. LD_{50} values falling between 10^6–10^7 were termed intermediate and were excluded from the statistical analysis. Number of HV and AV strains together gave 100%.

Systems and programs. The encoded data included the patient's sex, name, age, diagnosis, date of examination, type of specimen, place of isolation as well as 14 different properties of the isolates (O antigen, H antigen, presence of K1 or K5 antigens, lactose fermentation, haemolysin production, mannose resistant haemagglutinating capacity in human A erythrocytes, phage pattern, phage type, colicinogenicity, colicin type, lysogenicity, value of LD_{50}, result of mouse lung toxicity test).

Data were transmitted to and evaluated by use of personal computer analysing statistically the frequency of certain constituents. Results were examined for significance in $n \times n$ contingency tables by the chi^2 test [20]. The following data were analysed:

1. Type of specimen, diagnosis of patient, serogroup of strains, Col, HLY, MRA, K1, K5 positivity or negativity for which LD_{50} frequency distribution were tabulated.

2. Simultaneous occurrence of certain properties (i.e. MRA–K1, MRA–Col, MRA–HLY, K1–Col) for which LD_{50} frequency distribution was tabulated.

3. Simultaneous occurrence of certain serogroups (i.e. O18, O4–O6, O1–O2–O7) and certain properties (i.e. K1–Col, MRA–K5, MRA–HLY), or certain diseases (meningitis, sepsis, UTI) and certain properties (HLY^+ and HLY^-).

Results

E. coli with high virulence (HV) occurred the most frequently (58%) in different extraintestinal samples (Table I). The difference in the incidence of HV strains as compared to the incidence of avirulent (AV) ones between faecal and urinary samples was not significant ($p > 0.7$), but between faecal+urinary and other extraintestinal samples was significant ($p < 0.001$).

The distribution of *E. coli* strains according to different diseases and LD_{50} values is demonstrated in Table II. There was no significant difference in the occurrence between HV strains isolated from patients suffering from enteritis and healthy subjects ($p > 0.1$), or between those from healthy individuals and with urinary tract infection (UTI, $p > 0.3$). However, the incidence of HV strains in group of patients with sepsis ($p < 0.01$) or in group of patients with meningitis ($p < 0.001$) was significantly higher than in the above-mentioned groups.

The serogroup distribution of *E. coli* strains falling into different LD_{50} categories is presented in Table III. The incidence of strains belonging to HV group in the seven most frequent (O1, O2, O4, O6, O7, O18, O75) serogroups

Table I

Grouping of E. coli strains of different origin according to LD_{50}

Specimen	No. of strains in LD_{50} groups			Total
	$\leq 10^6$	10^6–10^7	$\geq 10^7$	
Faeces	29	81	171	281
Urine	16	30	83	129
Other extraintestinal sources	93	93	67	253
Total	138	204	321	663

Table II

Distribution of E. coli strains according to diseases and LD_{50} categories

Test groups	No. of strains in LD_{50} group			Total
	$\leq 10^6$	10^6–10^7	$\geq 10^7$	
Patients with enteritis	23	48	91	162
Healthy persons	42	61	107	210
Urinary tract infection	16	30	83	129
Sepsis	22	36	29	87
Meningitis	23	8	0	31
Other	12	21	11	44
Total	138	204	321	663

Table III

Serogroup distribution of E. coli strains in different LD_{50} categories

Serogroup	No. of strains in LD_{50} group			Total
	$\leq 10^6$	10^6–10^7	$\geq 10^7$	
O1	4	17	9	30
O2	28	9	7	44
O4	0	24	12	36
O6	16	33	14	63
O7	7	8	7	22
O18ac	55	12	13	80
O75	0	4	12	16
Others*	28	97	247	372
Total	138	204	321	663

* Other serogroups (see ref. [17]) and strains not typable and agglutinating spontaneously

Table IV

Correlation between different virulence markers and incidence of E. coli strains in different LD_{50} groups

Markers	No. of strains in LD_{50} group			Total
	$\leq 10^6$	10^6–10^7	$\geq 10^7$	
HLY^+	34	83	42	159
HLY^-	104	121	279	504
MRA^+	48	96	46	190
MRA^-	90	108	275	473
$K1^+$	75	34	15	124
$K1^-$	63	170	306	539
$K5^+$	18	14	5	37
$K5^-$	120	190	316	626
Col^+	91	91	83	265
Col^-	47	113	238	398
Col^+V^+	22	16	12	50
Col^+V^-	69	75	71	215

was very high (60%), significantly higher ($p < 0.001$) than in other serogroups (10%).

There was a marked correlation between the presence of different virulence markers (e.g. HLY, MRA, K1, K5, Col) and the frequency of strains belonging to the HV category (Table IV): 44% of HLY^+, 51% of MRA^+, 83% of $K1^+$, 78% of $K5^+$, 52% of Col^+ strains belonged to HV groups, whereas isolates that failed to have these virulence markers belonged less frequently (27, 25, 17, 27 and 16%, respectively) to HV categories. The difference in the incidence was statistically significant ($p < 0.01$–$p < 0.001$).

There was no significant difference in frequency of HV strains elaborating colicin V (65%) or other type of colicin (49%; $p > 1.1$).

In a previous study [17] a close association was observed between MRA–K1, MRA–Col, MRA–HLY and K1–Col properties. In the present work it was studied how do these simultaneously occurring properties influence the toxicity of *E. coli* strains for mice.

Correlations between MRA–K1 properties and LD_{50}. It was revealed that 71% of MRA^+ $K1^+$, 44% of MRA^+ $K1^-$, 88% of MRA^- $K1^+$ and 11% of MRA^-K1^- isolates belonged to the HV group (Table V). The difference between the incidence of $K1^+MRA^+$ and $K1^+MRA^-$ in HV category was not significant ($p > 0.05$). However, a significant difference was found between the frequencies in MRA^+K1^+ and MRA^+K1^- ($p < 0.02$), or between the frequencies in MRA^-K1^+ and MRA^+K1^- ($p < 0.001$) groups of isolates.

The simultaneous occurrence of K1–Col properties and their relation to toxicity for mice is shown in Table VI. The difference in the incidence of $K1^+Col^+$ (86%) and $K1^+Col^-$ (74%) strains in HV category was not statistically significant ($p > 0.1$). However, the difference between the frequency of strains with $K1^+Col^-$ and $K1^-Col^+$ (29%) properties was significant ($p < 0.001$).

Correlation between MRA–Col properties and LD_{50}. As demonstrated in Table VII, a significant difference was found in the occurrence of strains belonging to the HV group between MRA^+Col^+ (70%) and MRA^-Col^+ (46%;

Table V

Correlation between MRA-K1 markers and incidence of E. coli strains in different LD_{50} groups

Markers	No. of strains in LD_{50} group			Total
	$\leq 10^6$	10^6–10^7	$\geq 10^7$	
MRA^+K1^+	17	19	7	43
MRA^+K1^-	31	77	39	147
MRA^-K1^+	58	15	8	81
MRA^-K1^-	32	93	267	392

Table VI

Correlation between K1-Col markers and incidence of E. coli strains in different LD_{50} groups

Markers	No. of strains in LD_{50} group			Total
	$\leq 10^6$	10^6–10^7	$\geq 10^7$	
$K1^+Col^+$	61	28	10	99
$K1^+Col^-$	14	6	5	25
$K1^-Col^+$	30	63	73	166
$K1^-Col^-$	33	107	233	373

$p < 0.01$) or between MRA^+Col^+ and MRA^+Col^- (36%; $p < 0.001$). The difference in the incidence of HV strains between MRA^+Col^- and MRA^-Col^+ groups was not statistically significant ($p > 0.2$).

Correlation between MRA–HLY properties and LD_{50}. Table VIII shows that no significant difference could be demonstrated in the incidence between groups of HV strains with MRA^+HLY^+ (41%) and MRA^-HLY^+ (36%; $p > 0.3$), or between those with MRA^+HLY^- (63%) and MRA^-HLY^+ ($p > 0.3$). On the contrary, there was a significant difference in the incidence

Table VII

Correlation between MRA–Col markers and incidence of E. coli strains in different LD_{50} groups

Markers	No. of strains in LD_{50} group			Total
	$\leq 10^6$	10^6–10^7	$\geq 10^7$	
MRA^+Col^+	29	48	12	89
MRA^+Col^-	19	48	34	101
MRA^-Col^+	62	43	71	176
MRA^-Col^-	28	65	204	297

Table VIII

Correlation between MRA–HLY markers and incidence of E. coli strains of different origin in different LD_{50} groups

Markers	Origin of strains	No. of strains in LD_{50} group			Total
		$\leq 10^6$	10^6–10^7	$\geq 10^7$	
MRA^+HLY^+	Faeces	11	18	19	48
	Urine	1	6	5	12
	Other extraintestinal	10	36	7	53
	Total	22	60	31	113
MRA^+HLY^-	Faeces	7	14	7	28
	Urine	4	7	2	13
	Other extraintestinal	15	15	6	36
	Total	26	36	15	77
MRA^-HLY^+		4	6	4	14
	Urine	2	3	1	6
	Other extraintestinal	6	14	6	26
	Total	12	23	11	46
MRA^-HLY^-	Faeces	7	43	141	191
	Urine	9	14	75	98
	Other extraintestinal	62	28	48	138
	Total	78	85	264	427

Table IX

Correlation between K1, Col, O18 or MRA, K5, O18 markers and incidence of E. coli strains in different LD_{50} groups

Markers	Serogroup	No. of strains in LD_{50} group			Total
		$\leq 10^6$	10^6–10^7	$\geq 10^7$	
$K1^+Col^+$	O18	31	1	1	33
	Others	30	27	9	66
$K1^-Col^-$	O18	13	6	11	30
	Others	20	101	222	343
MRA^+K5^+	O18	8	5	0	13
	Others	3	4	0	7
MRA^-K5^-	O18	33	1	6	40
	Others	50	102	264	416

Table X

Correlation between K1, Col markers, serogroups O1, O2, O7 and incidence of E. coli strains in different LD_{50} groups

Markers	Serogroup	No. of strains in LD_{50} group			Total
		$\leq 10^6$	10^6–10^7	$\geq 10^7$	
$K1^+Col^+$	O1, O2, O7	22	19	4	45
	Others	39	9	6	54
$K1^-Col^-$	O1, O2, O7	2	5	12	19
	Others	31	102	221	354

of HV strains with MRA^+HLY^+ and MRA^-HLY^- (21%; $p < 0.01$), with the exception of strains originating from extraintestinal infection other than UTI, where 56% of MRA^-HLY^- isolates belonged to HV category.

In view of the close correlation existing between serogroup O18 and K1, Col possession on the one hand and between serogroup O18 and MRA, K5 positivity on the other [17], it seemed interesting to analyse their relation to mouse toxicity (Table IX). In serogroup O18 $K1^+Col^+$ group of strains belonged significantly more frequently to HV category ($p < 0.01$) than those in other serogroups. The same held true for $K1^-Col^-$ O18 ($p < 0.001$), or MRA^-K5^- O18 ($p < 0.001$) groups of isolates.

It has also been shown [17, 21–23] that $K1^+$ strains occurred mainly in serogroups O1, O2, O7 and this coexistence seemed to play an important role in the pathogenicity of *E. coli*. As seen in Table X, HV strains with $K1^+Col^+$ property occurred no significantly frequently (85%) in serogroups O1, O2, O7 as compared to those falling into other serogroups (87%; $p > 0.99$). Similarly there was no significant difference in the incidence in HV property of $K1^-Col^-$ strains belonging to O1, O2, O7 (14%) or other serogroups (12%; $p > 0.7$).

Table XI

Correlation between MRA, HLY markers, serogroup O4, O6 and incidence of E. coli strains in different LD_{50} groups

Markers	Serogroup	No. of strains in LD_{50} group			Total
		$\leq 10^6$	10^6–10^7	$\geq 10^7$	
MRA^+HLY^+	O4, O6	6	39	18	63
	Others	16	21	13	50
MRA^-HLY^-	O4, O6	2	3	2	7
	Others	76	82	262	420

Table XII

Correlation between HLY production, disease and incidence of E. coli strains in different LD_{50} groups

Diagnosis	Markers	No. of strains in LD_{50} group			Total
		$\leq 10^6$	10^6–10^7	$\geq 10^7$	
UTI*	HLY^+	2	9	6	17
	HLY^-	13	20	79	112
Sepsis	HLY^+	7	19	5	31
	HLY^-	15	17	24	56
Meningitis	HLY^+	1	2	0	3
	HLY^-	22	6	0	28

* Urinary tract infection

On the contrary, a significant difference was shown in the incidence of strains with $K1^+Col^+$ and $K1^-Col^-$ properties in HV category, independently of their serogroup distribution ($p < 0.001$).

In a previous study [16] a marked correlation was revealed also between MRA–HLY positivity and serogroups O4, O6. Analysing the correlation of these markers with the pathogenicity of strains for mice (Table XI) it was observed that those strains that possessed MRA^+HLY^+ properties and fell in serogroups other than O4, O6, belonged significantly more frequently (55%) to the HV category, than strains of serogroups O4, O6 (25%; $p < 0.05$). However, the difference in the incidence of strains belonging to serogroups, O4, O6 and those belonging to the other serogroups in the HV group of isolates was not statistically significant when they failed to have either MRA of HLY property ($p > 0.2$).

Several data indicated [4–6] that haemolysin production must be regarded as a virulence factor playing role in the pathogenesis of extraintestinal infections. Thus an appropriate analysis was made for showing the correlation between mouse toxicity and haemolysin producing capacity of strains considering the clinical diagnosis. Results are shown in Table XII. It was

revealed that strains isolated from patients with UTI and having HLY^+ property belonged significantly more frequently to HV group (25%) than strains not producing HLY (14%; $p < 0.001$). At the same time, in patients with meningitis, HLY^- strains fell more frequently into HV group than those with HLY^+ property.

Discussion

E. coli is a multifactorial pathogen causing disease in several body systems. There are different mechanisms of pathogenesis. The virulence of *E. coli* was examined as early as 1954 by Rowley [24]. He infected mice intraperitoneally with 14 h broth culture and estimated the LD_{50}. The most virulent strains were toxic for mice in dilution 1 : 100 (i.e. $< 10^6$ cells). Since his publication several other papers have given account of examinations of *E. coli* toxicity for mice, however, the methods differed from each other. Van den Bosch et al. [25] infected adult mice by intravenous injection and determined the viable counts in the mouse kidney. Later on, van den Bosch et al. [14] adapted several other procedures to show the different mechanisms of pathogenicity: lung toxicity assay, chicken embrio virulence test, and the urinary bladder injection method in suckling mice.

We applied intraperitoneal injection, and measured the LD_{50} for mice to understand better the role of different virulence markers. Our results concerning the high incidence of HV strains in extraintestinal specimens other than urine, or in patients with sepsis and meningitis corresponded to literary data [14, 21–23, 25] and supported our previous conception that there may be a parallelism between low LD_{50} and pathogenicity of strains associated mainly with extraintestinal diseases.

It has been known from previous studies [15] that *E. coli* serogroups O1, O2, O4, O6, O7, O18 and O75 are the commonest [26–28] and that an intact O antigen may be one of the virulence factors of the organism in UTI and also that O antigen determines the virulence of *E. coli* in meningitis [21, 29–32] as well as that strains belonging to serogroups O2, O6, O7, O18 cause bacteriaemia in rats [30] and other animal models [33]. In agreement with the data cited, we observed that strains of the most frequently occurring serogroups (especially O2 and O18) proved to be more pathogenic for mice than those belonging to the others.

Our result that isolates having different virulence factors (HLY, MRA, K1, K5, Col) are frequently virulent for mice and may also be frequently responsible for extraintestinal *E. coli* infections is also consistent with data of many other authors [6, 10, 14, 21, 32–36]. However, in contrast to data of Smith and Huggins [9, 37], who stated that all those strains that lacked the

Col V plasmid were much less virulent than the parent form, our survey revealed no significant difference in the incidence between HV strains producing colicin V and those that elaborated colicin other than V type. This result may be consistent with the role of certain colicins with iron uptake systems of bacteria [38, 40].

Although various candidate virulence markers have been discussed separately, the outcome of an *E. coli* infection depends not on one virulence factor acting alone but on a combination of these determinants acting together. The fact that MRA positivity and K1 positivity correlates, has been shown in a previous work [17]. While analysing how did these simultaneously occurring virulence markers influence the toxicity of an isolate in mice, it was revealed that only possession of K1 played an important role in pathogenicity. The same was demonstrated when the effect on virulence of the simultaneous occurrence of K1 and Col was examined. Namely, the incidence of strains having either K1 alone or K1 with MRA or Col and belonging to the HV group, was significantly higher than those that possessed only MRA or only Col positivity.

In a previous work [17] the frequent coexistence of K1 and Col in serogroups O1, O2, O7 and O18 originating mainly from extraintestinal sources offered an explanation for the extrainstestinal pathogenicity of these serogroups. Correlation of these phenomena with the high pathogenicity of strains described in this paper supports our previous conception, and is at the same time in agreement with the data of Smith and Huggins [37].

As to correlation between HV of strains and MRA–Col properties, it was found that isolates which possessed both virulence markers were more pathogenic in LD_{50} assay than those that had either MRA or Col positivity alone. Similarly, our survey also revealed that MRA^+HLY^+ strains were more virulent for mice than those that failed to have either MRA or HLY. The value of LD_{50} was independent of the presence of MRA, Col or HLY. It must be emphasized, however, that HV strains lacking the above-mentioned properties were isolated frequently from extraintestinal sources.

On the basis of results of the present study and also consistent with data of other authors a close association has been demonstrated between markers of pathogenicity and mouse toxicity of strains. However, in our previous work [16, 17] it was also discovered that bacteria can harbour different virulence factors simultaneously. On the basis of present analysis it must be emphasized that the virulence markers can be arranged in an order of importance. Antigen K1 should be considered as the most important property. All the other markers (MRA, HLY, Col) can strengthen the effect of each other. However, it remains to be answered whether these markers cover other, as yet undefined factors that are more directly responsible for distinct pathomechanisms of the diseases.

Strains belonging to serogroups O4 and O6 occur frequently in UTI. Besides, there is a close association between MRA, HLY possession and these serogroups [16]. The observation that MRA^+HLY^+ strains with HV occurred more frequently in serogroups other than O4 and O6, suggests that MRA and HLY properties play an important role also in the pathomechanism of extra-intestinal infections other than UTI.

As early as 1921 Dudgeon et al. [41] suggested that haemolytic activity might play a part in the virulence of *E. coli* in the human urinary tract. Similar observations were described by Minshew et al. [5] and van den Bosch et al. [14]. In agreement with their data. this study also indicates a close association among virulence for mice, HLY positivity and urinary origin of a strain. In their paper van den Bosch et al. [13] stated that HLY property did not seem to be an important virulence factor for the more virulent group III (i.e. strains causing extraintestinal diseases, mainly sepsis). In contrast to their observations our survey revealed that a high proportion of HLY^+ *E. coli* strains isolated from septic infections belonged to the HV group. However, strains isolated from patients with meningitis and belonging to the HV group were not frequently haemolytic. Therefore their conclusion ought to be completed with the statement that HLY property is not an important virulence factor for strains that cause meningitis, but it plays an important role in pathogenicity of strains that cause septic infections.

For the better understanding the mechanism by which *E. coli* causes extraintestinal infections, it must be taken into consideration that the pathogenesis of diseases may be multifactorial. Classifying diseases of man due to *E. coli*, in his paper Sussman [10] distinguished several different clinical forms: urinary tract infection, meningitis of newborn, septicaemia in the absence of meningitis, wound infection and haemolytic uraemic syndrome. In accordance with this classification one can also classify *E. coli* with different virulence factors into different pathogenetical groups. It seems worth-while to emphasize two distinct groups of diseases in this respect: meningitis in the newborn and septicaemia with different organotropism in adults.

On the basis of our present study and also of observations of several authors [2, 4, 7, 14, 17, 21, 42] it can be stated that in meningitis of "young" newborn (i.e. within one month of life) certain serogroups and K1 antigen may be characteristic of the isolates. The presence of S fimbriae [42] is a question to be answered. In this respect our previous finding [17] that 34 out of 36 O18ac, $K1^+MRA^-$ strains harboured fimbrial structures, and also our present observations that 18 out of 19 O18ac: K1 strains originating from CSF were isolated from newborns under one month of age, are remarkable. Besides, the observation [42] that both the neonatal brain and the K1 antigen are consisted of a sialic acid polymer which is very similar to S fimbrial receptors strongly suggests that in the pathomechanism of meningitis of young newborns,

both the S fimbria and K1 antigen play a significant role. In agreement with data of Korhonen et al. [42] 4 out of our 5 O7: K1 strains originated from CSF of newborns with fatal meningitis under one weak of age.

The close correlation between HV of strains and presence of K1 antigen, further on between serogroup O7 and O18 and HV, and between meningitis and HV, as well as the lack of correlation between MRA^+K1^-, MRA^+Col^- properties and the HV of the strains, beside, the high incidence in HV group of MRA^-HLY^- strains of extraintestinal origin, all support that LD_{50} in mice well reflects all those properties needed for causing meningitis in newborns, that is, serogroups O7, or O18ac, possession of K1 and lack of MRA and HLY capacity. At the same time, this correlation may indicate that clones exist with certain special properties and also that, association of these well characterized but in details not well known properties, in these clones play an important role in newborn meningitis.

Strains belonging to serogroup O78 without harbouring K1 antigen but frequently producing Colicin V [11, 12, 43, 44] isolated from also young newborns with fatal meningitis further complicates the question of pathomechanism.

The second large group that is remarkable in this respect, is the group of septicaemia associated with wound infection, abscess etc. In agreement with data of Korhonen et al. [42] on patients with pneumonia, we observed that strains isolated from blood of septicaemic patients belonged frequently to serogroups O4 and O6 and possessed MRA and HLY properties. Furthermore, in addition to patients with pneumonia, we had patients who developed septicaemia associated with renal abscess, gall bladder inflammation, endocarditis lenta, gluteal abscess or other phlegmonous processes of body cavities. In this respect, strains causing meningitis in older children or adults belonged also to this category. Isolates of this "sepsis and inflammation causing" group were very similar in characteristic to those that cause UTI: MRA positivity (i.e. presence of P fimbria), production of alpha haemolysin, possession of antigen K5 in serogroup O18. Unlike strains of "newborn meningitis" they occurred less unanimously in the HV group. Anyway, simultaneous occurrence of MRA^+HLY^+ properties, especially together with Col positivity is associated not only with an increased frequency of these strains in HV group, but also with the outcome of the disease they cause.

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A COMPARISON OF CEFONICID WITH OTHER BETA-LACTAMS REGARDING THE EFFECT OF HUMAN AND MOUSE SERA ON ANTIBACTERIAL ACTIVITY

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The effect of the addition of 50% human or mouse serum on the antibacterial activity of cefonicid, three first generation cephalosporins and ampicillin was studied. Human serum added to the test system considerably reduced the activity of cefonicid against *Staphylococcus aureus* and *Staphylococcus epidermidis* strains, and to a lesser degree against *Proteus mirabilis*, *Escherichia coli* and *Klebsiella pneumoniae* strains. Human serum also reduced, albeit to a lesser extent, the activities of cephalothin, cefazolin, and ampicillin, whereas it increased the activity of cephaloridine. In contrast, mouse serum did not or only insignificantly reduced the activity of cefonicid against some of the bacterial isolates; this is reflected in the excellent protective effect of cefonicid in experimental mouse infections.

Cefonicid (Monocid®, SK&F 75073, Fig. 1) is a second generation parenteral cephalosporin with a prolonged serum half-life of approximately four hours [1]. It is highly bound to serum proteins (about 98%) compared with cefazolin (86%). High serum protein binding usually affects the pharmacokinetics of drugs, resulting in high and prolonged serum levels and probably influences the bioavailablity of the free unbound portion of the drug with possible altered therapeutic potential. The purpose of this study was to provide experimental data to the above considerations.

Materials and methods

All the cephalosporins and ampicillin employed in these experiments were commercial preparations. Their aqueous solutions were sterilized by Millipore-filtration and added in doubling dilutions (0.1 to 200 μg/ml) to Mueller–Hinton broth with and without serum. The semi-automated microdilution-technique was used for the minimal inhibitory concentration (MIC) determinations. The test media were buffered to pH 7 by McIlvaine's citric acid–phosphate buffer [2] to insure a higher degree of uniformity in the assay. MICs were determined after overnight (about 18 h) incubation at 37 °C and recorded as the lowest concentrations of compounds (μg/ml) producing no visible growth.

The human and mouse sera were pooled batches and were heat inactivated at 58 °C for 30 min prior to use. These sera produced no inhibitory activity on the growth of the bacterial strains.

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The bacterial strains employed in this study were the same strains regularly used in our laboratories and published earlier [1, 3, 4]. The inocula for the MIC determinations were prepared by diluting broth cultures in the logarithmic growth phase to obtain the colony forming unit (c.f.u.) per ml as specified in each Table. The mouse protective effect of cefonicid (against various infections) expressed as ED_{50} in mg/kg was established with the conventional method [1, 4, 5].

Results and discussion

Table I presents the MIC data for cefonicid and cefazolin against selected Gram-positive and Gram-negative bacteria in Mueller–Hinton broth with and without 50% mouse or human serum. When mouse serum was added to the test system, the MIC values for both cefonicid and cefazolin usually did not change or increased with one dilution factor. However, in the presence of human serum the activity of cefonicid decreased eight to ten fold whereas cefazolin showed a twofold loss of activity. Cefazolin, in fact, has a greater activity than

Table I

In vitro activity of cefonicid and cefazolin against selected bacterial isolates in Mueller–Hinton broth without and with mouse or human serum

Bacterial strains[a]	Compound	MIC (μg/ml)		
		Mueller–Hinton broth	50% Mouse serum	50% Human serum
Staphylococcus aureus ATCC 25923	Cefonicid	1.6	3.1	25
	Cefazolin	≤0.1	0.2	0.4
Staphylococcus aureus 127 (Penicillin G resistant)	Cefonicid	3.1–6.3	3.1	50
	Cefazolin	0.2–0.4	0.2–0.4	0.4–0.8
Staphylococcus aureus 674 (Tour)	Cefonicid	0.8–1.6	1.6	25
	Cefazolin	≤0.1	≤0.1	0.2–0.4
Staphylococcus aureus 872 (Penicillin G sensitive)	Cefonicid	6.3	6.3	6.3
	Cefazolin	0.4	0.4	1.6
Staphylococcus aureus 873 (Penicillin G resistant)	Cefonicid	0.4	1.6	12.5
	Cefazolin	≤0.1	≤0.1	0.2
Escherichia coli 12140	Cefonicid	0.4	0.2–0.4	3.1
	Cefazolin	0.4–0.8	3.1	3.1–6.3
Escherichia coli 33779	Cefonicid	0.8–1.6	1.6	12.5–25
	Cefazolin	0.8–1.6	3.1	6.3
Proteus mirabilis 416	Cefonicid	≤0.1	0.2	3.1
	Cefazolin	3.1	6.3	12.5
Proteus mirabilis 442	Cefonicid	≤0.1	0.8	3.1
	Cefazolin	1.6	3.1	12.5
Klebsiella pneumoniae 4200	Cefonicid	≤0.1	≤0.1	1.6
	Cefazolin	0.8	0.8–1.6	3.1–6.3

[a] Inocula were for *S. aureus*, *E. coli*, *P. mirabilis* and *Klebsiella* sp. approximately 6×10^5, 3.4×10^5, 5×10^5 and 1.2×10^5 c.f.u./ml, respectively

cefonicid against *S. aureus* strains. The two cephalosporins have the same order of activity against *E. coli* strains, but cefonicid is more active against *P. mirabilis* and *K. pneumoniae* strains.

In another series of experiments, the influence of 50% human serum added to Mueller–Hinton broth was studied on the activity of cefonicid in

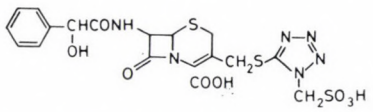

Fig. 1. Chemical structure of cefonicid

comparison with cephaloridine, cefazolin, cephalothin and ampicillin against multiple strains of *P. mirabilis*, the species most susceptible to cefonicid, as well as against *S. aureus* and *S. epidermidis*, the least susceptible species to cefonicid. In the anti-staphylococcus study ampicillin was not included.

Table II shows the MIC values of cefonicid, cephaloridine, cefazolin, cephalothin and ampicillin in Mueller–Hinton broth alone and with the addition of 50% pooled human serum against 24 *P. mirabilis* strains of which four are beta-lactamase producers. In Mueller–Hinton broth cefonicid has the greatest activity. Its mean MIC is 1.1 μg/ml with a range of 0.1 to 6.3 μg/ml. Only the beta-lactamase producing strains required concentrations of cefonicid as 3.1 or 6.3 μg/ml for inhibition. Ampicillin was not as active as cefonicid; its mean MIC value was 2.1 μg/ml, with a range of 0.8 to 2.5 μg/ml.

Table II

Effect of 50% human serum in Mueller–Hinton broth on the in vitro activity of cefonicid, cephaloridine, cefazolin, cephalothin and ampicillin against 25 P. mirabilis isolates[a, b]

Beta-lactam antibiotic	Assay medium	MIC range	Mean MIC
		μg/ml	
Cefonicid	Meller–Hinton broth	0.1–6.3	1.1
	50% human serum	1.6–50	7.0
Cephaloridine	Mueller–Hinton broth	6.3–>200	30.5
	50% human serum	6.3–>200	23.4
Cefazolin	Mueller–Hinton broth	12.5–>200	30.7
	50% human serum	25 –>200	53.1
Cephalothin	Mueller–Hinton broth	12.5–100	28.9
	50% human serum	12.5–100	36.5
Ampicillin	Mueller–Hinton broth	0.8–25	2.1
	50% human serum	0.8–>100	6.5

[a] Includes 1 strong and 3 weak beta-lactamase producing strains, and 20 β-lactamase negative strains

[b] Inocula ranged from 1×10^6 to 2.4×10^5 c.f.u./ml

Table III

Effect of 50% human serum in Mueller–Hinton broth on the in vitro activity of cefonicid, cephaloridine, cefazolin and cephalothin against 24 S. aureus and S. epidermidis isolates[a,b]

Cephalosporin	Assay medium	MIC range	Mean MIC
		μg/ml	
Cefonicid	Mueller–Hinton broth	1.6–12.5	5.7
	50% human serum	12.5–50	44.3
Cephaloridine	Mueller–Hinton broth	≤0.05–1.6	0.38
	50% human serum	≤0.05–0.8	0.11
Cefazolin	Mueller–Hinton broth	0.1–1.6	0.55
	50% human serum	0.4–3.1	1.27
Cephalothin	Mueller–Hinton broth	0.1–0.8	0.49
	50% human serum	0.8–1.6	1.00

[a] Twelve strains are beta-lactamase producer and the other 12 strains are beta-lactamase negative; none is methicillin resistant

[b] Inocula were 5×10^5 c.f.u./ml

The three control cephalosporins were much less active. Very few strains were inhibited by 6.3 μg/ml and the majority of strains were inhibited by 12.5 to 50 μg/ml. The beta-lactamase-producing strains were susceptible to 100 μg/m of cephalothin, but they were not inhibited even by 200 μg/ml of cephaloridine or cefazolin.

In the presence of human serum cefonicid's MIC increased about eight-fold, but these serum levels are obtainable in mouse and in man as well. Ampicillin's MICs are much less influenced by the presence of human serum. The generally poor MICs of cefazolin and cephalothin do not change significantly in the presence of human serum, while the MIC of cephaloridine appears to improve against certain isolates.

The effect of 50% human serum on the MIC values of the cephalosporins mentioned above against 24 strains of *S. aureus* and *S. epidermidis* are presented in Table III. Half of the strains produce beta-lactamase and the other half do not. None of the strains was known to be methicillin resistant. The data of the Table show that the in vitro activity of cefonicid was significantly influenced when 50% human serum was added to the Mueller–Hinton broth. The mean MIC in Mueller–Hinton broth was found to be 5.7 μg/ml with a range of 1.6 to 12.5 μg/ml, which increased in the presence of human serum to 44.3 μg/ml with a range distribution of 12.5 to 50 μg/ml, about an eight-fold loss in activity. Recently Barry et al. [6] also found that the addition of 50% human serum to Mueller–Hinton broth significantly reduced (about eight-fold) the bacteriostatic activity and almost eliminated the bactericidal activity of cefonicid in a test using 52 *S. aureus* isolates. Cefonicid was less active against beta-lactamase-producing than against beta-lactamase-negative strains [6].

The activity of cephaloridine, a very potent anti-staphylococcal agent (mean MIC 0.38 μg/ml; range ≤0.05 to 1.6 μg/ml) was increased (mean MIC 0.11 μg/ml; range ≤0.05 to 0.8 μg/ml) by the presence of 50% human serum in the test system. Its activity was also somewhat increased by human serum against *P. mirabilis* strains (Table II).

Cephalothin and cefazolin are potent anti-staphylococcal agents with relative stability to the chromosomal beta-lactamases. In Mueller–Hinton broth their mean MICs against these 24 isolates were found to be 0.49 μg/ml and 0.55 μg/ml, respectively; their MICs increased in the presence of 50% human serum to 1.0 and 1.27 μg/ml, respectively. This reduction in activity is insignificant in terms of the therapeutic consequences.

Table IV summarizes the in vitro activity (MICs in μg/ml) of cefonicid in Mueller–Hinton broth with and without 50% human or mouse serum, as well as its protective effect (ED_{50} in mg/kg) in experimental infections of mice with staphylococcal and Gram-negative bacterial strains regularly used in our laboratory for the chemotherapeutic evaluation of beta-lactam antibiotics. The data of the Table show that cefonicid is very active in vitro against the Gram-negative bacteria in Mueller–Hinton broth with MICs below 0.1 μg/ml, with the rare exception of the strong plasmid-mediated beta-lactamase producing strains of *E. coli* as found in other studies. It is, however, less active against strains of *S. aureus* and *S. epidermidis*. Human serum (50%) considerably reduced its activity against staphylococci and also against the Gram-negative bacilli. Since cefonicid, after therapeutic parenteral doses, produces high and prolonged serum levels, the MIC values obtained even with the addition of 50% human serum are easily achievable in the blood and most tissues.

The addition of 50% mouse serum to Mueller–Hinton broth does not significantly reduce the MIC values. In line with this finding, in infected mice,

Table IV

In vitro activity and in vivo effect of cefonicid against bacterial strains used in mouse infection — protection study

Assay[a]	MIC (μg/ml)						
	S. aureus 127	*S. aureus* 674 (Tour)	*E. coli* 12140	*E. coli* 33779	*P. mirabilis* 416	*P. mirabilis* 442	*K. pneumoniae* 4200
Mueller–Hinton broth	3.1–6.3	0.8–1.6	0.4	0.8–1.6	≤0.1	≤0.1	≤0.1
50% Human serum	50	25	3.1	12.5–25	3.1	3.1	1.6
50% Mouse serum	3.1	1.6	0.2–0.4	1.6	0.2	0.8	≤0.1
Mouse protection[b] ED_{50} (mg/kg)	11	8.7	1.6	2.1	0.3	0.24	0.55

[a] Inocula were the same as in Table I

[b] Mice were challenged intraperitoneally with a predetermined lethal suspension of bacterial cells in 5% hog gastric mucin and treated subcutaneously 1 and 5 h after infection

cefonicid produced excellent protective effects, with low ED_{50} values of about 2 mg/kg in *E. coli*; 0.3 mg/kg in *P. mirabilis*; 0.5 mg/kg in *K. pneumoniae* but only 10 mg/kg in *S. aureus* infections. Thus, the in vitro data (MIC values) can directly predict the therapeutic efficacy of cefonicid. Why human serum decreases the in vitro activity of cefonicid and mouse serum does not, is presently unanswered.

The meaning and significance of the decrease of cefonicid's activity in vitro when the culture broth containing 50% human serum, is not easy to answer. Cefonicid is highly bound to serum proteins but still diffuses to tissues and is effective in the treatment of soft-tissue infections [7] as well as osteomyelitis due to staphylococci, even when injected once-a-day for a prolonged period of time [8]. The effectiveness of cefonicid in the treatment of staphylococcal endocarditis has not been proven [9]. This hight be attributable to the location of the infection, where cefonicid cannot easily penetrate. Cefonicid seems not to be tightly bound to serum proteins, but rather it can be considered to be loosely bound to serum proteins allowing easy dissociation to yield free, unbound cefonicid. In this respect, cefonicid bound to serum proteins appears to represent rather a circulating reservoir for the antibiotic. It is unlikely that human serum proteins but not mouse serum proteins would influence the attachment of cefonicid to the penicillin binding proteins of the bacterial cell.

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A NOVEL APPROACH FOR UNDERSTANDING THE EFFECTS OF INTERFERONS — A WORKING HYPOTHESIS

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Experimental results accumulated during the last two decades prove that there are no mechanisms specific for interferon (IFN) action. Thus, IFN effects must be realized through the relatively limited number of metabolic pathways available for cell functions. Triggering the receptor system in association with membrane phospholipid breakdown, which plays an important role in signal transduction for a variety of biologically active substances, is suitable to explain generation of the pleiotropic IFN effects, too. Prevalence of one or the other of the "positive" and "negative" pathways of this signal transducing system depending on various known and up-to-now unknown factors may be responsible for the sometimes contradictory effects of IFNs.

Introduction

IFNs are a family of closely related proteins, which induce a wide-range of effects including establishment of an antiviral state, inhibition of cell proliferation, modulation of the cell surface and influence on the immune system [1].

The first step in IFN action is its interaction with specific receptors. These receptors trigger the induction of expression of different genes, the products of which mediate their various actions. It is not known, however, what mechanisms are taking place after interaction of IFN with its receptor responsible for induction of transcription. Thus, the following question arises (quoted from Sen [2]):

"Is there a 'second messenger' which amplifies and transmits the signal from the plasma membrane to the nucleus? Do all effects of IFN depend on this signal transmission or are some effects such as those on the plasma membrane direct effects?"

Here we focused our attention on these questions, taking also into consideration that there are several observations suggesting that IFN-dependent enzymes are involved in other regulatory systems as well [3–10].

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Surface action of IFN

In common with peptide hormones and neurotransmitters, the initial step in IFN action is its interaction with cell membrane components. These components are receptor proteins of the cell surface with a high affinity to IFN molecules [11–19].

It has been reported that two distinct classes of receptors exist for alpha, beta and gamma IFNs [20].

The receptors appear to be at least in part gangliosides with an oligosaccharide moiety at the functional binding site [21–23].

Evidence suggests that both glycoprotein and ganglioside components are important in the IFN binding site [24]. Very little is known, however, concerning the transfer of information from the receptor to the cell nucleus. The answer to the question whether IFN or its derivatives are implicated, is likewise not known.

Internalization and degradation of receptor-bound IFN has been reported [14, 19] and it was suggested [17] that following binding, IFN is internalized via the known pathway of receptor mediated endocytosis. It is important to note that not all cells behave similarly. L 1210 cells do not show any internalization and degradation of murine IFN alpha and beta [12].

Partially purified IFN microinjected directly into the cytoplasm or nucleus of cells failed to induce an antiviral state [25, 26]. In addition, the role of internalized and degraded IFN can be queried by experiments which revealed that methylamine — an inhibitor of internalization [14] — had no effect on the antiviral action of IFN [27] and chloroquine — an inhibitor of degradation [14, 17] — did not inhibit the induction of (2'–5')-oligoadenylate synthetase [14].

Surface action of IFN was also suggested by studies with IFN covalently coupled to sepharose beads [28]. IFN applied this way retained its antiviral activity.

Thus, most of the experimental data indicate that IFN acts through the cell surface, however, it can not be excluded that internalization of IFN may be also required for its biological activity [29]. For the sake of simplicity we accept that IFN acts primarily on the cell surface. In this case, after triggering of IFN receptors, second messenger system(s) should be involved in its actions. This theory gains also support from the fact that it is possible to transfer IFN-induced antiproliferative and antiviral activity from IFN-treated cells to untreated ones. Accordingly, it was hypothesized that secondary messenger molecules are transferred from the IFN-induced cells to the recipient ones which then express the antiviral and antiproliferative activity [30, 31].

Most tissues possess only two major types of receptors. One class triggers the production of cyclic AMP (cAMP), while the other initiates a signal cascade

reaction resulting in mobilization of calcium, activation of protein kinase C, release of arachidonic acid (for the synthesis of prostaglandins, thromboxanes and leukotrienes) and stimulation of guanilate cyclase to form cyclic GMP (cGMP) [32, 33]. Both of these receptor-controlled systems are obvious candidates for the transmission of an IFN signal. Although cAMP is known to influence a number of processes in association with IFN [34], this system does not appear to be responsible for all IFN effects. Thus, we focused our attention to the signal cascade system mentioned above.

Inositol trisphosphate and diacylglycerol second messengers. A number of extracellular signals which activate cellular functions through interactions with cell surface receptors have been reported to induce the breakdown of inositol phospholipids [33, 35, 36]. As it was shown recently, phosphatidylinositol 4,5-bisphosphate is cleaved following receptor stimulation by phospholipase C resulting in inositol 1,4,5-trisphosphate and diacylglycerol. Both inositol 1,4,5-trisphosphate and diacylglycerol appear to function as second messengers. Inositol 1,4,5-trisphosphate changes the interacellular level of calcium which is an important step in initiating cell proliferation [37]. In another line of the signal pathway protein kinase C activated by diacylglycerol plays a crucial role [33]. Tumour-promoting phorbol esters which have diacylglycerol-like structures are also able to stimulate this enzyme. One consequence of this stimulation is activation of a Na^+/H^+ carrier which exchanges protons for sodium ions [38]. Due to activation of this carrier the interacellular pH and sodium ion concentration rapidly increase. Such an increase of pH is a characteristic feature of the action of growth factors having an important role in stimulating of cell growth [39, 40]. Thus, the enzymatic reactions based on inositol lipid turnover represent a bifurcating signal pathway, activation of which results in stimulation of cell growth. This pathway represents positive signals, which in some tissues can be blocked by negative signals activating adenylate cyclase [33].

Diacylglycerol is only transiently produced in the membrane and is removed either by conversion to phosphatidic acid by a diacylglycerol kinase or by hydrolysis to monoacylglycerol by a diacylglycerol lipase [36]. This latter reaction results in the release of arachidonic acid which might be transformed to prostaglandins by cyclooxygenase and/or to leukotrienes by lypolygenase [41]. It is more likely that arachidomic acid peroxide and prostaglandin endoperoxide activate guanylate cyclase [42].

The role of cGMP as well as the mechanism by which cGMP is elevated is not clearly understood. It was suggested that cGMP serves as a negative enteracellular messenger [33, 43–45], accordingly cAMP and cGMP do not antagonize each other. Moreover, it is well known that prostaglandin E stimulates adenylate cyclase and thus increases the level of cAMP in fibroblasts [46, 47].

Possible relationships of inositol trisphosphate and diacylglycerol second messengers with IFN action.

Plasma membranes have been implicated as possible targets for many actions of IFN [1, 48, 49]. Time-dependent IFN-induced structural changes in the plasma membrane lipid bilayer were shown in cultured cells using spin label electron spin resonance techniques [50]. Treatment of human HeLa-S_3 cells in suspension culture with human β-IFN for 30 min caused an increase in the rigidity of the plasma membrane lipid bilayer. The plasma membrane rigidity of IFN-treated cells returned to the level of control cells within 3 to 5 h, but 24 h after beginning of treatment, the rigidity of the plasma membrane was increased again and remained so for at least 2 days. This IFN-induced perturbation of membrane structure proved to be species-specific. An other evidence obtained in S-180 mouse sarcoma cells treated with mouse β-IFN explains this increased membrane rigidity [51]. The composition of cellular phospholipids was altered as a result of treatment with mouse β-IFN. The unsaturated fatty acid content of all of the major phospholipids decreased, resulting in an increase in the relative proportions of the saturated acyl side chains. Fatty acyl side chains of membrane phospholipids are the principal structural components regulating membrane transitions. It must be stressed that arachidonate was consistently reduced in all phospholipid classes after IFN treatment. These changes were prevented by anti-interferon antibody and they were not observed after treatment with human IFN.

An increased membrane phospholipid hydrolysis involved in IFN action was described by Wallach and Revel, too [52]. Corticosteroids, prostaglandin E_1, cAMP and calf serum abrogated this effect, whereas aspirin and indomethacin increased the cell damage amplified by IFN. As corticosteroids prevent membrane lipid hydrolysis [53] and aspirin and indomethacin inhibit conversion of arachidonic acid to prostaglandins and thus prevent elevation of the level of cellular cAMP, it has been supposed that changes in membrane lipid catabolism could have a role in the phenomenon observed.

Figures 1, 2 and 3 show the known pathways associated with inositol phospholipid breakdown. Three basic ways can be distinguished; two of these may be considered as positive ones (Figs 2, 3) as they result in DNA synthesis and cell proliferation and the third way as a negative one (Fig. 1) with a consecutive inhibition of nucleic acid and protein synthesis.

IFNs are primarily known as "negative" signals inducing an antiviral state and antiproliferative effect. We discuss first, therefore, the pathway associated with these phenomena (Fig. 1). This is not a heavy task, since all of the details are summarized in Tovey's excellent review [34]. We give, therefore, only a short outline to show that IFN acts really through this pathway.

It has been shown that IFNs induce a very rapid and marked increase in the intracellular concentration of cGMP in mouse leukaemia L 1210 cells

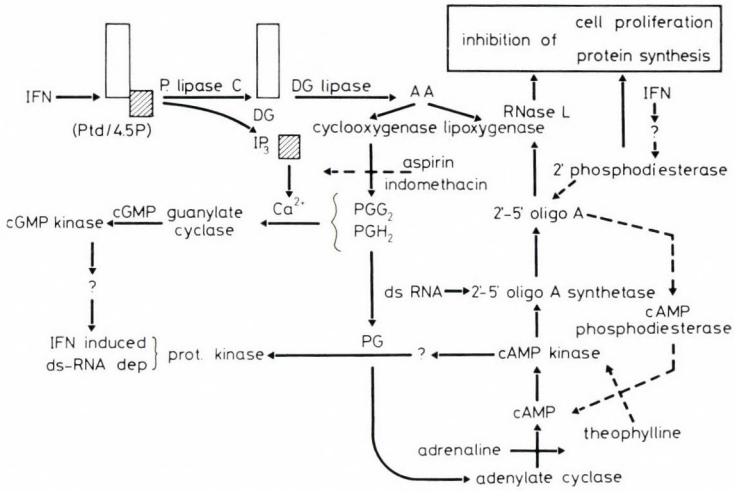

Fig. 1. A possible mechanism by which IFN induced antiviral and antiproliferative effects may be elicited. (PtdI 4,5 P) = phosphatidylinositol 4,5-bisphosphate; P. lipase C = phospholipase C; DG = diacylglycerol; AA = arachidonic acid; IP_3 = inositol, 1,4,5-trisphosphate; PG, PGG_2 and PGH_2 = prostaglandins; (2'–5')-oligo A = (2'–5')-oligoadenylate. The dashed lines represent the inhibitory aspects of the pathways

[54]. The accumulation of cGMP was transitory and as aspirin and indomethacin abrogated cGMP increase, this might be due to arachidonic acid release and prostaglandin synthesis. Induction of cGMP synthesis in IFN-treated L 1210 cells proved to be dependent on intracellular calcium.

cGMP production due to IFN-treatment appears to be an important factor in establishing the antiviral state of cells, as in IFN-resistant Daudi cells no increase of cGMP level, but an unchanged ability to induce (2'–5')-oligoadenylate synthetase and (2'–5')-phosphodiesterase was observed [55].

Inhibition of the establishment of IFN-mediated antiviral state by inhibitors of cyclooxigenase also indicates the role of cGMP, as this enzyme activates guanilate cyclase through prostaglandins [56]. The relationship between stimulation of prostaglandin formation and IFN action was confirmed also by other authors [57, 58].

A number of data indicate that cAMP which is known to be regulated by prostaglandins [59] is also associated with the antiviral and antiproliferative activity of IFN [34].

As no strict correlation was found between the capacity of IFN to induce cAMP and its antiviral and/or antiproliferative activity, Tovey came to the conclusion that changes in the intracellular concentration of cAMP is a consequence rather then cause of the IFN-mediated effects.

By administering adrenaline — an inactivator of adenylate cyclase — or teophylline — an inhibitor of cAMP phosphodiesterase — an increase of intra-

cellular cAMP levels with a consecutive increase of (2'–5')-oligoadenylate synthetase activity was described [9, 10].

These observations seem to be in contrast with Tovey's conclusion. In addition, they indicate — together with other data — [7, 8] that (2'–5')-oligoadenylate is not a unique, IFN-specific substance but one of the agents controlling the rate of cell division. Obviously, the cAMP-(2'–5')-oligoadenylate synthetase relationships are regulated, since (2'–5')-oligoadenylate synthetised in the cell by the action of IFN activates cAMP-phosphodiesterase, too [60]. This effect of (2'–5')-oligoadenylate represents a negative feedback mechanism in the regulation of the cAMP-(2'–5')-oligoadenylate system.

All these data are in line with the conception that this pathway of the inositol phospholipid breakdown dependent receptor system is at least partly responsible for some IFN effects.

Much less data have accumulated referring to relationships between other pathways of the signal cascade reaction and IFN action. It has been shown that IFNs release calcium from the intracellular membranes and thus free calcium ions become available to induce different mechanisms (Fig. 2) [24].

Effects of calcium are often mediated by the calcium-binding protein calmodulin. Following calmodulin activation several enzymes are activated, i.e. adenylate cyclase and cyclic nucleotide phosphodiesterase [61]. Calmodulin acts also directly on the regulatory subunit of cAMP-dependent protein kinase [62].

Arachidonic acid release is an other mechanism where calcium plays an important role, as both diglyceride lipase and phospholipase A_2 are stimulated by calcium. Phospholipase A_2 is a non-specific phospholipase cleaving arachidonate from phosphatidylinositol, phosphatidylcholine, and phosphatidyl-

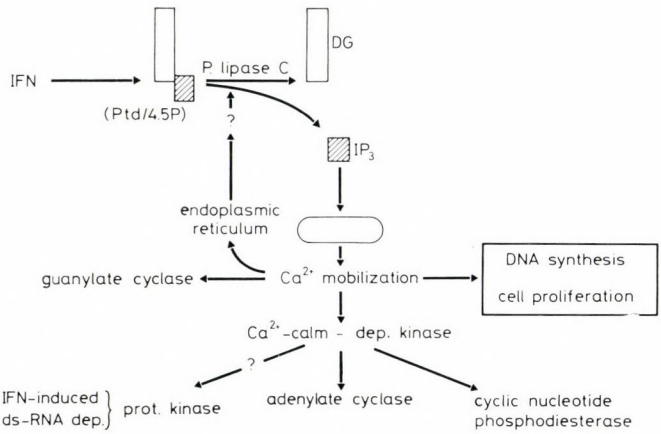

Fig. 2. A general scheme for the mobilization of membrane bound Ca^{++}. Ca^{++}-calm-dep. kinase = Ca^{++} calmodulin dependent protein kinase. Other abbreviations as in Fig. 1

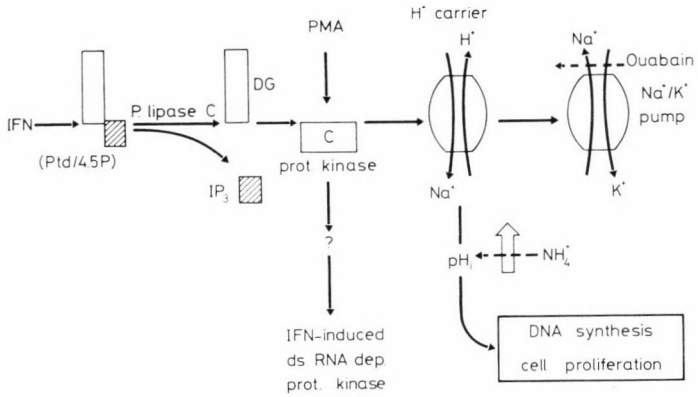

Fig. 3. Showing induction and function of protein kinase C. PMA = phorbol myristate acetate; pHi = interacellular pH. Other abbreviations as in Fig. 1

ethanolamine, thus enhancing the production of arachidonic acid [63]. These examples support the hypothesis that in IFN induced cells inositol trisphosphate is involved as a second messenger regulating the release of membrance-bound calcium.

In an other line of the signal pathway protein kinase C is the key enzyme (Fig. 3). This is a calcium-activated, phospholipid-dependent protein kinase [64], which is activated by diacylglycerol produced in the membrane during the signal induced turnover of inositol phospholipid. Tumour-promoting phorbol esters directly activate this enzyme, which probably serves as a receptor for phorbol esters [33]. We have predicted that if IFN utilises the inositol phospholipid-diacylglycerol protein kinase C pathway, then phorbol myristate acetate should have an effect on cells similar to that of IFN. Studying the cytopathic effect of vesicular stomatitis virus on UAC cells we could demonstrate that phorbol myristate acetate exhibits an antiviral effect synergistic with the antiviral effect of human IFN [65].

There is an other observation supporting the idea that protein kinase C may be involved in the regulatory action of IFN. It was shown that murine peritoneal macrophages treated with recombinant IFN-γ exhibited a substantial increase in protein kinase C activity. This activity was enhanced as much as 5-fold over that seen in untreated macrophages [67].

In addition, many other results suggest the IFN-like properties of phorbol ester. Phorbol myristate acetate and the biologically active phorbol esters have a variety of effects — including stimulation of synthesis of macromolecules, modulation of the metabolism of polyamines and cyclic nucleotides, stimulation of prostaglandin synthesis — similar to those of IFNs [67]. It is worth mentioning that tumour-promoting phorbol esters have been reported

to act as enhancers or inhibitors of IFN induction reminding to the priming and blocking effects of IFN [68, 69]. Immunmodulatory effects of phorbol esters are also known [70, 72]. IFN and phorbol esters enhance the cytotoxic activity of human blood lymphocytes and modulate natural killer (NK) activity [73, 74]. In addition, phorbol esters and IFN may induce discrete and early changes of the cytoskeleton structure [75, 76].

Tumour-promoting phorbol esters and synthetic diacylglycerol — potent activators of protein kinase C — mimic the action of mitogens elevating the intracellular pH; this intracellular pH shift is mediated by a Na^+/H^+ carrier and may serve as a common signal in the action of hormones which elicit the breakdown of inositol phospholipids [38]. In this respect it is important that IFN also elevates the interacellular pH by inducing proton efflux and induces an increased rate of sodium-dependent glycine uptake [24]. Thus, it is not surprising that ammonium chloride prevents the development of antiviral state in IFN-treated cells or abrogates it when already established [77], as NH^+ influx causes acid loading of the cells [78]. After removal of NH_4Cl the antiviral state became reestablished. It can be thus hypothesized that NH_4Cl inhibits development of the IFN-induced antiviral state by its effect on intracellular pH.

Ouabain — specific inhibitor of Na^+/K^+ transport — also inhibits establishment of the IFN-induced antiviral state [79]. This is in accordance

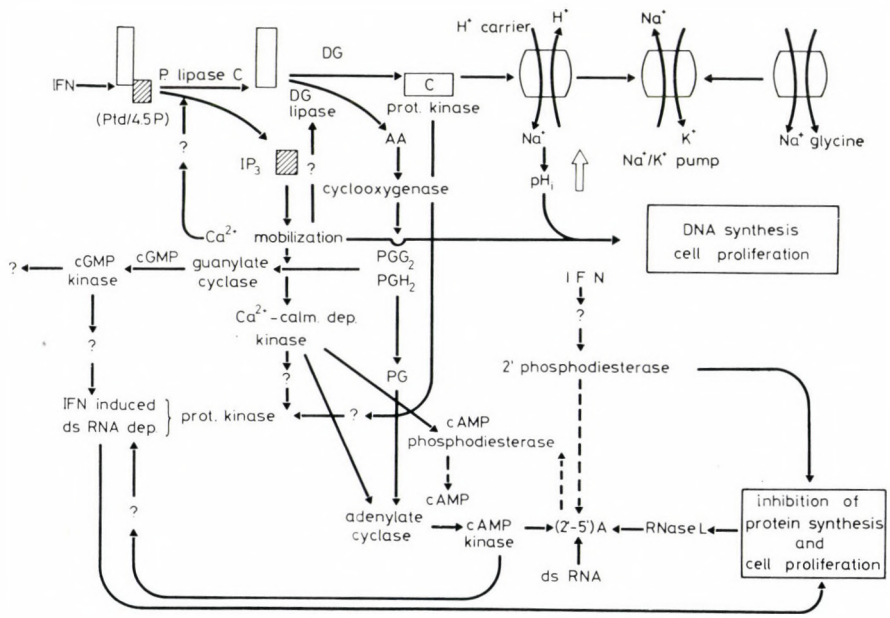

Fig. 4. A possible role of phosphatidylinositol degradation in IFN induced transmembrane processes. (2'–5') A = (2'–5')-oligoadenylate. Other abbreviations as in Figs 1, 2 and 3

with the observations described above, as elevated intracellular pH induces an increased Na^+/K^+ pump rate [78].

In general, IFN is considered to be an inhibitor of normal cell growth [80]. Triggering of inositol phospholipid breakdown by exogenous signals may induce, however, mechanisms resulting in stimulation of DNA replication and cell proliferation [32, 33, 36]. The question arises, whether IFN can act as such a signal? Some experimental data are in favour of giving a positive answer. Studying the effect of human leukocyte IFN on B cell proliferation it was found that 100–1000 U/ml IFN increased the spA Co 1 (*Staphylococcus aureus* strain Cowan 1) induced proliferation of B cells. Another subtype of IFN alpha and IFN beta, however, decreased the spA Co 1 induced proliferation [81]. The potential of IFN to either inhibit or enhance growth of tumours in mice induced by Moloney sarcoma virus transformed cells was also demonstrated. The effect of IFN proved to be dependent on the route and timing of IFN administration related to tumour cell inoculation [82].

There are also data indicating that IFN can directly stimulate proliferation of tumour cells in vitro, especially when low IFN doses (50–100 U/ml) were applied. The effect was dependent on the type and concentration of IFN used and the tumour cells studied [83, 84].

Conclusion

It can be stated that a considerable number of experimental data is in harmony with the hypothesis that triggering of the inositol phospholipid-diacylglycerol-protein kinase C signal transducing pathway by IFN might be responsible for several effects of IFNs. Naturally, we concentrated on results supporting this hypothesis, but are fully aware of the fact that other mechanisms, as for example activation of doublestranded RNA dependent protein kinase action, play also a role in IFN-action. Relationships of these mechanisms with different chains of the inositol phospholipid-diacylglycerol-protein kinase C signal transducing pathway should be revealed in oncoming researches. It should also be taken into consideration that the different biochemical pathways can be separated only for didactic purposes; in reality they are closely related and the end-result is dependent on the actual state of the cells, macro- and micro-environment, route and timing of IFN administration and different other factors (Fig. 4).

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NOTE ADDED IN PROOF

After submitting our manuscript an article by Yap W. H., Teo S., Tan Y. H. appeared in Science *234*, 355 (1986) strongly supporting our hypothesis.

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EFFECT OF LIGHT QUALITY, ANAEROBIOSIS AND GLUCOSE ON NITROGEN-FIXATION BY THE RICE FIELD CYANOBACTERIUM *AULOSIRA FERTILISSIMA* GHOSE

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Anaerobiosis enhanced the nitrogenase activity of the rice field alga *Aulosira fertilissima* both in light and dark. The activity was maximum in white light, lesser in red, blue and green light and least in the dark. Glucose stimulated the activity both in light and dark.

The ability to reduce the dinitrogen molecule is restricted to prokaryotes among which cyanobacteria play a major role principally because of their photosynthetic ability. Such trophic independence with regard to C and N, combined with the great adaptability to variations in environmental factors, permits these organisms to be ubiquitous and at the same time gives them a unique potential to contribute to productivity in a variety of agricultural and ecological situations. The rice field ecosystem is a microbial babylon with a variety of N_2-fixing microorganisms, the majority of which are cyanobacteria which grow well under the prevalent waterlogged and reducing conditions [1], and are considered to be potential contributors of fixed nitrogen to soil [2–5].

One such heterocystous form is *Aulosira fertilissima* which has been claimed to be the strongest N_2-fixer of Indian paddy fields [4]; the maintenance of fertility, higher crop yield and increased soil N_2 content in these fields seems to be due to the abundant growth of this alga. Surprisingly little, if any, work, has been done on the growth, physiology and nitrogenase activity of this very important organism. In view of the great potential agronomic importance of this alga we have isolated it in axenic cultures and studied its growth and nitrogenase activity.

Material and methods

Organism and growth. Aulosira fertilissima Ghose was isolated from the rice fields of Ramnagar (Lat. 25°18' N and Long. 83°11' E) near Varanasi. Clonal cultures were raised from single spores and made axenic by standard microbiological methods. Cultures were grown at 27 ± 1 °C, 2500–3000 lux. The organism grew best in the medium of Hughes et al. [6]. Its

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growth was estimated by extracting chl*a* in 80% aqueous acetone and measuring the absorbance at 663 nm.

Heterocyst frequency. The cultures were first made nonheterocystous by growing the alga in medium supplemented with 5 mM KNO_3. Experiments were then set up by transferring it into nitrogen-free medium, and the heterocyst frequency was measured as percent of the total cells by counting 10 filaments.

Nitrogenase activity. This was estimated by measuring the nitrogenase-catalyzed conversion of acetylene to ethylene [7]. The incubation time for the assay was 1 h. Gas samples were analyzed for ethylene in a CIS gas chromatograph (Baroda) fitted with a Porapak R column and hydrogen flame ionization detector. The nitrogenase activity under anaerobic conditions was measured after incubation of the alga under 99% N_2 and 1% CO_2 atmosphere.

Monochromatic light treatment. Exposure to monochromatic light was made in a wooden box (30 cm^2) lined internally with aluminium sheet and covered externally with black paper. The box had a 9 cm^2 aperture in the upper wall over which the desired filter was placed. The light source was a 150 W Philips comptalux lamp with a mercury coated reflector producing 100 $\mu W/cm^2$ of energy at the experimental site. Equal energy levels of the light treatments were obtained by adjusting the distance between the light source and the sample. Between the light source and the filter, a glass container was interposed through which running water flowed continuously. Three filters (blue 450 nm; green 545 nm; and red 650 nm) obtained from Carolina Biological Supply Co., Burlington, USA, were used.

Heterotrophy. D(+)Glucose was dissolved in double distilled water without heating, filtered through sterile Millipore filters (0.45 μm pore size) and aseptically added to the culture tubes to obtain the desired final concentration. One set of tubes was kept in the light and another set in the dark. At different time intervals nitrogenase activity was measured in both the sets. 3(3,4-Dichlorophenyl)-1,1-dimethyl urea (DCMU) was made up in ethanol to give a final concentration of 1×10^{-5} M.

Glutamine synthetase activity. The whole cell transferase activity of glutamine synthetase was measured by the method of Shapiro and Stadtman [8] as modified by Stacey et al. [9].

Nitrate reductase activity. In vivo nitrate reductase activity was measured by the method of Camm and Stein [10] as slightly modified by Kumar and Kumar [11].

Results

Growth and heterocyst frequency. The organism grew well in Hughes medium. The heterocyst frequency in exponentially growing cultures was found about 3.4% (Table I).

Induction and inhibition of nitrogenase activity. Following transfer of NO_3-grown material into nitrogen-free medium, nitrogenase activity (3.5 nmoles C_2H_4/μg chl*a*/h) occurred at about 24 h (Fig. 1) and reached a maximum (21.7 nmoles of C_2H_4/μg chl*a*/h) on the 6th day (Table II). The nitrogenase

Table I

Heterocyst frequency of Aulosira fertilissima

	Heterocyst frequency on days						
	0	1	2	4	6	8	10
Nitrate grown cultures transferred into nitrogen free medium	0	1 ±0.001*	1.8 ±0.02	2.6 ±0.04	3.4 ±0.04	3.3 ±0.01	3.2 ±0.02

* Per cent ±SE

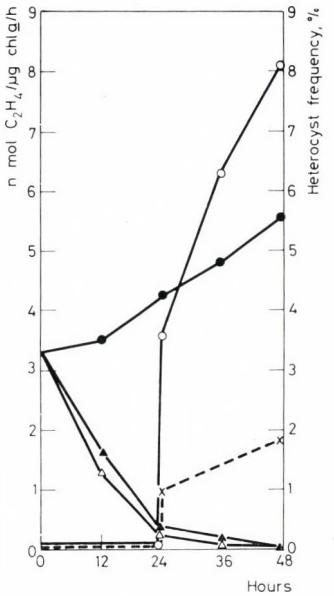

Fig. 1. Induction and effect of different combined nitrogen sources on nitrogenase activity. ● — ● Control; ○ — ○ induction of N_2ase activity; ▲ — ▲ Repression by nitrate (5 mM KNO_3); △ — △ Repression by ammonia (2 mM NH_4Cl); × --- × per cent heterocyst frequency

activity was inhibited by both nitrate and ammonium, the latter being more effective. Nitrate (5 mM KNO_3) inhibited the activity to 0.33 nmoles C_2H_4/µg chl*a*/h and ammonium (2 mM NH_4Cl) to 0.27 nmoles C_2H_4/µg chl*a*/h in 24 h

Table II

Effect of monochromatic light and anaerobiosis on nitrogenase activity

Light control*	6.3±0.11**	9.7±0.07	21.7±0.14	12.7±0.15	8.3±0.21
Light + anaerobiosis	11.5±0.15	14.6±0.11	32.8±0.12	19.2±0.13	5.9±0.08
Red	3.7±0.08	4.8±0.22	5.9±0.07	2.8±0.10	1.2±0.07
Red + anaerobiosis	5.3±0.27	6.2±0.10	9.3±0.11	3.7±0.09	0.6±0.13
Blue	5.4±0.07	3.2±0.11	1.8±0.14	1.2±0.21	1.0±0.15
Blue + anaerobiosis	9.5±0.08	5.2±0.22	2.2±0.07	1.6±0.07	0.5±0.10
Green	5.2±0.13	3.0±0.15	1.5±0.11	0.9±0.12	0.6±0.08
Green + anaerobiosis	5.9±0.09	3.6±0.29	2.2±0.11	1.7±0.10	0.5±0.13
Dark	3.3±0.21	2.4±0.11	2.2±0.14	1.7±0.11	0.7±0.07
Dark + anaerobiosis	3.7±0.08	2.6±0.15	2.4±0.13	2.1±0.09	0.5±0.21

* The value for light control at day zero was 2.97±0.07 SE
** Nitrogenase activity (nmoles C_2H_4/µg chl*a*/h) ±SE

as compared to controls (4.3 nmole $C_2H_4/\mu g$ chl*a*/h; Fig. 1). Anaerobiosis greatly enhanced nitrogenase activity till the 6th day which then gradually declined on the 8th and 10th day (Table II).

Effect of monochromatic light on nitrogenase activity. In red light nitrogenase activity was highest on the 6th day whereas blue and green light showed no such effect. After an initial stimulation on the 2nd day, the activity gradually decreased in blue and green light. Red light plus anaerobiosis increased the nitrogenase activity till the 6th day whereafter it gradually fell. Blue and green light plus anaerobiosis showed the same trend as aerobically grown treatments but the values were higher under anaerobiosis. The nitrogenase activity under all these monochromatic light conditions was less than in fluorescent light (Table II).

Heterotrophy. Glucose at 1–3% (w/v) greatly increased the nitrogenase activity which reached a maximum on the 6th day. Highest activity was obtained with 3% glucose while 4% and 5% did not cause any significant change in activity (Table III). In all cases nitrogenase activity was lower in darkness than in the light.

Effect of DCMU and glucose. Treatment with DCMU (1×10^{-5} M) inhibited nitrogenase activity but the same was enhanced in DCMU + 3% glucose. With glucose alone the activity was maximum. A similar trend was seen in the dark experiments. After a 6 h incubation in the dark, glucose (3%) was added to 2 samples, one being then incubated in the light and the other in

Table III

Effect of glucose on nitrogenase activity

Treatments	Nitrogenase activity on days				
	2	4	6	8	10
Light control*	6.7±0.05**	10.4±0.04	22.2±0.05	13.5±0.03	9.0 ±0.04
L + 1%	12.0±0.04	18.7±0.01	23.6±0.07	13.0±0.05	9.2 ±0.02
L + 2%	12.0±0.04	19.0±0.04	25.6±0.007	15.3±0.02	10.3 ±0.02
L + 3%	15.7±0.01	24.1±0.007	30.06±0.05	19.6±0.02	15.45±0.05
L + 4%	6.9±0.05	11.9±0.02	19.8±0.007	12.8±0.02	6.8 ±0.04
L + 5%	6.6±0.04	10.2±0.05	18.6±0.01	11.6±0.04	5.2 ±0.07
Dark control	3.5±0.03	2.4±0.02	1.3±0.005	0.6±0.005	—
D + 1%	4.0±0.005	4.2±0.02	4.4±0.05	4.0±0.04	2.1±0.05
D + 2%	4.5±0.05	4.7±0.005	4.9±0.02	4.2±0.07	2.3±0.02
D + 3%	6.3±0.007	7.5±0.04	8.9±0.02	6.8±0.02	4.5±0.007
D + 4%	4 ±0.06	3.7±0.04	3.8±0.05	1.9±0.02	0.5±0.007
D + 5%	3.5±0.05	3.3±0.02	2.8±0.007	1.4±0.06	0.2±0.05

* Nitrogenase activity in light control on day zero was 3.2±0.02 SE
** Nitrogenase activity (nmoles $C_2H_4/\mu g$ chl*a*/h) ±SE

Table IV

Effect of DCMU on nitrogenase activity

In light

Treatment	Nitrogenase activity at hours			
	0	6	12	24
Light control	3.18 ± 0.03**	3.78 ± 0.02	3.81 ± 0.02	3.97 ± 0.01
Light + 3% glucose	3.18 ± 0.03	4.86 ± 0.05	5.16 ± 0.04	6.96 ± 0.04
Light + DCMU + 3% glucose	3.18 ± 0.03	2.63 ± 0.01	2.98 ± 0.02	3.54 ± 0.02
Light + DCMU	3.18 ± 0.03	1.9 ± 0.01	0.8 ± 0.01	0.5 ± 0.01

In dark

Treatment	Nitrogenase activity at hours			
	0	6	7	8
Dark control	3.18 ± 0.03	1.8 ± 0.02	0.9 ± 0.02	0.6 ± 0.007
Dark + 3% glucose	3.18 ± 0.03	3.27 ± 0.05	4.33 ± 0.02	4.52 ± 0.02
Dark + DCMU	3.18 ± 0.03	1.7 ± 0.04	0.8 ± 0.03	0.5 ± 0.03
Dark–light + *3% glucose	3.18 ± 0.03	1.8 ± 0.02	2.5 ± 0.04	3.1 ± 0.05
Dark–dark + *3% glucose	3.18 ± 0.03	1.8 ± 0.005	2.1 ± 0.007	2.4 ± 0.01

* Cultures were grown in the dark for 6 h and then incubated for 1 and 2 h in light and dark with 3% glucose

** Nitrogenase activity (nmoles C_2H_4/μg chl*a*/h) ±SE

the dark. Surprisingly, there was stimulation in nitrogenase activity in both the sets (Table IV).

Glutamine synthetase activity was found to be 0.824 nmoles γ-glutamyl hydroxamate/μg chl*a*/30 min. Nitrate reductase activity reached a maximum of 0.29 μg nitrite/μg chl*a* on the 2nd day.

Discussion

The *nif* genes which encode the machinery of N_2-fixation are subject in all N_2-fixing prokaryotes to end product repression by ammonia [12]. Removal of exogenous combined N_2 source which depletes the intracellular ammonia pool, is necessary to trigger transcription and translation of genes and thus elicits N_2-fixation (i.e., induction). From our data it appears that the induction of nitrogenase activity under aerobic conditions in light is closely co-related with the appearance of mature heterocysts and the development of nitrogenase activity. With the appearance of heterocysts (1%) at 24 h there was induction of nitrogenase activity (Fig. 1) on the 6th day when heterocyst frequency was maximum (3.4%) and the nitrogenase activity was highest. When grown in the presence of 5 mM nitrate or 2 mM ammonia, *Aulosira* filaments lack hetero-

cysts. Ammonia was a more effective repressor of heterocyst differentiation than nitrate. When nitrogenase activity was measured under anaerobic conditions (see Stewart and Pearson [13]) there was considerable increase of specific activity. This may be due to the fact that under anaerobic conditions both vegetative cells and heterocysts synthesize nitrogenase. Red light stimulated growth and hence the nitrogenase activity, in contrast to blue and green light. The inhibition of nitrogenase activity with blue light seems consistent with the observations that blue light inhibits CO_2-fixation and N_2-metabolism [14]. Green light inhibits growth and hence the nitrogenase activity. Fluorescent light was most favourable for nitrogenase activity as it permits maximum photosynthesis. The stimulation by red light could be due to the fact that it supports heterocyst differentiation [15, 16], whereas green light is inhibitory. The nitrogenase activity increased greatly with the addition of 3% glucose. The reason for this could be the direct assimilation of the substrate. That glucose provides the reductant for N_2-fixing process is also shown by the addition of DCMU which blocks PSII system leaving PSI functional. Under these conditions ATP synthesis by cyclic photophosphorylation continues but the generation of photoreductant (NADPH) is arrested and N_2-fixation declines. With the addition of glucose there is rapid recovery of nitrogenase activity. Enhanced nitrogenase activity in glucose was also observed in the dark. Nitrogenase activity also occurred in samples without glucose addition. The present observations with *Aulosira* suggest that both the rate and duration of nitrogenase activity in the dark may be affected by the rate of photosynthesis and carbon assimilation during the light period. The decline in nitrogenase activity after transfer of *Aulosira* from light to dark can be associated with the speedy depletion of energy and reductant supplies for nitrogenase action. Continued nitrogen fixation in the dark would therefore depend on mobilization of reserve products and on the rate at which carbon moves from its reservoir to the site of nitrogenase activity. Addition of glucose would be expected to support and stimulate nitrogenase activity. This is borne out by our data. The nitrogenase activity observed in the dark without the addition of glucose may be due to a large endogenous pool of reductants present in *Aulosira*. The high glutamine synthetase activity can be correlated with high nitrogenase activity; consequently, the nitrate reductase activity is low.

Most cyanobacteria are believed to be obligate photoautotrophs. However, isolated reports on the growth of individual strains on organic media [1, 18] together with the results of surveys of the heterotrophic potential [19, 20] have provided ample proof that some cyanobacteria are capable of growth on exogenous low molecular weight organic compounds.

Herein we report that *Aulosira fertilissima* is not an obligate photoautotroph but a facultative heterotroph which can grow and fix N_2 in the

presence of glucose both in light and dark. The results obtained in this study are consistent with the earlier findings [17] on the stimulation of N_2-fixation by organic substrates.

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IN VIVO AND IN VITRO EFFECT OF TOXIC AND RADIO-DETOXIFIED ENDOTOXIN PREPARATIONS ON CHEMILUMINESCENCE OF PERITONEAL CELLS OF MICE

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Chemiluminescence (CL) of peritoneal cells was investigated one and five days after injection of toxic and radio-detoxified endotoxin. The CL was triggered by toxic and radio-detoxified endotoxin. The radio-detoxified endotoxin has less pronounced in vivo activating effect than the toxic endotoxin. As triggering agent the toxic endotoxin was more effective than the radio-detoxified endotoxin. However, the radio-detoxified endotoxin has preserved some in vivo activating and in vitro triggering effect.

Bacterial endotoxins (LPS) are potent activators of the immune response. Among other effects they influence the function of macrophages such as the enzymatic function and the oxidative metabolism [1, 2].

In vivo and in vitro treatment of peritoneal macrophages with endotoxin displays a greatly enhanced ability to generate superoxide (O_2^-) in response to stimulation by opsonized zymosan or by phorbol mystrat acetate (PMA) [1, 3]. Such activated macrophages also exhibit H_2O_2 release [4, 5]. The influence of intraperitoneal and subcutaneous injection of LPS on peritoneal and spleen cells has been investigated in LPS sensitive and resistant mice by CL of latex particles [6]. LPS is able to generate chemiluminescence by polymorphonuclear leukocytes (PMN) directly, not only through the activation process by previous incubation with LPS [7].

The adjuvant effect of LPS preparations, detoxified by different chemical methods was investigated as early as during the sixties. It seemed that the adjuvant activity was connected with the method of detoxification. According to Nowotny [8], treatment with boron trifluoride induces an expressed decrease of adjuvant activity. On the other hand, detoxification by potassium methylate does not influence the adjuvant capacity of endotoxin [8, 9]. We have observed a pronounced decrease of adjuvant activity of LPS detoxified by potassium

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methylate. As a possible cause of this, it maybe supposed that the chemical detoxification was brought later to a standstill.

The toxic and other deleterious effect of LPS can be decreased by gamma irradiation. The diminution of the lethal effect was dependent on the dose of irradiation [10–12].

We examined the effect of toxic and radio-detoxified endotoxins on peritoneal cells. The toxic and radio-detoxified endotoxins were used as in vivo activators and as in vitro triggering agents. Radio-detoxified endotoxin was used, because these preparations as adjuvants enhanced the immune response similarly to the effect of toxic LPS [13, 14]. The macrophages play an essential role in the immune response which can be influenced by various doses of endotoxins. By CL testing, the question whether the toxic and radio-detoxified LPS influences the respiratory burst in the same way and manner, could be answered.

During these experiments luminol amplified CL was used, which reacts mainly with hydrogen peroxide (H_2O_2) and probably the singlet oxygen ($^1O_2$), but it seems not to react with hydroxyl radicals (OH·) [15].

Materials and methods

Animals. Three months old C57B1 × $DBA_2(F_1)$Lati (Laboratory Animals Breeding Institute, Gödöllő) hybrid mice of both sexes were used. The animals were treated with 100 μg of toxic or radio-detoxified endotoxin preparation intraperitoneally, then sacrificed on the first or on the 5th day.

Peritoneal cells were obtained by lavaging the peritoneal cavity of mice with isotonic NaCl solution. Peritoneal cells of 6–10 mice were pooled before cell separation by Ficoll-Uromiro gradient centrifugation (1.071 density). The cells were washed and resuspended in TC 199 medium containing Tris buffer and glucose (pH 7.2). The final concentration of the cells was 10^6 or 4×10^6 per ml.

Endotoxin (LPS) preparation. Endotoxin was prepared by warm phenol–water method from *Escherichia coli* O89 strain [16].

Detoxification of LPS preparation. For detoxification, LPS was dissolved in distilled water (10 mg/ml) and irradiated using a ^{60}Co-gamma source (Sovatom) by the method of Previte et al. [17], modified by us. The doses of detoxification were 50 or 150 kGy.

Chemiluminescence (CL). Luminol dependent CL was measured in the Beckmann LS 100 liquid scintillation counter, out of coincidence mode at room temperature. The reaction was done in dark, in dark adapted plastic scintillation vials. The vials contained Luminol at 4×10^{-8} M final concentration. The reaction volume in the test tubes was 1.1 ml containing 1, 10 or 100 μg endotoxin preparation and 5×10^5 cells on the first day, and 2×10^6 cells on fifth day after in vivo endotoxin treatment.

Statistical analysis. Mean ±SE was determined and mathematical significance was calculated by Student's *t* test.

Results

CL of resident peritoneal cells. The in vitro effect of toxic and radio-detoxified endotoxin preparations on the peritoneal cells of untreated animals were investigated. Table I demonstrates that the untreated cells produce only

Table I

Chemiluminescence of peritoneal cells from control mice
Cells were triggered in vitro by 10 μg of toxic or radio-detoxified endotoxin

Type of in vitro triggering	CPM×10³, time in min				
	15	20	25	30	45
PBS	8.8±0.8	8.7±0.8	9.7±0.8	9.3±0.9	9.3±1.1
LPS	7.9±0.8	8.0±0.4	9.1±0.3	8.4±0.4	7.1±0.4
50 kGy LPS	8.2±0.6	8.3±0.9	9.0±0.9	9.2±1.3	9.1±1.3
150 kGy LPS	7.7±0.7	7.6±0.6	7.8±0.5	7.9±0.5	7.5±0.5

Type of in vitro triggering	CPM×10³, time in min				
	60	75	90	105	120
PBS	9.9±1.4	10.1±1.3	12.0±1.7	10.8±1.1	10.3±0.9
LPS	7.3±0.6	7.6±0.9	8.1±0.8	8.7±1.1	9.0±1.1
50 kGy LPS	9.3±1.1	9.7±1.8	10.7±2.0	10.5±1.8	10.9±1.6
150 kGy LPS	8.0±0.8	8.9±1.1	9.2±1.4	9.5±1.3	9.6±1.5

Results represent mean ±SE of at least 4 vials. Experimental animals were untreated. The peritoneal cells were prepared as given in "Materials and methods". Vials contained Luminol, 2×10^6 cells and 10 μg of LPS preparation

a very low level of CL. Adding toxic or radio-detoxified endotoxin preparations to the cells, no higher CL values were induced as compared with phosphate buffered saline (PBS).

The background CL of peritoneal cells of in vivo treated animals. Animals were injected with 100 μg of toxic or radio-detoxified LPS intraperitoneally; an untreated group served as control.

The background level of CL in normal animals was low (Fig. 1). However, in animals treated with toxic endotoxin a significant elevation of background CL was observed on the first day. Radio-detoxified endotoxin caused an expressed, but less pronounced increase in the background level than the toxic endotoxin. On the fifth day the activity of peritoneal cells was lower than on the first day after treatment. The elevation of the background levels of both LPS treated groups was statistically significant, in comparison to the untreated controls.

The CL of endotoxin-activated peritoneal cells in the presence of toxic or radio-detoxified endotoxin preparations. The effect of different doses of toxic or radio-detoxified LPS on cells obtained from LPS treated mice was also investigated. On the first day after endotoxin treatment the higher in vitro dose of LPS (10 μg) could only moderately elevate the CL. One μg LPS had a pronounced effect and induce a high CL response (Fig. 2). As to the effect of

150 kGy radio-detoxified LPS, at the higher (10 μg) in vitro dosis the CL was elevated in the late period of CL response, but at 1 μg the peak appeared in the early period with a second peak at the end of the first hour (Fig. 2). In the in vitro presence of toxic LPS the early peak was expressed.

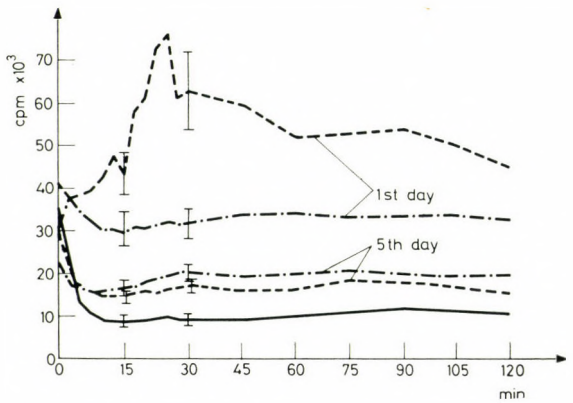

Fig. 1. Chemiluminescence of peritoneal cells from control mice and from animals treated in vivo with toxic or radio-detoxified LPS, without in vitro triggering agent. On the first day 5×10^5, on the fifth day 2×10^6 cells per vials were investigated. Each point represents the mean of at least 11 vials. In vivo treatment: ——— untreated control, – – – LPS, ·–·–·– 150 kGy LPS

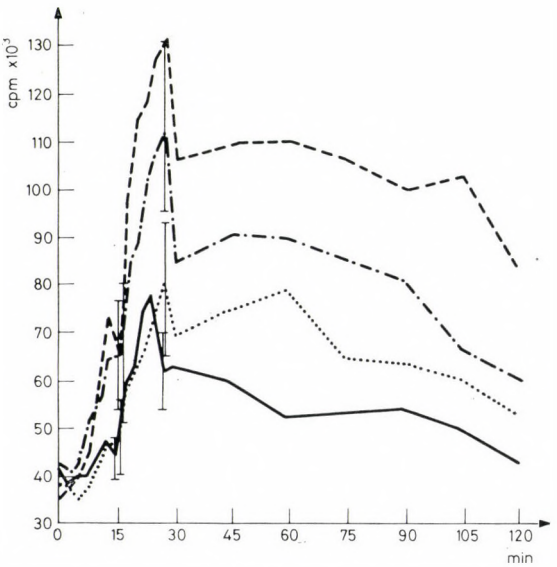

Fig. 2. Chemiluminescence of peritoneal cells from animals treated on the previous day with 100 μg of toxic LPS. Cells were in vitro triggered with 1 μg of toxic or radio-detoxified LPS. Each point represents the mean of at least 5 vials. In vitro triggering: ——— PBS, – – – LPS, ·–·–·– 50 kGy LPS, ····· 150 kGy LPS

On the fifth day after the in vivo treatment of mice, peritoneal cells were less sensitive to the in vitro triggering of LPS preparations. Although for in vitro investigation a four-fod amount of cells was necessary, the response was lower. The effects of triggering toxic endotoxin doses showed no marked differences. Various doses of radio-detoxified LPS had different effects. Ten μg of 150 kGy radio-detoxified LPS had a slight effect (Fig. 3).

CL of radio-detoxified LPS-activated peritoneal cells in the presence of toxic or radio-detoxified LPS. For in vivo treatment we used an LPS preparation detoxified by 150 kGy ^{60}Co-gamma irradiation. On the first day after the rreatment the basic level of CL was lower than that observed after toxic LPS treatment (see Fig. 1). The addition in vitro of toxic or radio-detoxified prepatations in a dose of 10 μg (Fig. 4), hardly enhanced the CL of peritoneal cells.

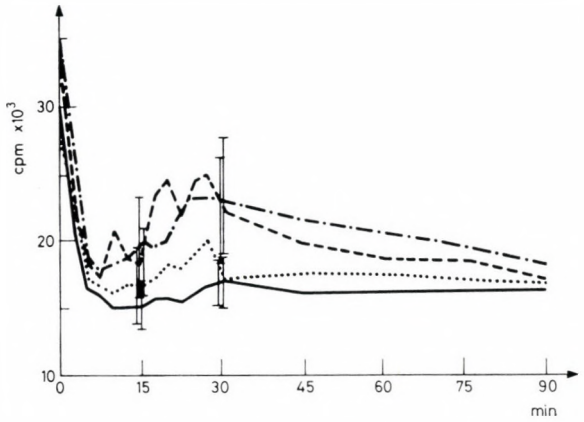

Fig. 3. Chemiluminescence of peritoneal cells after adding in vitro 10 μg of toxic or radio-detoxified LPS preparations. Mice were treated 5 days earlier with 100 μg of toxic LPS intraperitoneally. Each point represents the mean of at least 5 vials. In vitro triggering: ——— PBS, – – – LPS, ·–·–· 50 kGy LPS, ····· 150 kGy LPS

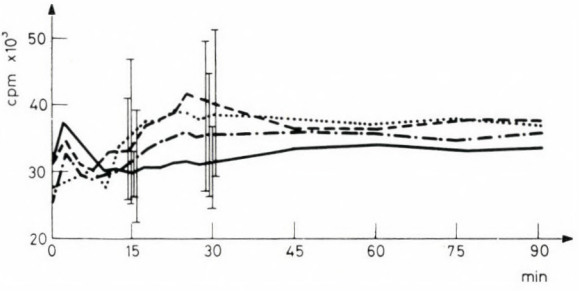

Fig. 4. Chemiluminescence of peritoneal cells after adding in vitro 10 μg toxic or radio-detoxified LPS preparations. Mice were treated on the previous day with 100 μg of 150 kGy radio-detoxified LPS intraperitoneally. Each point represents the mean of at least 5 vials. In vitro triggering: ——— PBS, – – – LPS, ·–·–· 50 kGy LPS, ····· 150 kGy LPS

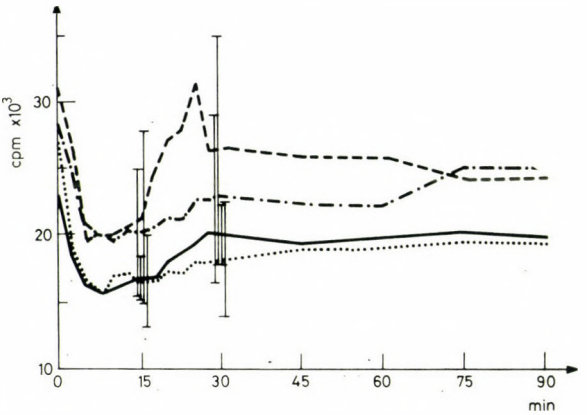

Fig. 5. Chemiluminescence of peritoneal cells in the in vitro presence of 100 μg of toxic or radio-detoxified LPS preparations. Mice were treated 5 days earlier with 100 μg of 150 kGy radio-detoxified LPS intraperitoneally. Each point represents the mean of at least 5 vials. In vitro triggering: ——— PBS, – – – LPS, ·–·–· 50 kGy LPS, ····· 150 kGy LPS

At 1 μg dose all preparations were ineffective. On the fifth day after the in vivo treatment with radio-detoxified LPS, the in vitro CL activity of the cells was even lower than on the first day's reaction. One or 10 μg LPS did not induce any CL in the cells, only the high dose of LPS (100 μg) could evoke a slight CL response (Fig. 5).

Discussion

Endotoxin treatment causes significant changes in animals [5, 18]. For example it induces tolerance against repeated high dose treatment and stimulates the non-specific resistance. Detoxified LPS possesses the tolerance inducing effect as well [13]. Our aim in the present study has been to answer the question how toxic or radio-detoxified LPS modifies the macrophage functions measurable with CL.

The activating effect of LPS treatment on the mononuclear phagocyte system was proven by phagocytosis of activated cells in the presence of phorbol mystrate acetate (PMA) or complement coated zymosan [3, 19]. PMN's triggering effect of LPS on CL was observed by Herkins et al. (cit. Galanos [20]). There is no explanation in the literature, how cells are activated in vivo by detoxified LPS and how these cells respond to endotoxins as triggering agents.

According to our results, resting macrophages do not respond immediately by an increase of CL either to the radio-detoxified LPS or the toxic one in vitro (Table I). The action of LPS is probably not a direct effect but it works through the activation of macrophages during longer incubation. Our results show that the experimental animals were pathogen-free, as CL responses of our

untreated animals were low. These results are in good agreement with the report of Metzger et al. [19], who considered the lack of spontaneous or PMA inducible CL increase in thioglycolate induced cells as the confirmation of pathogen-free state. Bryant and Hill [21] also found only slightly higher CL of macrophage monolayers in non-primed animals in the presence of opsonized zymosan than the background CL. In our experiments pretreatment of toxic or radio-detoxified LPS significantly increased the spontaneous CL at the first day following the treatment (Fig. 1). On the 5th day the increase was still significant, but less marked. Our results were not supported by the finding of Bryant and Hill [21] who have studied animals pretreated with *Corynebacterium parvum* and found no significant differences in the background CL values between macrophage monolayers taken from *C. parvum* treated and control animals.

In the first day following pretreatment with toxic LPS, CL increased significantly. This fact was observed in the presence of 1 μg and 10 μg of LPS. It may be assumed that on the first day the macrophages become activated. This activated macrophage population has an increased activity in the production of oxygen radicals and phagocytosis. This increased reactivity manifests itself in a higher CL in the presence of triggering LPS. The triggering effect of detoxified LPS preparations was decreased. This decrease seemed to depend on the increase of the dose of irradiation (Fig. 2).

On the 5th day after treatment with LPS the CL in vivo, the activity of the cells decreased significantly (Fig. 2). Our results are in agreement with the results of Feuillet-Fieux et al. [6], who found that in high responder mice the effect of LPS on the fourth day after intraperitoneal injection was less extensive than on the second day, measured by CL of latex particles.

It is difficult to find a correct explanation of our results. One possibility is that peritoneal cells can be irresponsive to a repeated triggering agent because of the developed endotoxin tolerance on the 5th day. But it may also be supposed that the results on the 5th day correlate with the decrease of the enzymatic activity or enzyme content. It seems to be similar to the results of Seim [22], who, during in vitro differentiation of human monocytes, observed the decrease of luminol dependent CL, possibly caused by the decrease of myeloperoxidase activity of cells. Kalager et al. [23] found differences in CL and enzyme activity of peritoneal leukocytes induced by the injection of caseinate, depending from the harvesting intervals. Furthermore, it is possible that the distribution of cells is functionally not the same on the 5th day as on the first day. However, no significant change could be seen in the distribution of cells morphologically. Animals pretreated with toxic endotoxin produced less pronounced CL response to radio-detoxified preparations than to toxic ones. It may be assumed that the in vivo activating effect of detoxified LPS is less than that of the toxic LPS. Other experimental results [12–14] support

that detoxified preparations with intact tolerance inducing capacity have in many respect only moderate effectiveness.

Our data showed a partial decrease of CL of peritoneal cells collected one day after treatment with radio-detoxified LPS in comparison with CL of cells originating from toxic LPS pretreated animals (Fig. 3). However, detoxified preparations could trigger significantly CL of cells in vitro. The diminished activity of cells show the fact that a higher dose of LPS should be used for the in vitro triggering, because a lower dose fails to induce any CL response. On the 5th day CL could be induced only with a high dose of toxic LPS (Fig. 5). Radio-detoxified LPS induce small or not any response.

Summarizing, detoxified LPS preparations have less pronounced activating and triggering effect both in vivo and in vitro than toxic LPS. However, the in vivo effect was not completely missing in radio-detoxified LPS preparation, which can probably activate the macrophages to some extent.

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THE ROLE OF GENES LAC1 AND LAC2 IN THE BIOSYNTHESIS OF LACTOSE METABOLISM ENZYMES BY *KLUYVEROMYCES LACTIS*

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By crossing different *Kluyveromyces lactis* strains, the role of genes LAC1, LAC2 and of gene C were analyzed. These genes are involved in the biosynthesis of enzymes for the metabolism of lactose and galactose. They control the biosynthesis of the lactose and galactose transport, of beta-galactosidase and of the three enzymes of the Leloir pathway. The presence of at least one of the LAC gene is required for the biosynthesis to occur. The gene C seems to code for a negative factor which blocks the expression of the LAC1 and LAC2 genes in the absence of an inducer.

Kluyveromyces lactis Van der Walt can assimilate lactose as carbon source. Lactose is hydrolyzed into glucose and galactose by the intracellular β-galactosidase (E. C. 3.2.1.23). The β-galactosidase is inducible by lactose and galactose [1, 2]. The maximum activity level of β-galactosidase is obtained following 9 cellular generations in the presence of the inducer. It is from 100 to 150 times greater than when the cells are grown on a medium without an inducer [2]. Herman and Halvorson [3] studied the genetic control of the synthesis of β-galactosidase by crossing *Kluyveromyces lactis* (a) Y 1140 and (α) Y 1118 strains. The segregation for the β-galactosidase activity and no-activity galactosidase character suggested that there are two separate genes for β-galactosidase. The genotypes attributed were LAC1 lac2 for strain (α) Y 1118 and lac1 LAC2 for strain (a) Y 1140. Gene LAC1 seems to be tied to the structural gene for β-glucosidase. Tingle and Halvorson [1] showed that the LAC1 lac2/lac1 LAC2 diploids have an enzymatic activity equal to that of the parent strains. These authors isolated mutants of Y 1118 strain with a reduced galactosidase activity. These strains have the LAC1 lac2 genotype. However, the Lac1 lac2/lac1 LAC2 diploids showed an activity 50% lower than expected. In opposition to the first case where the authors observed a gene dosage effect, the mutation on lac1 was partially epistatic on the LAC2 gene. Sheetz and Dickson [4] isolated several mutants unable to assimilate lactose which they named lac$^-$. Seven complementation groups were named LAC3 to LAC9. These strains are also unable to grow on galactose. The LAC4 gene was shown to be the structural gene for β-galactosidase [5]. Also Riley and Dickson [6]

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isolated and identified *K. lactis* mutant strains which are defective for galactokinase (ATP-D galactose 1 phosphotransferase, E. C. 2.7.1.6.), transferase (uridine diphospho-glucose-D-galactose-1-phosphate uridyltransferase, E. C. 2.7.7.10.) and epimerase (uridine diphosphoglactose-4-epimerase). The corresponding genes were respectively named GAL1, GAL7 and GAL10 due to a similarity to genes responsible for the metabolism of galactose in *Saccharomyces cerevisiae*. The order of these genes seems to be identical to that found for *S. cerevisiae*. Dickson et al. [7] obtained constitutive mutants (lac10^6) for β-galactosidase. They showed that LAC10 has a negative control on β-galactosidase as well as on galactokinase, transferase and lactose permease.

In the present work, we studied the influence of the LAC1 and LAC2 genes and of a gene C (constitutive character) on the regulation of the biosynthesis of the enzymes involved in the metabolism of lactose and galactose.

Materials and methods

Strains and production of spores. The two original haploid heterothallic parental strains of *K. lactis* Y 1140 (a lac1 LAC2) and Y 1118 (αLAC1 lac2) were obtained from strain CBS Centraalbureau voor Schimmelcultures Delft 2359 and CBS 6315, respectively. Two other strains, Y 11630 (a lac1 LAC2 his$^-$) and Y 11631 (α LAC1 lac2 lys$^-$) were kindly supplied by Alberta Hermann, U. S. Department of Agriculture North Regional Research Laboratory, Peoria, Illinois, USA. All subsequent studies involved these strains or cultures derived from mating between CBS 2359 $\times$ CBS 6315 or Y 11630 $\times$ Y 11631.

Media and growth conditions. The complete medium (YME) contained 0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1% glucose [8]. Minimal medium (MM) contained Difco Yeast Nitrogen Base (0.7%), with carbon 0.5% source (glucose, glycerol, lactose, or galactose). Minimal medium with L-amino acid (MMaa), was MM supplemented with 0.022% lysine and 0.022% histidine. Spore isolates and stock cultures were maintained on yeast-malt-extract (YME) described by Wickerham [8]. Liquid cultures were incubated with shaking at 28 °C. Solid medium contained 3% agar. Cell growth was monitored by optical density measurements in an Elvi colorimeter at 420 nm. 1 OD unit corresponds to 200×10^6 cells/ml and 1.8 mg dry matter/ml.

Genetic analysis. Haploid strains were grown on YME solid medium for 24 h then they were crossed by replica-plating. After incubation for 3 to 10 days to allow mating and sporulation, asci appeared. Ascus dissection was performed according to the technique described by Johnston and Mortimer [9]. Asci were suspended in 1 ml of physiological saline (NaCl 0.9%) with 1 ml of gut juice of *Helix pomatia*. After 10 min, the asci were dissected on YME medium using the De Fonbrune micromanipulator. Genetic analyses were carried out only on asci with all four spores surviving on different media: MMaa + glucose; MMaa + lactose; MMaa + galactose; MMaa glucose + lactose and MMaa glucose + galactose. Segregation analysis of lysine and histidine was performed on MM + glucose + histidine and MM + glucose + lysine.

Preparation of enzyme extracts. The galactokinase, uridyl transferase and epimerase assays were performed on enzyme extracts prepared from ground cells. The cells were harvested during their active growth phase. After washing twice with distilled water the pellet was dispersed in 25 ml distilled water. The cells were harvested so that this suspension showed an OD of 40 units. Ten ml of this suspension were ground with glass beads in a Braun apparatus for 5 min. A jet of CO_2 snow was used every 10 s for cooling. Following separation of the glass beads, the mixture was centrifuged at 50 000 *g* for 25 min. The resulting supernatant was frozen. After thawing the extract was centrifuged at 140 000 *g* for 45 min. This final supernatant constituted our enzyme extract. Protein assays were performed at each step of the extraction. In all cases only a little activity loss (less than 5%) occurred during the freezing step.

The assay of β-galactosidase activity was performed on permeabilized cells. The cells were centrifuged out, washed twice with distilled water and resuspended in Z buffer (50 mM NaH_2PO_4; 10 mM KCl; 1 mM $MgSO_4$; 50 mM mercapto-ethanol; pH 7) described by Miller [10]. The volume used was adjusted so that the OD measurement was between 0.1 and 0.5. Then

0.5 ml of an ethanol/toluene (4/1) mixture was added to 5 ml of the above suspension and the whole mixture was mixed for 10 s and incubated at 30 °C for 10 min. The β-galactosidase assay was then immediately performed, with *O*-nitro-phenyl-β-D-galactoside (ONPG).

Assay and induction of the galactosidase and the three enzyme of Leloir pathway, in cells rendered permeable by treatment with dimethylsulfoxide (DMSO) was performed as described by Adams [11].

Enzyme assays. The assay of β-galactosidase was performed with cells previously permeabilized with toluene. One ml ONPG (4 mg/ml) was added to 5 ml of a permeabilized cell suspension. At regular interval 1.2 ml of the reaction mixture was taken and 0.5 ml of sodium carbonate (1 M) was added to this sample. The *O*-nitrophenyl liberated was measured at 420 nm. One OD unit corresponds to 1.33 μM liberated substrate. One unit β-galactosidase corresponds to the amount of enzyme required for the hydrolysis of 1 n mole ONPG per min at 30 °C and pH 7.

The assay of galactokinase, of galactose-1-phosphate uridyltransferase and of uridine diphospho-galactose 4 epimerase were performed according to the methods described by Robichon-Szulmajster [12]. One OD unit corresponds to 55.61 μmole galactose esterified by the galactokinase. One OD unit at 340 nm corresponds to 0.479 μmole of transformed substrate in the case of the uridyltransferase and 0.239 μmole substrate for the epimerase.

The lactose transport activity was determined as follows: the cells were harvested by centrifugation at 2000 *g* for 5 min and washed twice with distilled water, then resuspended in a volume of water so as to correspond to 3 units OD; 2.5 ml of the suspension were incubated at 30 °C in the presence of D-glucose ^{14}C lactose Amersham CFA 278, 1 μCi or of D-1-^{14}C galactose Amersham CFA 435, 1 μCi; the concentration of lactose or of galactose in the reaction tube was 1 mM; 0.5 ml samples were taken during 10 min; each sample was filtered with a Millipore 0.45 μ membrane and washed 3 times with lactose (10 mM) or galactose (10 mM) at 4 °C; the filter membranes were recovered and dissolved with 0.5 ml soluene 100 (Packard) for 12 h; following the addition of 12 ml of Instagel (Packard) scintillation liquid, the radioactivity was measured.

Protein assay was performed by the Biuret reaction according to Strickland [13].

Results

Segregation of the LAC and GAL phenotypes and β-galactosidase activity. Two types of crosses were performed, one with the *K. lactis* a 2359 and α 6315 strains, and the other with the *K. lactis* a Y 11630 his$^-$ and α 11631 lys$^-$ strains. In the latter case, we verified that the segregation of the genetic markers was normal. Each spore obtained was tested for growth on MM + lactose and MM + galactose. The presence or absence of β-galactosidase activity was also determined after culturing on MM + glucose in Petri dish according to the procedure described by Herman and Halvorson [14]. The resulting segregation is shown in Table I. Spores growing on lactose were classed as Lac$^+$, those growing on galactose were Gal$^+$, and those showing a β-galactosidase activity were βgal$^+$. Two types of spores were obtained, those with the Lac$^+$ gal$^+$ βGal$^+$ phenotype and those with the Lac$^-$ Gal$^-$ βgal$^-$ phenotype. The segregation obtained agrees with that described by Herman and Halvorson [3]. All the spores without β-galactosidase activity were unable to metabolize galactose. In order to determine whether the gene(s) LAC1 and/or LAC2 control(s) the biosynthesis of enzymes involved in the metabolism of lactose and galactose, we studied and compared the activities of the β-galactosidase, the 3 enzymes of the Leloir pathway [15], the lactose and galactose transport systems of the parent strains and of the spores with different genotypes.

Table I

Genetical analysis of the segregation of genes LAC1 and LAC2

	Genotypes	Phenotypes
Parent strains		
2359 and 11630	lac1 LAC2	Lac$^+$ Gal$^+$ β gal$^+$ (+)
6315 and 11631	LAC1 lac2	Lac$^+$ Gal$^+$ β gal$^+$ (+)
Possible recombinants	LAC1 LAC2	Lac$^+$ Gal$^+$ β gal$^+$ (+)
	lac1 lac2	Lac$^-$ Gal$^-$ β gal$^-$ (—)

Different types of asci resulting from the cross of two strains with lac1 LAC2 and LAC1 lac2 genotypes

	Parental ditypes	Tetratypes	Non parental ditypes
Genotypes	lac1 LAC2	lac1 lac2	lac1 lac2
	lac1 LAC2	lac1 LAC2	lac1 lac2
	LAC1 lac2	LAC1 LAC2	LAC1 LAC2
	LAC1 lac2	LAC1 lac2	LAC1 LAC2
Phenotypes	4$^+$/0$^-$	3$^+$/1$^-$	2$^+$/2$^-$
2359$\times$6315	3	7	3
11630$\times$11631	9	19	2

Enzymatic study of the parent strains. The measurements of the activity of β-galactosidase, the three enzymes of the Leloir pathway, and of lactose and galactose permeases were performed using cells grown during 15 h on MM + glycerol (non-induced) and on MM + galactose (induced). The assay for β-galactosidase activity was performed using cells which were permeabilized with toluene. The Leloir pathway enzymes were assayed using the ground cell supernatant. The grinding yield, i.e. proteins in the ground cell supernatant as percent of whole cell proteins, was always between 55 and 60%

Following growth on glycerol (Table II), the activity of the three enzymes of the Leloir pathway was nearly nil for all strains tested. However, the activity of β-galactosidase and the rates of penetration of lactose and galactose were greater in strain 2359 than in the other three strains (ten-fold for β-galactosidase and three-fold for lactose permease). The β-galactosidase and the lactose and galactose permeases seemed to be partially constitutive in strain 2359.

Following growth in the presence of an inducer, the β-galactosidase activity was about twice greated for strain 2359 as compared to the other strains tested. The galactokinase and the transferase are also more active for this strain. It is noteworthy that only strain 2359 has a transport system for galactose.

Table II

Enzymic activity of parent strains in the absence (value above the bar) and in the presence (value below the line) of an inducer

Strains	β-Galactosidase[a]	Galactokinase[b]	Transferase[b]	Epimerase[b]	Lactose trar sport[c]	Galactose transport[c]
2 359	20 430 / 1 512	2274 / 0	84 / 0	130 / 0	0.7 / 0.3	1.66 / 1.20
6 315	9 100 / 130	101 / 0	23 / 2	400 / 0	0.5 / 0.1	* / *
11 630	7 078 / 185	530 / 0	28 / 4	100 / 5	2.0 / 0.1	* / *
11 631	6 700 / 129	210 / 0	40 / 1	40 / 2	0.5 / 0.1	* / *

[a] Activity expressed as n moles of ONPG hydrolyzed per mg protein, per min
[b] Activity expressed as n moles substrate transformed per mg protein, per min
[c] μMoles substrate penetrating the cells per min, per g dry matter. Penetration was measured during the first 10 min
* Non detectable

Genetic analysis of the constitutive character. The above results required further study of the constitutive character of the β-galactosidase and other enzymes of strain 2359. Strain 6315 was crossed with strain 2359. The 9 resulting asci (36 spores) were assayed for their β-galactosidase activity in the absence of an inducer. The spores with a high activity (more than 1000) were named C^-, those with a low activity (less than 500) were named C^+.

The study of this crossing corresponds in fact to the study of the segregation of three genes: LAC1 and LAC2 and gene C coding for the constitutive character. Twelve genotypically different asci were obtained, 6 of which were phenotypically distinguishable (Table III). Among the 9 asci tested, 5 different phenotypes were obtained corresponding to the expected phenotypes. The hypothesis that the consitutive character is coded by a gene seems probable.

Another complementary study was performed comparing the number of spores of different expected phenotypes and those obtained from the cross of strain 2359 (genotype lac1 LAC2 C^-) and 6315 (genotype LAC1 lac2 C^+). The results are summarized in Table IV. Among the 36 spores to be tested, there would be 18 spores C^- and 18 spores C^+ if the constitutive feature were monogenic. However, 11 of the resulting spores were lac1 lac2 without any β-galactosidase activity. The constitutive feature study had to be carried out on the remaining 25 spores. If the genes are unlinked, there should be equal numbers of C^+ and C^- spores. The experimental results were 12 spores C^- and 13 spores C^+. The hypothesis concerning the monogenic character for the constitutive character is thus confirmed.

Table III

Genetical analysis of the 3 genes LAC1, LAC2 and C

Liaison type between LAC1/LAC2	Non parental ditype NPD		Parental ditype PD			
Genotype	LAC1 LAC2 C$^+$ LAC1 LAC2 C$^-$ lac1 lac2 C$^+$ lac1 lac2 C$^-$	LAC1 LAC2 C$^-$ LAC1 LAC2 C$^-$ lac1 lac2 C$^+$ lac1 lac2 C$^+$	LAC1 LAC2 C$^+$ LAC1 LAC2 C$^+$ lac1 lac2 C$^-$ lac1 lac2 C$^-$	LAC1 lac2 C$^+$ LAC1 lac2 C$^+$ lac1 LAC2 C$^-$ lac1 LAC2 C$^-$	LAC1 lac2 C$^+$ LAC1 lac2 C$^-$ lac1 LAC2C$^+$ lac1 LAC2 C$^-$	LAC1 lac2 C$^-$ LAC1 lac2 C$^-$ lac1 LAC2 C$^+$ lac1 LAC2 C$^+$
	—	+	—		+	
Phenotype	+	+	—		+	
	0	0	0		—	
	0	0	0		—	
Theorical frequency	1/12	1/12	1/12		3/12	
Number of asci	0	2	1		1	

	Tetratype TT					
Genotype	LAC1 lac2 C$^+$ LAC1 LAC2 C$^+$ lac1 LAC2 C$^-$ lac1 lac2 C$^-$	LAC1 lac2 C$^-$ LAC1 LAC2 C$^+$ lac1 LAC2 C$^+$ lac1 lac2 C$^-$	LAC1 lac2 C$^+$ LAC1 LAC2 C$^-$ lac1 LAC2 C$^+$ lac1 lac2 C$^-$	LAC1 lac2 C$^+$ LAC1 LAC2 C$^-$ lac1 LAC2 C$^-$ lac1 lac2 C$^+$	LAC1 lac2 C$^+$ LAC1 LAC2 C$^+$ lac1 LAC2 C$^-$ lac1 lac2 C$^+$	LAC1 lac2 C$^-$ LAC1 LAC2 C$^-$ lac1 LAC2 C$^+$ lac1 lac2 C$^+$
		+			+	
Phenotype		—			+	
		—			—	
		0			0	
Theorical frequency		3/12			3/12	
Number of asci		1			4	

β-Galactosidase activity was measured following spore culture on MM + glycerol
(—) Weak activity (less than 500)
\+ Strong activity (more than 1000)
0 No activity

Table IV

Genetical analyses of different spore types resulting from the cross of strain 2359 (lac1 LAC2 C⁻) with strain 6315 (LAC1 lac2 C⁺)

Genotype	Phenotype β-galactosidase activity in absence of inducer	No. of spores obtained	Theorical % spores	Observed % spores
lac1 lac2 C⁻ lac1 lac2 C⁺	0	11	25	30
lac1 LAC2 C⁺ LAC1 lac2 C⁺ LAC1 LAC2 C⁺	— (less than 500)	13	37.5	36
lac1 LAC2 C⁻ LAC1 lac2 C⁻ LAC1 LAC2 C⁻	+ (more than 1000)	12	37.5	33

Study of the Leloir pathway enzymes and the lactose and galactose transport systems. The enzyme assays were performed for 8 different genotype spore obtained from the two above crosses. The culture conditions were the same as for the parent strains when the spores would grow on galactose. Those which could neither use lactose nor galactose were grown on MM + glycerol. After three generations, galactose was added (5 g/l) to the medium. Enzymes assays were performed following 15 h of culture in the presence of the inducer. For these latter strains, we verified that lactose and galactose penetrated into the cells by passive transport. Three groups of spores could be distinguished from each other according to their enzymic activities and their genotypes (Table V). The lac1 lac2 spores have no activity in the absence of inducer and a very

Table V

Enzymatic activities of galactokinase, transferase and epimerase of strains with different genotypes following growth without inducer (MM + glycerol) and with an inducer (MM + galactose)

Genotype	No. of strains tested	Galactokinase*		Transferase*		Epimerase*	
		MM + glycerol	MM + galactose	MM + glycerol	MM + galactose	MM + glycerol	MM + galactose
lac1 lac2 C⁻ lac1 lac2 C⁺	11	0	0	0	10	0	4
LAC1 lac2 C⁺ lac1 LAC2 C⁺ LAC1 LAC2 C⁺	10 10	0	415±230	2±1	71±20	3.3±1	12±5
LAC1 lac2 C⁻ lac1 LAC2 C⁻ LAC1 LAC2 C⁻	8 5	0	1085±190	7.1±2	128±20	6.2±3	36±10

* Activity expressed as nmole substrate transformed per mg protein, per min

The results given are means and standard deviations calculated from all spores of each group

weak activity if they were associated with C^+ or C^-. The presence of at least one of the LAC1 or/and LAC2 gene seems to be required for the induction of the enzyme systems studied.

Among the spores with the lac1 LAC2; LAC1 lac2; LAC1 LAC2 genotypes, two groups could be distinguished according to the C^+ or C^- character. Significant differences could be observed for the galactokinase, transferase and epimerase activities between the two groups. The activity level was found to be about twice greater for C^- spores than for C^+ spores either in the presence or in the absence of an inducer.

The transport systems for lactose and galactose were then studied. The assays were performed on cells which were grown as previously described. The results are summarized in Table VI. Again, the spores with the lac1 lac2 genotype were shown to have no lactose or galactose permease activity. The spores with the LAC1 lac2; lac1 LAC2 and LAC1 LAC2 all had a lactose permease activity inducible by lactose and galactose. On the other hand, as only strain 2359 has a galactose transport system, among the descendants of the 2359 × 6315 cross there were spores with a galactose permease activity. The study of the galactose transport system of strain 2359 was undertaken separately (unpublished results). The influence of C on the two transport systems was difficult to demonstrate. The analytical techniques used were not sufficiently sensitive for such a study.

The two C^+ and C^- alleles were defined according to the β-galactosidase activity following culture in the presence of an inducer. The action of gene C

Table VI

Lactose transport activity and galactose transport activity after growth without (MM + glycerol) or with inducer (MM + lactose) or (MM + galactose)

Genotypes	No. of spores tested	MM + glycerol		MM + lactose		MM + galactose	
		Lactose transport	Galactose transport	Lactose transport	Galactose transport	Lactose transport	Galactose transport
lac1 lac2 C^- lac1 lac2 C^+	11	*	*	*	*	*	*
LAC1 lac2 C^+ lac1 LAC2 C^+	7	*	*	0.5 à 1.2	* ou 0.1 à 0.2	0.5 à 2	* ou 0.2 à 0.3
LAC1 LAC2 C^+	6	*	*	1 à 2	* ou 0.1 à 0.6	1.5 à 3.5	* ou 0.2 à 0.3
LAC1 lac2 C^+ lac1 LAC2 C^-	7	* ou 1 à 2	* ou 1 à 1.5	1 à 2	* ou 1 à 1.5	1.5 à 3.5	* ou 0.5 à 1
LAC1 LAC2 C^-	5						

The rates of galactose and lactose penetration were obtained by measuring the penetration of this sugar during the first 10 min. Lactose and galactose uptake are expressed as micromoles of lactose or galactose taken up per g of dry weight and per min

* Assay non significant

Table VII

β-Galactosidase activity of strains with different genotypes following growth in the absence (MM + glycerol) and in the presence (MM + galactose) of an inducer

Genotypes	No. of strains tested	β-Galactosidase (1)	
		MM + glycerol	MM + galactose
lac1 lac2 C⁻ lac1 lac2 C⁺	11	0	423±266
lac1 LAC2 C⁺ LAC1 lac2 C⁺ LAC1 LAC2 C⁺	 10 10	370±150	 9 209±2884 18 254±1990
lac1 LAC2 C⁺ LAC1 lac2 C⁻ LAC1 LAC2 C⁻	 8 5	1757±573	 22 630±1264 25 920±1428

[1] Activity expressed as nmoles ONPG hydrolyzed per mg protein, per min

[2] The results given are means and standard deviations calculated from all spores of each group

is in fact more general and also affects the activity of the galactokinase transferase and epimerase enzymes. The presence of LAC1 and/or LAC2 was also shown to be required for the synthesis of the enzymes of the Leloir pathway and of the lactose and galactose transport system. In order to clarify the role of the C, LAC1 and LAC2 genes and their association another study of β-galactosidase was undertaken using spores of 8 different genotypes.

Study of the β-galactosidase activity and of the differential rate of biosynthesis of β-galactosidase. The β-galactosidase activity was assayed on cells which were grown as previously described. Following culture on galactose, 5 levels of activity were observed for the following gene associations: C⁺ or C⁻ lac1 lac2; C⁺ LAC1 LAC2; C⁺ LAC1 lac2 or C⁺ lac1 LAC2; C⁻ LAC1 LAC2; and C⁻ LAC1 lac2 of C⁻ lac1 LAC2 (Table VII). The spore with the genotypes lac1 lac2 C⁻ or C⁺ have a very low level activity, similar to the basal level of the C⁺ LAC1 and/or LAC2 spores when these latter were grown on glycerol. Strains with the C⁻ genotype have a β-galactosidase activity 2.4 to 2.8 times greater than those of the C⁺ genotype with the harvest activity. However, within the C⁺ group or the C⁻ group there were appreciable differences when the genotypes were LAC1 LAC2 or LAC1 lac2, lac1 LAC2. The enzymatic activity was twice greater among LAC1 LAC2 C⁺ strains compared to strains with the LAC1 lac2 C⁺ and lac1 LAC2 C⁺ genotypes. A gene dosage effect was observed with the LAC1 LAC2 association.

A complementary study was performed in order to determine the differential rate of biosynthesis of β-galactosidase ($\Delta E/\Delta DO$) for each genotype following cell growth on glycerol and following the addition of galactose as inducer. Table VIII summarized the results obtained. The differential rate of biosynthesis following cell growth on glycerol was 10-fold greater for strains

Table VIII

Differential rates of biosynthesis of β-galactosidase (ΔE/Δ OD) of strains with different genotypes grown on glycerol and glycerol + galactose

Genotypes	No. of spores tested	ΔE/Δ DO		$K = \frac{\Delta E/\Delta \text{ DO gal}}{\Delta E/\Delta \text{ DO gly}}$
		Grown on glycerol	Grown on glycerol + galactose	
LAC1 lac2 C$^+$ lac1 LAC2 C$^+$	3	211±117	2 316±1032	11
LAC1 LAC2 C$^+$	6	211±117	6 633±1877	31
LAC1 lac2 C$^-$ lac1 LAC2 C$^-$	1	2000	17 900	9
LAC1 LAC2 C$^-$	3	2000±178	18 533±3300	9

E = enzymatic activity per ml culture
K = inductibility factor

with C$^-$ genotype compared to those with C$^+$ genotype. Following growth on galactose, the activity appeared to be maximum among C$^-$ spores whereas its level varied among C$^+$ spores depending on their genotypes lac1 LAC2; LAC1 lac2 or LAC1 LAC2. Among these latter strains, the induction factor was 2.8-fold more higher for the LAC1 LAC2 strains compared to those with the LAC1 lac2 and lac1 LAC2 genotypes.

A gene dosage effect was shown by Tingle and Halvorson [1] for diploids from the Y 1140 (lac1 LAC2) and Y 1118 (LAC1 lac2) cross. In our case, an identical phenomenon was observed among haploid strains with the LAC1 LAC2 C$^+$ genotype. This effect was masked in spores with the C$^-$ genotype. The C$^-$ gene alone was responsible for activity and induction levels close to maximum.

Conclusion

The roles of genes LAC1 LAC2 and C in the biosynthesis of enzymes for the metabolism or lactose and galactose were analyzed. Herman and Halvorson [3] perviously showed that genes LAC1 and LAC2 controlled the regulation of biosynthesis of β-galactosidase. Results reported in the present work indicate that these two genes also control the biosynthesis of the three enzymes of the Leloir pathway, the lactose and the galactose transport system when present. A gene dosage system was demonstrated for β-galactosidase.

Dickson et al. [7] demonstrated the presence of a gene LAC10, the product of which was at least partially required for the regulation of the biosynthesis of β-galactosidase, galactokinase and lactose permease. These authors proposed the hypothesis that a negative regulation system was involved concerning gene

LAC10. The gene C studied in the present work could be the same LAC10: further complementation tests on different strains are to be performed in order to confirm this hypothesis. The following regulation scheme is proposed according to our results. Gene C would code for a repressor (negative factor) whereas LAC1 and LAC2 would code for a positive factor affecting directly or indirectly the biosynthesis of β-galactosidase, the three enzymes of the Leloir pathway and the transport system. When the C^+ allele was present and in the absence of an inducer, genes LAC1 and LAC2 could not be expressed. However, in the presence of an inducer, the repression by C^+ was partially lifted so that the activities of the different enzymes were not at their maximum level. When the C^- allele was present, the repression was weaker and the enzyme activity levels were higher than in C^+ strains. In the presence of an inducer, the repression was completely lifted, the enzyme activities were then at their maximum levels. In strains with gene C^+, in the presence of an inducer, the partial repression of genes LAC1 and LAC2 could be compensated by the simultaneous presence of these two genes; their products would be twice greater and the biosynthesis of the different enzymes would increase. This would explain the gene dosage effect observed for the LAC1 LAC2 strains. LAC1 and LAC2 would code for a factor indispensable for the expression of structural genes of the different enzymes or of their products Among the strains with the genotype lac1 lac2, this factor would not be synthetized, this would explain the absence of activity of the enzymes studied.

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A NEW HYDROXAMATE SIDEROPHORE FOR IRON SUPPLY OF *SALMONELLA*

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A new hydroxamate siderophore was isolated from *Salmonella stanleyville* strain 207/81. The organism exhibited an unusual siderophore pattern in failing to synthesize enterobactin and aerobactin. The purified siderophore was characterized by cross-feeding, paper chromatography, thin layer chromatography and paper electrophoresis. In respect to iron supply of *Salmonella* strains there were relationships between this and other well-known siderophores, e.g. enterobactin, aerobactin, rhodotorulic acid and ferrichrome. The new hydroxamate siderophore was detected in all *Salmonella* strains tested

One of the mechanisms for acquisition of iron by bacteria is the excretion of iron specific chelators, termed siderophores, the function of which is to sequester and transport iron via specific transport proteins into the cell. In *Salmonella* strains two types of siderophores were found [1, 2]. There are strains which produce enterobactin alone and some which produce enterobactin and aerobactin. In screening investigations of *Salmonella*, in respect to their "siderophore pattern" strains with unusual properties were detected. Beside others, the strain *Salmonella stanleyville* 207/81 was unable to synthesize enterobactin or aerobactin. Investigations on the effective iron supply system, presented in this paper, led to a new hydroxamate, later found in all *Salmonella* strains tested.

Material and methods

Bacterial strains. The bacterial strains applied as indicator strains in the bioassays are listed in Table I, those for investigating the new hydroxamate siderophore are presented in Table II.

Bioassays for siderophores and relationships between siderophores. In order to characterize the properties of the siderophores functionally, we decided to study the ability to cross-feed different well characterized indicator strains. The bioassays for enterobactin and aerobactin were performed as described by Luckey et al. [3] and Rabsch and Reissbrodt [4]. The hydroxamate bioassay was used as described by Powell et al. [5] with *Arthrobacter flavescens* JG-9 as indicator strain. Investigations with *S. stanleyville* 207/81 as indicator strain was performed in tris-succinate medium supplemented with glucose (0.5%) as described by Rabsch and Reissbrodt [4]. The filter paper discs for cross-feeding test were loaded with 5 μl from each of the siderophore solution ($\sim 10^{-3}$ M). Relationships between siderophores in the cross-feeding test were observed by reading the bioassay plates after a further incubation at room temperature for 24 h. Relationship between two siderophores was indicated by a new zone of growth of the indicator strain outside the first growth zones and between both siderophores. On the other hand, an inhibition of one of the first growth zones was also considered as a relationship. Such relationships were found also without the first growth zones, only from the siderophore which diffused into the agar medium.

Chemicals. Rhodotorulic acid, ferrichrome, N^6-acetyl-N^6-hydroxylysine and aerobactin were gifts of J. B. Neilands (Berkeley, California, USA). Desferal (deferriferrioxamine B

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mesylate) was obtained from Ciba-Geigy AG, Basel, Schweiz, 2,3-dihydroxybenzoic acid (DHBA) was a gift of H. Feist (Institut für Tierseuchenforschung, Jena, DDR). Enterobactin was self prepared as described by Young et al. [6].

Isolation of the new hydroxamate. The *Salmonella* strains tested were incubated in 200 ml shaken liquid tris-succinate medium supplemented with glucose (0.5%), pH 7.4, at 37 °C for 24 h. The cell suspension was centrifuged at 5000 rpm and concentrated in a rotary evaporator to 10 ml. Extraction was performed according to Simpson and Oliver [7], ion-exchange chromatography as described by Braun [8].

The concentrate was extracted with 10 ml of phenol–chloroform (1:1) and the extraction repeated once more. After addition of 50 ml of diethylether, the phases were separated by addition of 10 ml of water. The organic solvents were evaporated, the aqueous solution was run through an Amberlite XAD-2 column (Serva, Heidelberg, GFR) (1×14 cm). Then it was washed with water, NaCl solution (2%) and sodium desoxycholate solution (2%). After extensive washing with water, it was eluted with ammonia solution (25%): methanol (1:1). After evaporation the residue was dissolved in water and mixed with acetic acid (1%). The sedimented DNA was removed by centrifugation. The rough extract was concentrated. Isolation and purification of the new hydroxamate was performed by preparative paper chromatography, carried out on FN4-Papier (VEB Spezialpapierfabrik Niederschlag, Erzgebirge, DDR) with solution I: ammonia solution (25%): ethanol: water (5:80:15) and solution II: ammonium formiate (5%) in 0.5% formic acid. Thin layer chromatography was carried out on cellulose TLC (Serva, Heidelberg, GFR) with ammonia solution (25%):ethanol:water (5:80:15). Paper electrophoresis was performed on FN4 paper in borate buffer pH 9.3, 300 V, 30 mA, 90 min. As spray reagent for siderophores, ethanolic $FeCl_3$ solution was used.

The colours of the spots after spraying with $FeCl_3$ was red-violet in the case of the new hydroxamate, all the other hydroxamates were violet-red, N^6-acetyl-N^6-hydroxylysine was blue-violet. Enterobactin and 2,3-DHBA showed up as dark-violet spots.

Hydroxamates were determined by the Csáky test [10], and phenolic type siderophores by the method of Arnow [11]. The estimation of proteins was performed as described by Lowry et al. [12]. For investigation of the amino acid composition, the substance was hydrolyzed with 6 M HCl at 100 °C for 18 h and then separated by thin layer chromatography on Cellulose DC Alufolie (E. Merck, Darmstadt, GFR) with n-butanol : acetic acid : water (80:20:20); the spray reagent was ethanolic ninhydrine solution (0.2%).

The microbiological activity of the separated substances was determined by cross-feeding test on different indicator strains in the same manner as described by Rabsch et al. [9]. Paper strips were placed on the bioassay plates. After incubation overnight the growth of the indicator strains around the separated effective siderophores was evaluated.

Results

Using different bioassays in screening investigations of *Salmonella* strains, different siderophore patterns were found (Table III). Especially strain *S. stanleyville* 207/81 showed unusual properties. This strain formed neither enterobactin nor aerobactin and could cross-feed only the indicator strain *S. typhi-murium* enb-7. For growth, glucose supplemented liquid medium poor in iron was necessary. The effective hydroxamate siderophore could be isolated from the supernatant of *S. stanleyville* 207/81 culture in glucose supplemented tris-succinate medium by methods described above. Later, the isolation of this hydroxamate was possible from all *Salmonella* strains tested (Table III).

This hydroxamate, formed under low iron conditions, was able to cross-feed the mutant *S. typhi-murium* enb-7, the indicator strains *Arthrobacter flavescens* JG-9 and *S. stanleyville* 207/81, but not the mutants *Escherichia coli* LG1522 or *S. typhi-murium* TA2700 (Table IV). The substance promoted the

Table I

Indicator strains used in the experiments

Strain	Cross-feeding with siderophores or siderophore precursors	Origin
S. typhi-murium LT2 enb-7	enterobactin, 2.3-dihydroxybenzoic acid (DHBA), ferrichrome, Desferal®, rhodotorulic acid	J. B. Neilands[1]
S. typhi-murium LT2 TA 2700	enterobactin	J. B. Neilands[1]
E. coli LG 1522	aerobactin, rhodotorulic acid	P. H. Williams[2]
Arthrobacter flavescens JG-9	many hydroxamates	P. J. Szaniszlo[3]

[1] University of California, Berkeley, USA
[2] University of Leicester, England
[3] University of Texas, Austin, USA

Table II

Bacterial strains used in the experiments

Wild strains	Source	Origin
S. typhi-murium 80/82	hospital infection	Rabsch et al. [9]
S. typhi-murium 330/84	food poisoning	Rabsch et al. [9]
S. stanleyville 207/81	diarrhoea of cattle	this study
S. memphis	unknown	J. B. Neilands[1]
S. austin	unknown	J. B. Neilands[1]
S. typhi-murium 45/78	hospital infection	this study
S. typhi-murium 1028/78	hospital infection	this study
S. typhi 92/84	from a carrier	this study
S. montevideo 7770/85	hospital infection	this study
S. heidelberg 5436	hospital infection	this study
S. typhi-murium 363/84	salmonellosis of a pigeon	this study

[1] University of California, Berkeley, USA

growth of *S. stanleyville* 207/81 (Fig. 1a) and inhibited the growth of *Salmonella memphis* (Fig. 1b).

Cross-feeding properties and the R_F-values of the hydroxamate isolated from any of the *Salmonella* strains listed in Table IV were the same, as well as cross-feeding activity and the red-violet colour of the separated spots. In paper electrophoresis the hydroxamate migrated towards the cathode at pH 9.3 (Fig. 2). The new hydroxamate showed different properties as compared to other siderophores or siderophore precursors (Tables IV and V). Surprisingly it

Table III

Siderophore pattern of different Salmonella strains

Strain	Indicator strains*		
	S. typhi-murium enb-7	*S. typhi-murium* TA 2700	*E. coli* LG 1522
S. typhi-murium 330/84	+	+	−
S. typhi 92/84	+	+	−
S. montevideo 7770/85	+	+	−
S. heidelberg 5436	+	+	−
S. typhi-murium 363/84	+	+	−
S. typhimurium 32/79	+	+	+
S. memphis	−	−	+
S. typhi-murium 80/82	+	−	+
S. stanleyville 207/81	+	−	−

* For siderophores see Table I

Table IV

Cross-feeding of siderophores and siderophore precursors including the new hydroxamate compound from different Salmonella strains

Siderophore precursor	Cross-feeding with				
	Arthrobacter flavescens JG-9	*E. coli* LG 1522	*S. stanleyville* 207/81	*S. typhi-murium* enb-7	*S. typhi-murium* TA 2700
Ferrichrome	+	−	+	+	−
Rhodotorulic acid	+	+	+	+	−
Desferal®	+	−	+	+	−
Aerobactin	−	+	−	−	−
N^6-acetyl-N^6-hydroxylysine	−	−	−	−	−
Enterobactin	−	−	+	+	+
2,3-Dihydroxybenzoic acid	−	−	−	+	−
New hydroxamate from					
S. typhi-murium 80/82	+	−	+	+	−
S. typhi-murium 45/78	+	−	+	+	−
S. typhi-murium 1028/78	+	−	+	+	−
S. stanleyville 207/81	+	−	+	+	−

was possible to extract the hydroxamate from the supernatant by ethylacetate at pH 1.5.

The hydroxamate-siderophore was positive in the Csáky test, negative, in the Arnow test and gave the Folin reaction. Serine or lysine were not found but isoleucine/leucine, phenylalanine and valine were present.

Beside the first growth zones with the siderophores tested, a second growth zone appeared after further staying at room temperature for 24 h

Table V

Behaviour of different siderophores and siderophore precursors in the paper chromatography, thin layer chromatography and paper electrophoresis

Method	R_f-values for							
	New *Salmonella* hydroxamate	Enterobactin	2,3-Dihydroxybenzoic acid	Aerobactin	N^6-Acetyl-N^6-Hydroxylysine	Rhodotorulic acid	Ferrichrome	Desferal®
PC sol. I	0.65	n.d.	n.d.	0–0.2*	0.57	n.d.	n.d.	n.d.
sol. II	0.95	0.53	0.63	n.d.	n.d.	n.d.	n.d.	n.d.
TLC	0.55	n.d.	n.d.	0–0.15**	0.64	0.67	0.64	0.87
Paper electrophoresis	cathode-side, 1.7 cm**			anode side, 3.3 cm**				

* Aerobactin migrated in association with LPS, two activities were found on the paper chromatography strip, but only one activity after electrophoresis

** Migration length

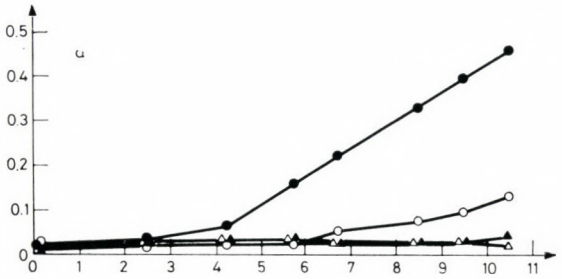

Fig. 1a. Promotion of the growth of *S. stanleyville* 207/81 by the new hydroxamate in tris-succinate medium. Ordinate, extinction at 620 nm; abscissa, time in hours. ● with the new hydroxamate and with glucose; ○ with glucose only; ▲ with the new hydroxamate but without glucose; △ without new hydroxamate and glucose

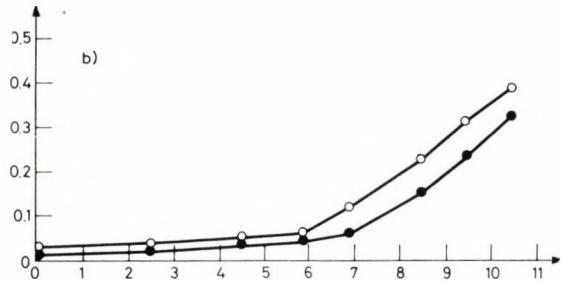

Fig. 1b. Inhibition of the growth of *S. memphis* by the new hydroxamate in tris-succinate medium. Ordinate, extinction at 620 nm; abscissa, time in hours. ● with the new hydroxamate; ○ without the new hydroxamate

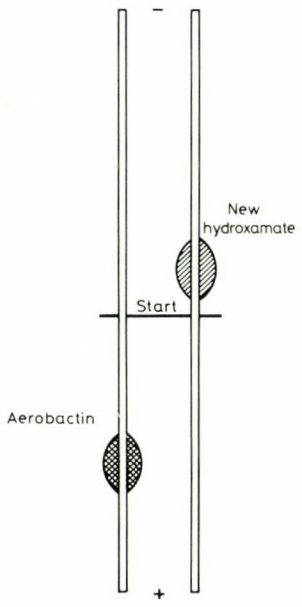

Fig. 2. Two hydroxamates, separated by paper electrophoresis in the same run (see Materials and methods). Left strip: aerobactin, cross-feeding with *E. coli* LG 1522; right strip: new hydroxamate, cross-feeding with *S. stanleyville* 207/81

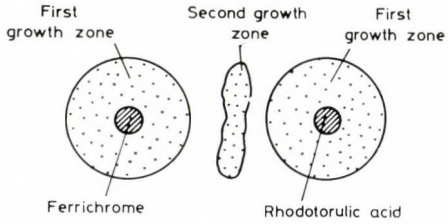

Fig. 3. Relationship between ferrichrome and rhodotorulic acid is indicated by a second growth zone in cross-feeding test with the indicator strain *S. stanleyville* 207/81

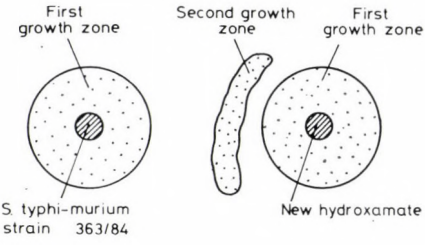

Fig. 4. Relationship between siderophores excreted by *S. typhi-murium* 363/84 and the new hydroxamate in cross-feeding test with the indicator strain *S. stanleyville* 207/81

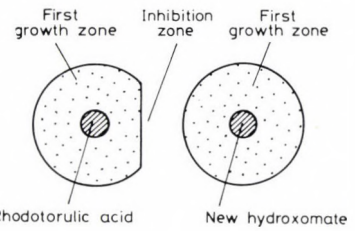

Fig. 5. Inhibition by the new hydroxamate of the growth zone formed around the rhodotorulic acid filter paper disc in cross-feeding test with the indicator strain *S. stanleyville* 207/81

(Figs 3 and 4). On the other hand, there were inhibitions of the first growth zones between rhodotorulic acid and the new hydroxamate (Fig. 5). Such relationships between different siderophores were found in the most cases with the indicator strain *S. stanleyville* 207/81, but also with the mutant *S. typhimurium* enb-7.

Discussion

Some *Enterobacteriaceae* species such as *Escherichia coli* (Warner et al. [13]), *Shigella sonnei* (Perry and San Clemente [14]), *Aerobacter aerogenes* (Gibson and Magrath [15]), *Salmonella typhi-murium* (Colonna et al. [16], Rabsch et al. [9]) synthesize more than one type of siderophore. *Salmonella* strains produce either enterobactin or aerobactin alone or both (McDougall and Neilands [2]).

The low amounts of enterobactin formed by host-adapted *Salmonella* strains, e.g. *S. typhi* (Rabsch and Reissbrodt in preparation) led to the consideration for further siderophores involved in the process of iron supply. In some wild strains of salmonellae unusual siderophore patterns were found (Table II). By extraction and purification of the supernatant of the enterobactin- and aerobactin-negative strain *S. stanleyville* 207/81, a new hydroxamate siderophore was isolated. This hydroxamate siderophore was found in other *Salmonella* strains tested, too. As indicated chemically and by cross-feeding tests this substance was different from enterobactin or aerobactin, as well as from their precursors. In liquid tris-succinate medium poor in iron the formation of this hydroxamate was dependent on supplementation with glucose (0.5%). This was in agreement with the growth of *S. stanleyville* 207/81. Different complex formation constants among siderophores explain the different effectiveness of the hydroxamate for promotion or inhibition of growth of *Salmonella* strains. This is in coincidence with the relationships between the siderophores observed in the cross-feeding tests (Figs 3–5). Between the siderophores iron is exchanged in dependence of complex formation constants, pH and time. One of the siderophores can mediate the transfer carrier of iron to an

other siderophore (Raymond et al. [17]). Regarding the observations on relationships between the siderophores, the following preliminary conclusion can be drawn for the complex formation constants: enterobactin > new hydroxamate of salmonellae > aerobactin > rhodotorulic acid.

The significance of the amino acid content in the new hydroxamate is unclear. All three amino acids lack the ε-amino group necessary for formation of hydroxamic acid group. As a prerequisite to an exact chemical formulation of the molecule, a further purification is necessary. Beside this, *S. stanleyville* 207/81 can be applied in the cross-feeding tests to investigate strains for this hydroxamate. The uptake system for this hydroxamate in respect to the receptors in the cell wall is unknown until now. *S. typhi-murium* enb–7 contains receptors for many siderophores (Table I). The aerobactin receptor is not involved in the uptake of the hydroxamate siderophore, as(i) the cross-feed is independent of cloacin added to the disc; (ii) the aerobactin dependent *S. memphis* cannot use this hydroxamate. The importance for the cell of the new hydroxamate described in this paper is unclear until now.

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DETECTION OF *CHLAMYDIA TRACHOMATIS* IN THE FEMALE GENITAL TRACT BY FLUORESCEIN-LABELLED MONOCLONAL ANTIBODY

(A NOTE)

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Endocervical smears were examined in a high-risk group of women using the Chlamyset^{R} "Direct Chlamydia Antigen Detection Test". From the 66 samples 42 were positive for *Chlamydia trachomatis* with highest incidence in the infertile group and in patients complaining of recurrent discharge. No correlation has been found between chlamydial endocervical infection and infected vaginal smears.

The pathogenic role of *Chlamydia trachomatis* has been described in a number of disease patterns. Its clinical importance has been known for a long time in tropical endemic trachoma involving blindness. Its role in lymphogranuloma venereum has been recognized later. During the last two decades attention has been called to its part in urogenital infections [1–5].

According to several authors [6, 7] chlamydiae are the most frequent sexually transmitted pathogens in industrial countries. Reports from the US and Western Europe estimate the occurrence of *C. trachomatis* infection 1–7% in healthy males and 5–20% in women [8]. Predominance of the infections showed interdependence with the social and economic conditions, as well as the sexual activity of the patients [4, 8].

In Hungary several reports have been published on the occurrence of *C. trachomatis* infection [3, 9–14], but no report has been found on examinations in gynecological patient material.

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Materials and methods

The "Direct Chlamydia Antigen Detection Test" (Chlamyset^R, Orion Diagnostica, Espoo, Finland) was used. The reagent contains freeze-dried, monoclonal fluorescein-labelled *C. trachomatis* antibody produced in mice. Evans blue was used for contrast-staining. The antibody reacts with all *C. trachomatis* serotypes but not with *C. psittaci* [15, 16] and certain discrepancies can be detected in reactivity [17].

Specimens were obtained from the cervix and the squamocolumnar junction of 66 pregnant and gynecological patients. A small aseptic dacron swab (Chlamyset) was rotated several times in the cervix to remove cellular elements. Smears were prepared on special slides (Chlamyset), allowed to dry, then fixed in methanol for 5 min. Incubation was performed at room temperature in moist chamber for 15 min by using 30 μl fluorescein-isothiocyanate-conjugated monoclonal antibody. After a short wash in distilled water, a coverslip was applied by means of 20 μl adhesive solution containing 50% glycerol and 50% 0.1 M tris pH 8.5. The preparations were examined with Zeiss Fluovall fluorescent microscope by immersion at $\times$1000 magnification. Positive result was indicated by at least 10 apple green fluorescent disks against the counterstained red background.

Results and discussion

Table I shows the occurrence of *C. trachomatis* in patients with different degrees of purity of the vaginal flora and with *Trichomonas vaginalis* positive findings. No correlation was found between the constitution of the vaginal flora and *C. trachomatis* positivity.

Table II shows the occurrence of *C. trachomatis* positivity according to various clinical conditions. Highest positivity was found in secondary infertility and in patients with therapy resistant discharge.

C. trachomatis is an intracellular obligate parasite [10]. In the female genital tract it occurs most frequently in the cervix [4, 5] and is responsible for endometrial and tubal infections and for inclusion conjunctivitis of newborns [18].

Mucopurulent discharge is a sequela of endocervical chlamydial infection. Sometimes the process subsides without any clinical symptoms. *C. trachomatis* is a frequent finding in cervical ectropion [5, 19].

Table I

Occurrence of chlamydial cervical infections

	No. of patients				
	*Vaginal smears**			*T. vaginalis* positive	Total
	I	II	III		
C. trachomatis positive	11	14	13	4	42
C. trachomatis negative	6	8	8	2	24
Total	17	22	21	6	66

* Degree of alteration in normal vaginal flora

Table II

Occurrence of chlamydial infections in various clinical conditions

	No. of patients		
	C. trachomatis		Total
	positive	negative	
Erythroplasia	4	4	8
Pregnants free of complaints	3	6	9
Pregnants with colpitis	4	4	8
Secondary infertility	6	1	7
Adnexitis after artificial abortion	8	3	11
Recurrent fluor	17	6	23
Total	42	24	66

The present studies included samples from patients obtained directly before reconstructive operation due to extensive erythroplasia of the os uteri and negative vaginal discharge. Out of the eight patients positive result was obtained in four (Table II).

During pregnancy self-purification of the vagina becomes reduced in consequence of the suppression of lactobacilli and thus susceptibility to infections increases. In three out of nine pregnants free of complaints and in four out of eight endocervical *C. trachomatis* was shown (Table II).

Endometritis, salpingitis, and adnexitis can be sequelae of endocervical chlamydial infection [20]. The role of the organism in secondary infertility is well known. *C. trachomatis* was shown in 6 of the 7 patients with secondary infertility, as well as in 8 out of the 11 patients with adnexitis following artificial abortion (Table II).

The major part of gynecological outpatient consultation consists of patients complaining of vaginal discharge. Twenty-three of these patients were treated repeatedly due to recidivation. In spite of a normal or partly decreased lactobacillus content of the vaginal smears, they complained of a relapse of discharge. Chlamydial infection has been confirmed in 17 cases (Table II).

The exceedingly rapid performance of the test requiring altogether 30 to 40 min is of great advantage. It enables the examination of a large amount of samples with adequate sensitivity and specificity.

Väänänen et al. [17] found that sensitivity of the direct staining method with Chlamyset® Antigen reagent amounted to 89.3% and specificity to 98.7% when compared with cultivation. Similar results have been reported by Meurman et al. [16].

In view of the widespread occurrence of vaginal discharge, as routine examinations used commonly in gynecology will clear the aetiology only partly, it is advisable to make examination for the presence of chlamydiae.

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ISOLATION FROM FOOD AND CHARACTERIZATION BY VIRULENCE TESTS OF *YERSINIA ENTEROCOLITICA* ASSOCIATED WITH AN OUTBREAK*

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(Received December 15, 1985)

A *Yersinia enterocolitica* outbreak associated with "pork cheese" is described. The phosphate–sorbitol–bile method was combined with KOH pretreatment for isolation of the agent from food. The pathogenicity of the food and faecal isolates was verified with virulence tests: the calcium-dependent populations carried a 42 Mdal plasmid, showed HEp-2 penetration and disintegration and were autoagglutinable.

It has been known for more than half a century that *Yersinia enterocolitica* is pathogenic for man. Methods for the diagnosis of yersinia enteritis had already been available by the end of the 'sixties in a number of European countries including Hungary. Nevertheless, clinical diagnosis of yersiniosis still meets difficulties [1, 2]. The disease may be accompanied by various complications [3–5], and may have a number of extraintestinal manifestations without enteritis, such as thyroid illness [6], cholangitis [7], pharyngitis [8], arthritis, urethritis, glomerulonephritis [9], septicaemia associated with respiratory illness of adults [10], pneumonia [11] and muscle suppuration [12]. The symptoms may present considerable differential diagnostic problems in medicine and surgery [8, 13, 14].

More recently an intensive research work all over the world, aimed at characterizing the bacterium, has contributed to the carification of the epidemiology of *Y. enterocolitica* infections. As emphasized by several authors [15–17], yersinia enterocolitis should be regarded as a zoonosis. Morris and Feely [18] and Lee [19], among others, have pointed out that *Y. enterocolitica* may spread via the food chain. Numerous authors have called attention to its

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* Parts of this paper were presented at the 12th "Balaton" Public Health Days, Siófok, 1984, and at the Annual Meeting of the Hungarian Society for Microbiology, Nyíregyháza, August 22–24, 1984.

possible presence in fresh [20–23] and pasteurized [24] milk. Wauters' suggestion [25] that the domestic pig is the most important reservoir has been supported by others [26–29]. Aldová et al. [30] isolated so-called "clinical" and "environmental" strains of *Yersinia* from various foodstuffs, drinking waters and surface waters.

Reports from Hungary [31] and abroad [32] suggest that detection of *Y. enterocolitica* in foodstuff, in itself, does not prove the pathogenicity of this bacterium and its transmission by foodstuff. Strains isolated from foodstuff or water, either "clinical" or "environmental" ones, should be tested for virulence [33].

Based on epidemiological investigations, a number of authors [34–37] have reported *Y. enterocolitica* outbreaks presumably conveyed by food. However, due to the lack of reliable methods, isolation of this organism encounters difficulties even if samples from the suspect food are available. In the literature we found only three outbreaks in the course of which *Y. enterocolitica* had been isolated from samples of the food consumed by the patients. All the three were extensive outbreaks, occurred in the USA and were associated mainly with strains of serogroup O8 [38–40].

In the present report we describe an outbreak associated with the consumption of "disznósajt" (in literal translation, "pork cheese"). "Pork cheese" is a meat product containing small pieces of boiled chitterlings stuffed into a coat prepared from the stomach of the hog; after filling the product is processed at a temperature not above that of boiling water.

This was the first yersinia outbreak in Hungary, in which the causative agent was isolated from the suspect food.

Material and methods

Bacterial strains. The variants E14651 Ca^{++}-dependent (Ca-dep.) and E14651 Ca^{++}-independent (Ca-indep.) were isolated from the strain *Y. enterocolitica* E14651, which had been isolated from "pork cheese". The *Y. enterocolitica* B20187 is a representative of the three strains isolated from faecal samples during the outbreak under study; they appeared identical with one another in all the tests applied by us. We isolated a Ca-dep. and a Ca-indep. variant from strain B20187. *Y. enterocolitica* strain X8312 (serogroup O3, biotype 4) is a reference strain maintained in the laboratory. The strain *Escherichia coli* K_{12} HB101 (p.JB3JI) is a strain carrying a 40 Mdal plasmid [41].

Nutrient media. Medium 1. Phosphate–sorbitol–bile buffered solution (PSB), [42]. *Medium 2.* Rappaport's enrichment medium [43]. *Medium 3.* Deoxycholate-citrate (DC agar [43]). *Medium 4.* MacConkey's Tween 80 agar [44]. *Medium 5.* Simple broth [43]. Media used in Ca^{++}-dependence tests: in lack of MOX agar [45] containing bound calcium, we elaborated media 6–8 for this purpose. *Medium 6.* Calcium-free blood agar base: Bacto peptone (Difco), 10 g; Lab-Lemco Powder (Oxoid), 15 g; NaCl, 3 g; $Na_2HPO_4 \cdot 2\ H_2O$, 2 g; agar (Difco), 15 g; distilled water, 1000 ml; autoclaved at 115 °C for 30 min; pH 7.4. *Medium 7.* Blood agar base (BA) containing Ca^{++} (BA + Ca^{++}): 184 ml calcium-free blood agar base melted and cooled to 45 °C and 16 ml of 0.2 M $CaCl_2$ added. *Medium 8.* Blood agar base containing bound calcium (BA + Ca^{++} + MOX): 220 ml of BA + Ca^{++} agar solution cooled to 45 °C + 16 ml of 0.25 M calcium oxalate + 8 ml of 0.5 M $MgCl_2$ [45]. *Phosphate buffer diluent:*

Trypticasin (tryptic digest of casein, Reanal, Budapest), 1 g; $Na_2HPO_4 \cdot 7\,H_2O$, 10.9 g; KH_2PO_4, 3.5 g; distilled water, 1000 ml, pH 7.0; autoclaved at 115 °C for 30 min [46].

Isolation of bacteria. The media and methods used in examining bacterial contamination (except *Y. enterocolitica* contamination) of "pork cheese" are described in a Hungarian guide [46]. From faecal samples, *Y. enterocolitica* was isolated on DC agar medium.

For isolation of *Y. enterocolitica* from "pork cheese" samples three different methods were used. *Method I.* Twenty-five g of the sample were placed into 225 ml of Rappaport's enrichment medium and incubated at 37 °C for 48 h, subcultured on DC agar plates, which were incubated at 26 °C for 48 h. *Method II.* Twenty g of the sample were homogenized in 180 ml PSB solution. Two ml volumes were measured into 100 ml PSB solution in a 250 ml flasks (0.2 g sample/100 ml) [47]. The enrichment media were incubated at 4 °C for 6 days (Tables I, II/1) or for 14 days (Tables I, II/2). Then untreated cultures and cultures treated with KOH as described by Aulisio et al. [48] were plated on MacConkey's Tween 80 medium and DC agar. Incubation lasted at 26 °C for 48 h. *Method III.* Of each PSB enrichment culture incubated at 4 °C for 6 days or 14 days (see Method II) 1 ml was transferred into 100 ml of Rappaport's enrichment medium and incubated at 26 °C for 48 h, then untreated and KOH-treated cultures were inoculated on MacConkey's Tween 80 and DC plates; these were cultured at 26 °C for 48 h. Methods I, II and III were used parallel in the experiments and when food poisoning was suspected.

Examination of temperature-sensitive Ca^{++}-dependence. Very minute amounts of 48 h cultures of *Y. enterocolitica* grown on DC medium for 48 h were transferred into simple broth and the cultures were incubated at 26 °C for 18 h. Then, a tenfold dilution series was prepared up to 10^{-6}. From each dilution 0.1 ml was spread with a glass rod on the surface of BA plates (Series I and II). Series I was incubated at 26 °C, Series II at 37 °C, both for 48 h. Then the colonies were counted and marked on the bottom of the Petri dish, and the plates were re-incubated, both Series I and II at 26 °C for 48 h. On the plates preincubated at 26 °C (Series I) the colonies remained unchanged both in size and number, whereas in Series II the colonies showed some further growth, and new colonies about 1 mm in diameter appeared. These and bacteria uncapable of forming colonies under such conditions were regarded as the Ca-dep. population. The total number of colony-forming units was determined after 48 h incubation on DC medium.

Detection of the 42 Mdal virulence plasmid. Agarose gel electrophoresis [49] was used. Colonies of Ca-indep. isolates of *Y. enterocolitica* grown on BA-agar at 37 °C and minute Ca-dep. colonies developing after reincubation at 26 °C on the same medium served for this purpose. Strain p.JB3JI (40 Mdal) was used as plamid control.

Penetration into HEp-2 cells and toxicity tests. The methods published by Kay et al. [50] were used, except that 18 h cultures of yersinia isolates of 10^8 c.f.u. were obtained in simple broth instead of brain heart infusion browth. The reaction was regarded positive when bacteria were visible by light microscopy in the HEp-2 cells washed off with phosphate buffer 90 min after infection. Cellular disintegration indicated positive toxicity reaction.

Autoagglutination test. The test as modified by Lee et al. [51] was used, except that the 48 h *Y. enterocolitica* isolates were pre-incubated on BA agar instead of DNase agar. The autoagglutination test was performed at both 37 °C and 26 °C and read after 16 h. The test was accepted as positive if the bacterial cells were settled and the supernatant was clear or transparent at the end of the incubation at 37 °C whereas in the tubes incubated at 26 °C the medium was uniformly turbid.

Mouse pathogenicity test. Y. enterocolitica broth (26 °C, 18 h) was centrifuged and the cells were resuspended in saline up to a dilution of 10^5–10^8 cells per ml. With the dilutions CFLP mice of about 30 g body weight were inoculated, each with 0.5 ml, intraperitoneally. The mice were observed for a week after inoculation. The test was negative if the mice remained free of symptoms.

Results

Clinical and epidemiological findings. At the end of December, 1983, a suspect case of food poisoning was reported. The two and a half-year-old patient suffered from abdominal pain, vomitus, severe diarrhoea, mild sore throat, and fever up to 39 °C. The parents had been ill a week earlier with sore throat, extremital pain and fever up to 40 °C as leading symptoms, and

abdominal tenderness. The physician's diagnosis was influenza. A pediatrician raised the suspect of food poisoning and the parents conceived the idea that the illness of the child might be attributed to "pork cheese" prepared from a pig slaughtered by the family on December 3. The child consumed of the "pork cheese" a few days before the onset of his illness, the parents did it somewhat earlier. The "pork cheese", 3.5 kg in weight, was "heat-treated" for half an hour and smoked after stuffed. It was allowed to stand at an airy place without cooling. Two or three weeks later the parents, and soon thereafter relatives in four families, began to eat the "pork cheese". Epidemiological examination showed that, besides the parents and their child, 14 persons had consumed the "pork cheese" and five of them became ill. Their symptoms were approximately the same as those shown by the parents. The case incidence per number of family members for the five households was as follows: 3/4; 2/4; 2/5; 1/3; 0/2.

Isolation of yersiniae. Y. enterocolitica was isolated from faecal samples of the child who was actually suffering from enteritis as well as from two faecal samples of seven adults who had recovered by the time of sampling. The isolates belonged to serogroup O3, biotype 4 and phage type VIII; they were resistant to ampicillin, sensitive to chloramphenicol, neomycin, polymyxin B, tetracycline, co-trimoxazole and gentamicin.

"Pork cheese" available for sampling was still present in refrigerators of two households. The bacterial counts per g of sample were as follows: total viable counts, 10^8; coliforms, 10^7; "faecal" *Escherichia coli*, 10^3; *Bacillus cereus*, 10^4; *Streptococcus faecalis*, 10^5; *Staphylococcus aureus*, 10^4.

The methods of isolation of *Y. enterocolitica* from "pork cheese" samples and the results of the tests are shown in Table I. Suspected colonies of yersinia were not seen in the miscellaneous flora grown of MacConkey's Tween 80 and DC plates inoculated with cultures enriched in Rappaport's medium (Method I). Due to massive growth of other bacteria, yersinia isolation also failed when Method II was applied so that the 6-day and 14-day cold PSB enrichments were not treated with KOH before spread on DC agar or MacConkey's Tween 80 medium. When, however, the cold enrichments were treated with 0.25% KOH for 2 min before spread, the thinning of the background flora allowed yersinia colonies to appear; the colonies formed an almost confluent layer in the first segment of the plates, and isolated colonies appeared in the second. A pretreatment of the inocula with 0.5% KOH solution for 15 s completely destroyed the concomitant flora and allowed only one or two yersinia colonies per plate to grow. Using Method III, we failed to isolate yersinia when cold pre-enrichment was followed by post-enrichment in Rappaport's medium either with or without KOH treatment.

The "pork cheese" stored in the refrigerator of one of the related families yielded *Y. enterocolitica*. The adults in the family had suffered from a severe

Table I

Isolation of Yersinia enterocolitica from samples of the "pork cheese" associated with the outbreak

Method	Amount of sample inoculated	Enrichment			Yersinia colonies grown at 28 °C, for 48 h on					
					MacConkey's Tween 80 agar			DC agar		
		medium (ml)	°C	time, days	KOH pretreatment					
					nil	0.25%, 2 min	0.5%,15 s	nil	0.25%, 2 min	0.5%, 15 s
I	25 g	Rapp. (225)	36	2	n.t.	n.t.	n.t.	—[b]	n.t.	n.t.
II/1	0.2 g	PSB (100)	4	6[a]	—[b]	+++	+	—[b]	+++	+
II/2	0.2 g	PSB (100)	4	14[a]	—[b]	+++	+	—[b]	+++	—
III/1	II/1 6-day[a]	Rapp. (100)	26	2	—[b]	—[c]	—	—[b]	—[c]	—
III/2	II/1 14-day[a] 1 ml	Rapp. (100)	26	2	—[b]	—[c]	—	—[b]	—[c]	—

Rapp. = Rappaport's enrichment medium
n.t. = Not tested
[a] = Used also as inoculum in Method III
[b] = Yersinia-negative, massive miscellaneous flora
[c] = Yersinia-negative, poor miscellaneous flora
— = No growth of bacteria
+++ = Scattered yersinia colonies on segments I and II, poor miscellaneous flora
+ = One or two yersinia colonies in segment I; no other bacteria

disease diagnosed as influenza one week earlier. The faecal samples taken from patients simultaneously with those taken from the "pork cheese" were negative. The strain isolated from the "pork cheese" was identical in serogroup, biotype and phage type with the three strains obtained from faecal samples of the index child and two adult patients. This fact and the high fever accompanying the disease suggested that the yersinia contamination of the "pork cheese" accounted for the outbreak.

Virulence tests. To check the above assumption, we examined the virulence of the strain isolated from the "pork cheese" in comparison with the strain B20187 of faecal origin. We employed six virulence tests, the first of which was testing for Ca^{++} dependence.

Figure 1 shows a confluent growth on the Ca^{++}-free plates inoculated with the 10^{-3} dilution of strain E14651 and incubated at 26 °C. In contrast, only scattered, Ca^{++}-indep. colonies grew from the same dilution on plates incubated at 37 °C.

As shown in Fig. 2, the growth on BA + Ca^{++} plates inoculated with the 10^{-6} dilution of the same culture, the colony number was approximately the same at 26 °C and 37 °C. The yersinia strains isolated from patients showed

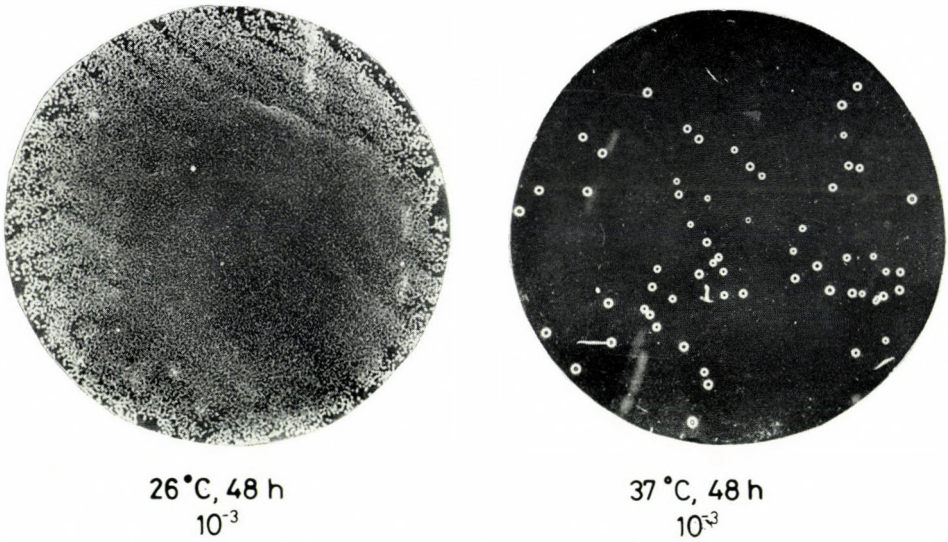

Fig. 1. Temperature-sensitive Ca^{++} dependence of *Y. enterocolitica* strain E14651 on a calcium-free nutrient medium (BA)

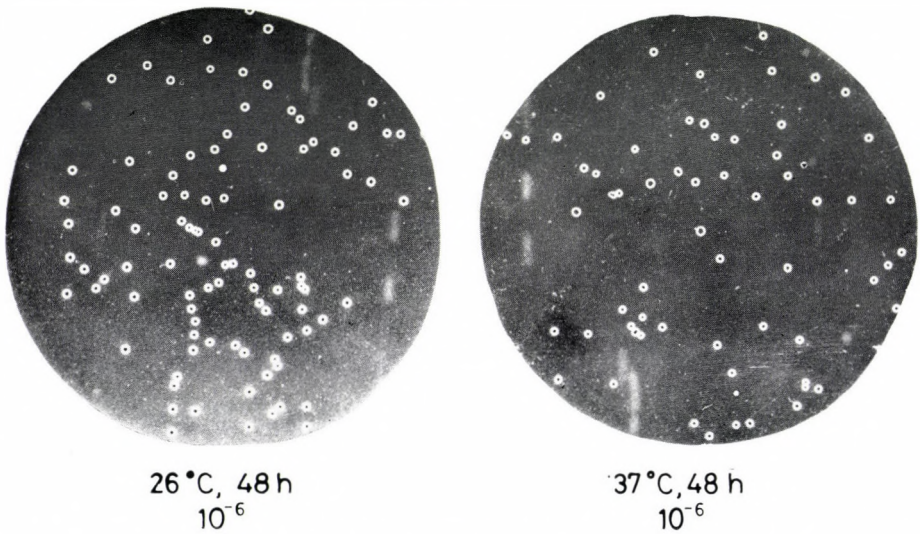

Fig. 2. Temperature-insensitive colony formation of *Y. enterocolitica* strain E14651 on a medium supplemented with calcium (BA + Ca^{++})

the same temperature-sensitive Ca^{++} dependence as the food-isolate (Figs 1 and 2).

In Fig. 3 the temperature-sensitive Ca^{++} dependence of the strains of different origin is shown numerically. There was no considerable inter-strain difference at the same temperature if the colonies were growing on the same

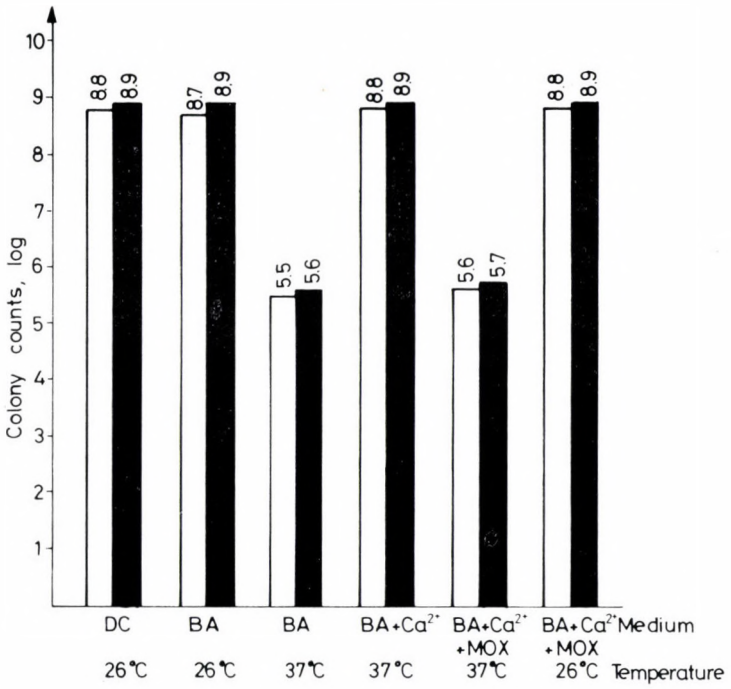

Fig. 3. Temperature-sensitive Ca^{++} dependence of two *Y. enterocolitica* isolates on three different media. Open columns, strain E14651 isolated from food; solid columns, strain B20187 isolated from faeces. For abbreviation of media see Materials and methods

medium. For further comparison, the colony number obtained on DC medium after incubation at 26 °C for 48 h is shown. Colony growth on BA and BA + Ca^{++} + MOX was optimal at 26 °C, whereas at 37 °C the number of colony-forming yersiniae was lower by three exponents, because only the Ca-indep. mutants of the populations grew on these media at 37 °C. In the presence of Ca^{++}, colony formation was undisturbed even at 37 °C.

Figure 4 shows how to isolate Ca-dep. and Ca-indep. colonies. Strain E14651 isolated from "pork cheese", formed two kinds of colony: the large ones grew at 37 °C, i.e. they were Ca-indep. mutants; the minute colonies, on the other hand, were Ca-dep., as they appeared only after re-incubation at 26 °C for 48 h. We prefer our BA medium to the MOX agar [52] because, using the former, we were able to differentiate the Ca-dep. and Ca-indep. mutants from each other with great certainty, and because it was the BA medium from which we succeeded in isolating both Ca-dep. and Ca-indep. colonies, irrespective of the origin (food or faeces; child or adult) of the strains.

In possession of Ca-dep. and Ca-indep. lines, we attempted to detect the virulence plasmid in them. In these experiments, Eckhardt's agarose gel

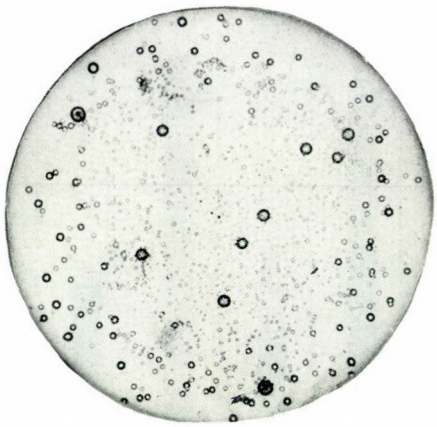

Fig. 4. Ca-dependent and independent colonies of *Y. enterocolitica* strain E14651 on calcium-free medium (BA) after incubation at 37 °C for 48 h then at 26 °C for 48 h. Large colonies are Ca-independent, minute colonies are Ca-dependent

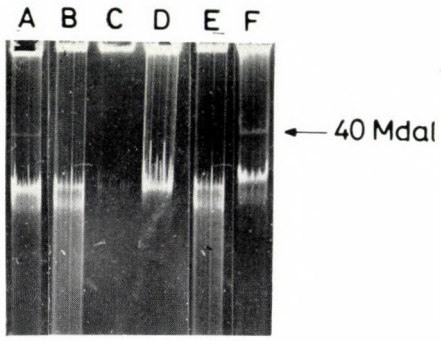

Fig. 5. Examination of *Y. enterocolitica* isolates for the virulence plasmid on Eckhard's agarose gel. A = p.E14651 (Ca-dep.); B = E14651 (Ca-indep.); C = p.B20187 (Ca-dep.); D = B20187 (Ca-indep.); E = X8312 (Ca-indep.); F = p.JB3JI (plasmid control)

electrophoresis was used. Lane "F" of Fig. 5 shows the electrophoretic pattern of strain p.JB3JI having the 40 Mdal plasmid. In the electrophoretograms of the Ca-dep. isolates (lanes "A" and "C") the band indicates the presence of a 42 Mdal plasmid. Similar bands fail to appear in lanes "B" "D" and "E". Lanes "B" and "D" correspond to the Ca-indep. mutants that had lost their plasmid spontaneously, while lane "E" is the electrophoretogram of the reference strain X8312, which is maintained in laboratory passages.

Table II summarizes the results of the virulence tests carried out with the different isolates and laboratory strains of *Y. enterocolitica*. In brief, the Ca-dep. strains carried the 42 Mdal virulence plasmid; they penetrated HEp-2 cells; due to their toxic effect, disintegrated the HEp-2 cells, and gave positive autoagglutination. For the Ca-indep. mutants, on the other hand, only the

Table II

Results of virulence tests for Y. enterocolitica isolates

Isolate	Plasmid of 42 Mdal	HEp-2 penetration	HEp-2 disintegration	Auto-agglutination	Mouse toxicity (10^5, 10^8 i.p.)
E14651 Ca-dep.	+	+	+	+	—
E14651 Ca-indep.	—	+	—	—	—
B20187 Ca-dep.	+	+	+	+	—
B20187 Ca-indep.	—	+	—	—	—
X8312	—	+	—	—	—

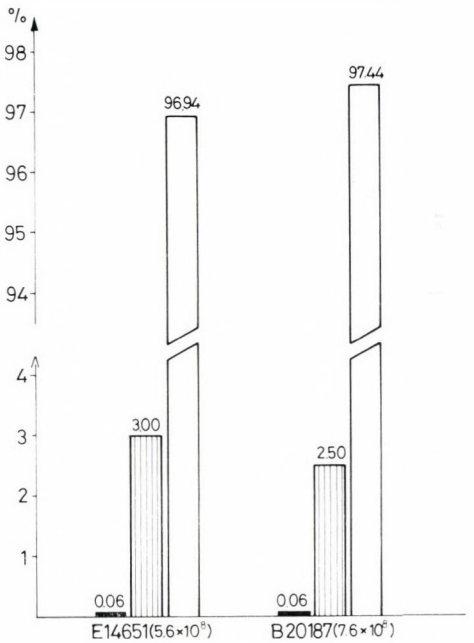

Fig. 6. Ca-dependent and independent colonies in per cent on the total number of colony formers in cultures on calcium-free medium (BA) of *Y. enterocolitica* strain E14651 isolated from food and strain B20187 isolated from faeces. Solid columns, Ca-independent; shaded columns, Ca-dependent growing in small colonies; open columns, no colonies on BA medium

HEp-2-penetration test was positive. Mice inoculated either with the Ca-dep. strains or the Ca-indep. ones, had remained symptomless by the end of the one-week observation period.

The fact that three of the virulence tests ran parallel with the Ca^{++} dependence promted us to estimate the degree of virulence of yersinia strains on the basis of the degree of their Ca^{++} dependence. For this purpose, we

examined the ratio of the numbers of Ca^{++}-indep. minute colonies grown at 26 °C and colony forming units not developing under the experimental conditions.

Figure 6 shows that strains isolated from foodstuff and from faeces exhibited nearly the same value. For both strains only 0.06% of the colonies were Ca-indep. after incubation at 37 °C, however, on further incubation at 26 °C an additional 3.0 and 2.5% of the respective organisms formed minute colonies; however, the majority of Ca-dep. were lost during the 48 h on Ca-free medium. Of the Ca-dep. viable count originating from foodstuff or faeces, 3 and 2.5%, respectively, of the population formed minute colonies.

Discussion

It is a considerable difficulty in isolating *Y. enterocolitica* from foodstuff that yersiniae are usually overgrown by a wide variety of other bacteria. Recently, many reports have proposed newer and newer methods for isolation of yersiniae from foodstuffs. We have tried to apply methods of a number of authors in yersinia screening tests since 1980, and succeeded in isolating *Y. enterocolitica* serogroup O3 biotype 4 from raw hog's tongue and flesh. The experience thus obtained prompted us to combine and modify methods available in the literature.

The use of Method II described in the present report permitted the isolation of *Y. enterocolitica* from "pork cheese". The theoretical limit of sensitivity of the method is 5 viable yersinia cells per g. We failed to isolate yersinia by using Rappaport's enrichment medium either directly or after cold enrichment. The failure might be explained by some observations reported by Doyle et al. [28, 53]. They emphasized that — in case of serogroup O3 and O9 — Rappaport's enrichment medium fails unless the yersinia count is sufficiently high. Schiemann's [54] experiments suggesting that *Y. enterocolitica* shows an increased KOH tolerance at relatively low temperatures prompted us to assume that pretreatment before subculturing with KOH of the inoculated Rappaport medium at 26 °C would reduce the number of colony-forming units more than a direct treatment of cultures incubated at 4 °C. Accordingly, our Method II seems to be suitable for routine use, since isolation of yersinia from "pork cheese" succeeded even in presence of a background flora of 10^8/g.

In the virulence tests, our isolates, originating from either faeces or food, agreed well with the criteria of virulent *Y. enterocolitica* strains [55, 56, 57]. Examining the virulence plasmid of *Y. enterocolitica*, Portnoy et al. [58, 59] proved that a fragment of the plasmid, i.e. the "virulence factor" accounts for Ca^{++} dependence. If so, our isolate of food origin must be of

high virulence, as virulent as the isolates obtained from patients. This means that the role of "pork cheese" in the outbreak under study is clear and that the "heat treatment" of the "pork cheese" had been unsatisfactory.

The mouse pathogenicity tests were negative for all the strains tested. This is not surprising because literary data have suggested that certain *Y. enterocolitica* strains including strains of serogroup O3 biotype 4, may be apathogenic for mice. Experiments of Smith et al. [60], Bakour et al. [61] and Browne et al. [62] have shown that special conditions are needed to demonstrate some mouse pathogenicity of these strains. On the other hand, the negative virulence tests of the reference strain (B8312) or yersinia were somewhat unexpected, though, literary data [58] suggest that *Y. enterocolitica* strains may lose their virulence factor together with some features characteristic of the virulent *Y. enterocolitica*. This behaviour of the reference strain suggests that even fresh isolates may lose virulence while maintained in the laboratory.

In the yersinia outbreak reported here, the leading symptoms in adults — upper respiratory symptoms, high fever and extremital pain — were not accompanied by enteritis. Thus, these cases were different from the yersinia infections diagnosed in Hungary so far.

Yersinia enteritis accompanied by pharyngitis has been mentioned by several authors. Tacket et al. [34] reported a mass outbreak of yersinia enteritis supposedly transmitted by pasteurized milk. In the outbreak, besides many intestinal and extraintestinal cases, pharyngitis requiring hospital care occurred in 14 cases; these cases showed no symptoms of enteritis. Although the outbreak observed by us included few cases, it agrees well with the outbreak reported by Tacket et al. in that the child suffered from enteritis whereas extraintestinal symptoms were predominant in the adult cases.

It may be assumed, accordingly, that the human pathogenicity spectrum of *Y. enterocolitica* is considerably wider than it has been registered in Hungary on the basis of the laboratory and clinical routine work that mainly aimed at clarifying the aetiology of enteritides suspect of yersinia.

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SPECIFICITY OF FUNGAL LIPASE IN HYDROLYTIC CLEAVAGE OF OIL

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Lipase isolated from the fungus *Fusarium oxysporum* f. sp. *lini* has shown specificity to saturated acids. In vitro hydrolytic splitting of cotton seed, ground-nut and fungal *(F. oxysporum)* oil substrates by the enzyme preparation from this fungus, revealed preferential fatty acid specificity. The enzyme attacked the triglyceride molecule, liberating 90–97% of saturated fatty acids, irrespective of length of the carbon chain.

Specificity of microbial lipases has been investigated by many workers [1–7]. These enzymes are known to differ in their site of attack and hence can be useful in studying the triglyceride structure. Previous studies [1–5, 8] on microbial lipases have shown unique specificity towards the triglycerides. Thus, lipase from *Pseudomonas fragi* is similar to pancreatic lipase in that it attacks the 1,3 position of triglycerides. The lipase from *Staphylococcus aureus* attacks both 1,2 and 1,3 positions, while lipase produced by *Geotrichum candidum* is stereospecific and exhibits a high degree of specificity towards unsaturated fatty acid linkages particularly for oleic acid. Lipase isolated from uredospores of *Puccinia graminis* produces 1,2 and 1,3 diglycerides from triolein [9].

During the course of our investigation on the effect of various culture conditions on biotechnological production of oil by *F. oxysporum*, it was considered worthwhile to find possible variations in specificity of lipase produced. Studies on the effect of various culture conditions such as source of carbon and nitrogen, C : N ratio, pH, temperature, and period of incubation, the fatty acids profile showed great variability. Structural variations during in vivo biosynthesis of triglycerides were also observed, which could be attributed to the specificity of lipase in esterifying a particular type of fatty acid. Moreover, accumulation of certain fatty acids in free form, indicated inefficiency of lipase in utilizing these fatty acids in the formation of triglycerides.

These observations prompted us to make preliminary studies on in vivo specificity of crude lipase isolated from a biomass produced by *F. oxysporum* on culturing in chemically defined media.

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Material and methods

Organism and culture conditions. The fungus *F. oxysporum* f. sp. *lini* (Bolley) Synder and Hansen strain CBS 197 was obtained from the Indian Type Culture Collection, Division of Mycology and Plant Pathology, Indian Agriculture Research Institute, New Delhi. Cultures were maintained on slants of potato–dextrose agar at 28 °C ± 2 °C and subcultured every fortnight.

The fungus was grown in Czapek–Dox medium [10] with minor modification. The basal nutrient medium consisted of glucose, 15 g; ammonium nitrate, 0.4 g; potassium dihydrogen phosphate, 0.1 g; potassium chloride, 0.05 g; magnesium sulphate, 0.05 g; ferrous sulphate, 0.01 g per 100 ml medium at pH 5.9.

Preparation of crude enzyme. After 14 days of incubation, the mycelial mat was removed, filtered and washed thoroughly with distilled water to free it from traces of culture medium. The felt was dried at low temperature (0–5°) and repeatedly extracted with low boiling petroleum ether (40–60°) in a Soxhlet extractor. The fat-free cake obtained in this manner was dried in air and pulverized (60 mesh) at low temperature (0–5°). This powder was suspended in phosphate buffer (0.2 M, pH 6.7) and used as crude enzyme preparation.

Determination of lipase specificity. The specificity of crude lipase preparations was determined by identifying and assaying the fatty acids liberated by hydrolytic cleavage of neutral oil substrates of known composition [11]. Cotton seed, ground-nut and fungal *(F. oxysporum)* oil substrates were emulsified in a Waring blender, to which was added (for each flask) 8 ml phosphate buffer (0.2 M, pH 6.7) containing 0.5 ml of 1% gum arabic, 1% $CaCl_2$ and 500 mg oil. After shaking thoroughly for 5 min, 8 ml emulsion was transferred to 50 ml Erlenmeyer flasks and buffer (2 ml) containing enzyme (5 mg) was added. The contents were incubated for 3 h, at 37 °C ± 1 °C. Three replicates plus two controls, one without enzyme and the other without substrate, were prepared. After addition of 0.5 ml of 20% sulphuric acid to terminate the reaction and 50 ml water, the reaction product was extracted with ether followed by evaporation of the solvent.

Isolation and identification of fatty acids. The hydrolytic product was taken up in 50 ml warm ethanol and excess 1% potassium hydroxide, in order to saponify the liberated fatty acids. Alcohol was evaporated off and 50 ml water was added. The fatty acids were regenerated by adding 20% sulphuric acid, and extracted with ether. The ether layer was dried over Na_2SO_4, the solvent was evaporated, and the residue weighed to a constant weight.

Fatty acid esters were prepared according to the method described in reference [12] and analyzed by GLC, equipped with a flame ionization detector. The column was packed with 15% 1,4-butandiol succinate on acid washed 100/120 chromosorb P. The flow of N_2 carrier gas was 40 ml/min and that of H_2, 15 ml/min. Temperatures of column, injection port and detector were 195, 270 and 270 °C, respectively. Identification of fatty acids was made by comparing retention time of reference standards and quantitative assay was done by measuring the area under peaks [13].

Results and discussion

The results summarized in Table I show that the hydrolytic cleavage by this fungal enzyme results in preferential liberation of saturated fatty acids from all the three substrates, irrespective of length of the carbon chain. The hydrolysis takes the usual course, regardless of the location of the fatty acids in the triglyceride.

In spite of the presence of large proportions (52.0–81.9%) of unsaturated fatty acids in the triglyceride molecule of the substrates, only negligible amounts of it are liberated by this enzyme.

In all the three substrates viz., groundnut, cottonseed and fungal oils, palmitic acid has been found to be the major acid liberated.

Table I

Fatty acid specificity of crude lipase enzyme isolated from F. oxysporum

Sl. No.	Fatty acids	Cotton seed oil		Ground nut oil		*F. oxysporum* oil	
		a	b	a	b	a	b
1	14 : 0	1.1	10.9	—	1.3	0.2	3.0
2	16 : 0	24.5	68.3	10.8	69.3	44.5	55.0
3	16 : 1	0.4	—	—	—	—	—
4	18 : 0	3.2	14.4	2.6	8.0	4.3	39.5
5	18 : 1	18.0	—	48.6	6.6	39.6	2.0
6	18 : 2	51.4	4.0	33.3	4.3	11.8	0.5
7	18 : 3	0.6	—	—	—	0.6	—
8	20 : 0	0.8	2.0	3.3	6.7	—	—
9	22 : 0	—	0.4	1.4	3.8	—	—
Total saturated acids		29.6	96.0	18.1	89.1	48.0	97.5
Total unsaturated acids		70.4	4.0	81.9	10.9	52.0	2.5

a = Fatty acids (wt %) in triglycerides
b = Fatty acids (wt %) liberated after enzymatic hydrolysis

Table II

Total saturated and unsaturated fatty acid 2-monoglyceride fraction
(Pancreatic lipase hydrolysis)

Substrate oil	Fatty acid liberation from 2-position	
	saturated	unsaturated
Groundnut	11	89
Cottonseed	1	99
F. oxysporum	14.2	85.8

Within a period of 3 h of incubation the enzyme releases 90–97% of the total saturated fatty acids. Thus this enzyme, in conjunction with other lipases, can be useful in determining the fine structure of triglycerides.

In the 2-monoglyceride fraction of oils, derived from cottonseed, groundnut and fungus *(F. oxysporum)* by the pancreatic lipase hydrolysis (Table II), it was observed that the saturated acid component varies between 1–14%.

The fungal lipase hydrolyses the triglycerides of cottonseed, groundnut and fungal oil, yielding a mixture of saturated (96, 89.1 and 97.5%) and unsaturated (4, 10.9 and 2.5%) acids. The high percentage of saturated acid obtained after the enzyme hydrolysis, indicates that these might have been derived, in part from 2-position of the triglyceride molecule. This suggests that the fungal enzyme, though saturated acid specific, is non-specific towards the primary and secondary positions of the triglyceride molecule.

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EFFECTS OF HERPES VIRUS INFECTIONS ON THE CHEMILUMINESCENCE INDUCED BY ZYMOSAN PHAGOCYTOSIS IN MOUSE PERITONEAL MACROPHAGES

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The chemiluminescence (CL) induced by zymosan phagocytosis was tested in mouse peritoneal macrophages infected with three different types of herpes viruses: herpes simplex type-1 (HSV-1), human cytomegalovirus (HCMV) and murine cytomegalovirus (MCMV). The intensity of CL was tested in various intervals of virus infections. In the first eight hours zymosan induced chemiluminescence decreased in all the three systems. By the 24th hour, the macrophages infected with HCMV had almost completely recovered from the early defect, while in the macrophages infected with both HSV-1 and MCMV, the chemiluminescence induced by zymosan remained impaired.

Macrophages are known to be important in the host's immune defense system, and a number of publications have dealt with the effect of herpes viruses on these cells [1–6]. The peritoneum of the animal shows an impaired clearance of exogenous carbon particles by macrophages when infected 7–9 days previously [7]. Also chemiluminescence is augmented during phagocytosis of opsonized particles in mice infected with 10 p.f.u. (plaque forming unit), 3, 6 and 13 days previously, suggesting an increased level of metabolic activation. A reduction in phagocytosis of *Staphylococcus aureus*, as soon as 24 h after in vitro infection has been reported [8]. With increasing replication of virus and decreasing macrophage survival, phagocytosis activity declines further, even in those cells that remain viable [4, 9].

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As chemiluminescence induced in phagocytic cells is estimated to be a sensitive method for the characterization of the phagocytic activity [10, 11], in this study we have compared the effect of three types of herpes viruses on CL induced by zymosan phagocytosis in mouse peritoneal macrophages. Two of these viruses, herpes simplex type-1 and murine cytomegalovirus, can multiply in the mouse peritoneal macrophages, whereas human cytomegalovirus is unable to do so. According to our results, the infections with HSV-1 and MCMV involve a prolonged decrease in the phagocytosing, chemiluminescence producing activity of macrophages, but HCMV results in only a transient impairment.

Materials and methods

Viruses. Murine cytomegalovirus (MCMV) strain "Smith" was obtained from Guy Hospital, London, through the courtesy of Professor C. A. Mims. Strain AD-169 of human cytomegalovirus (HCMV) was kindly supplied by Dr. H. K. Andersen, Medical University of Aarhus, Denmark. Herpes simplex virus type-1 (HSV-1) strain "HIL" was kindly made available by Professor F. Rapp, Medical University of Pennsylvania, Hershey, USA.

Cell cultures and medium. Human embrionic fibroblast cells (HEF) were prepared from aborted human embrios. Mouse embrionic fibroblasts (MEF) were isolated from Balb/c embrios. The cell cultures were used for virus propagation and titration between the second and tenth passages. The growth medium consisted of Minimal essential medium (MEM) supplemented with 10% heat-inactivated (56 °C, 30 min) foetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.075% $NaHCO_3$ [12].

Virus propagation. The HEF cell monolayers were infected with HCMV at a multiplication of 0.2 p.f.u. per cell. The MCMV and HSV-1 strains were propagated in MEF cultures after infection of 1 p.f.u. virus per cell. The viruses were absorbed for 2 h at 37 °C and the medium was changed. The virus stocks were harvested by freezing then clarifying the suspensions by centrifugation at 2000 g for 20 min. Virus stocks were quick frozen and stored at —70 °C.

Determination of virus yield. The infected cultures were incubated at 37 °C for various lengths of time. The infected cells were removed from the glass and disrupted by ultrasonic vibration (MSE ultrasonic apparatus: 1.5 A, 1 min, 4 °C) in culture medium and clarified by centrifugation at 2000 g for 20 min. The supernatants were titrated on cell monolayers seeded in plastic Petri dishes. After 2 h adsorption, the infected cultures were overlayered with culture medium containing 0.3% Bacto agar (Difco). When the CPE developed, the cultures were fixed with formalin, then after removing the agar overlay, stained with 0.1% crytal violet and the plaques were counted.

Cytopathic effects (CPE) induced by the herpes viruses were examined under the light microscope. In each case 1000 cells were counted and the per cent of rounded cells was expressed.

Mouse peritoneal macrophages. The peritoneal cavity of Balb/c mice of both sexes weighing 25–30 g was washed out with medium RPMI-1640 supplemented as described in the case of MEM. We used sterile conditions throughout the procedures. Macrophages gained in this way were washed and cultured further after infection with the viruses at different intervals of cultivation. The cells were cultured and tested for CL in the same glass liquid scintillation cuvettes.

Measurement of chemiluminescence. After cultivation, the macrophages were washed twice with Hanks balanced buffer solution (HBSS). Finally, the cells (monolayers) were covered with HBSS containing 10^{-4} M of luminol. One mg of zymosan-A (Sigma) was added to 10^6 cells. To the controls no zymosan was added. After 10 min of incubation at 37 °C, the luminol amplified CL was measured by a Nuclear Chicago liquid scintillation counter at the "coincidence off" mode. The emmission of photons (expressed in cpm) was measured in each sample 5 times at 5 min intervals and the whole number of photons measured in a sample was regarded as a value characteristic of that culture. The CL measurements took place on triplicates of each culture [10, 11].

Results

The zymosan-induced CL of mouse peritoneal macrophages infected with HCMV after a starting increase in the second hour, decreases deeply at the 4th hour. After this time, a gradual recovery of the cells can be observed till the 24th hour of infection. The cells produce almost as much photons as the non-infected ones. In the macrophages infected with MCMV, the zymosan-induced CL starts to decrease at the 4th hour, reaching one of the lowest points at the 8th hour and remaining at the same low level thereafter. In the case of macrophages infected with HSV-1, after a starting elevation, the zymosan-induced CL decreases until the 4th hour. This transient defect is almost completely respored by the cells up to the 8th hour of infection. But after this time, an expressed decrease follows, leading to the lowest values of CL measured in the macrophages infected by any of the three viruses. Comparing the per cent of cells showing cytopathic damages at the 24th hour of infections, the greatest number of defected cells can be found in the cultures of HSV-1 infected macrophages. All these date are shown in Fig. 1.

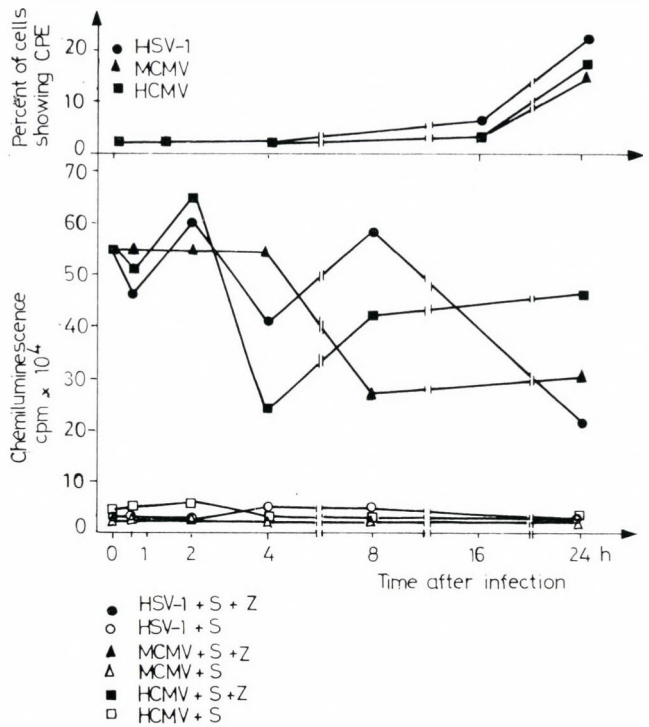

Fig. 1. Chemiluminescence induced by zymosan in murine peritoneal macrophages infected with three different types of herpes viruses

No production of infective HCMV viruses occurs only the presence of inoculated viruses can be demonstrated. On the other hand, MCMV and HSV-1 start to multiplicate after the 12th hour of infection in the macrophages, and the peaks of the virus titres can be found at the 48th hour (HSV-1) and the 72nd hour (MCMV). Besides, HSV-1 and MCMV involve the highest number (percentage) of cells with cytopathic defects. Such data are demonstrated in Fig. 2.

When macrophages are infected with ultraviolet light irradiated (96 000 erg/s/mm^2) viruses, no replication occurs. The zymosan induced CL of these cells does not differ from the CL measured in the non-infected control cultures.

Discussion

Our results show that the herpes viruses can induce remarkable defects in the phagocytic functions of macrophages. The type and severity of the virus effects depend on the degree of multiplication of the infective viruses in the cells. Since HCMV can not multiply in the mouse peritoneal macrophages at

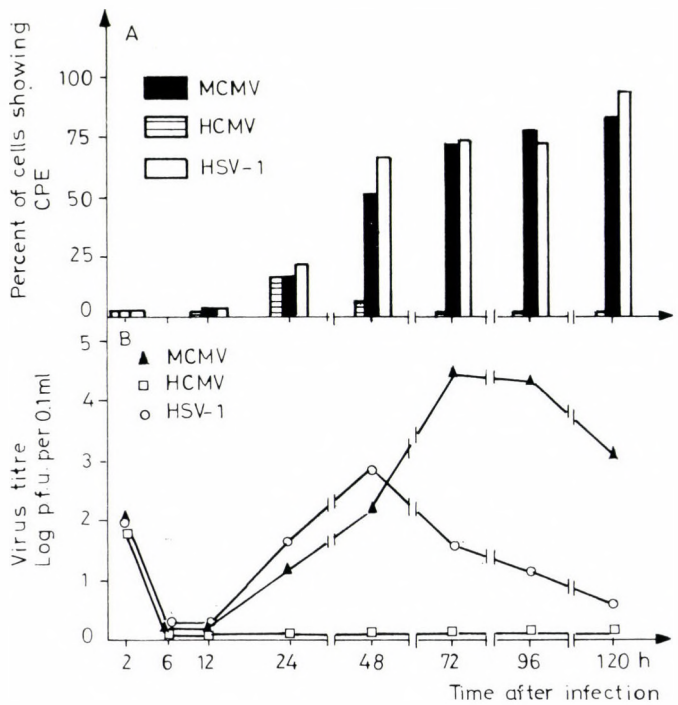

Fig. 2. Virus titres and cytopathic effects in murine peritoneal macrophages during the infections

all, it produces only an early and transient impairment in the phagocytic activity of macrophages. By the 24th hour, the cells can almost completely recover from the effect of this virus. Among the three types of viruses we used, MCMV multiplies the best in the mouse peritoneal macrophages, involving a long-lasting and definite damage in the phagocytic function and the morphology of these cells. But up to the first 48 hours, HSV-1 shows the highest rate of multiplication, that is why this virus causes the most severe defect in the phagocytic function and production of CL of macrophages infected with any of the three viruses in the first day of infections. Our results confirm the previous observation on the defective phagocytic capacity of the macrophages infected with HCMV 24 h after the infection [8]. It seems likely that after the early impairment, on the influence of cell products deriving from the infected cells (interferon, interleukins), a general activation of macrophages can take place in vivo after the third day of infection [4, 10, 13, 14]. Our results underline the aspect that the severity of the damage in the macrophages infected with several types of viruses shows a rather good parallelism with the multiplication rate of the virus in the macrophage [4].

The measurement of zymosan-induced CL has found to be a sensitive method for the measurement of phagocytic activity and metabolism. This method can be useful in the characterization of phagocytes. For example, in AIDS patients a defect of zymosan-induced CL was found beside the T cell deficiency [15]. Our results confirm the usefulness of this method in virus infections [15–18]. Besides, they provide some further evidence for the pathogenetic effects of herpes viruses not only on the lymphocytes [2, 3, 19–21], but on the monocytes, macrophages, too [1, 19, 21].

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DIFFUSION OF METRONIDAZOLE THROUGH THE DENTINAL TUBULES OF EXTRACTED TEETH

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Passing of metronidazole from the root canal of extracted gangrenous teeth through the dentinal tubules was proved by agar diffusion and minimum inhibitory concentration assay. The findings explain the excellent clinical experience with metronidazole in root treatment.

In search for bactericidal drugs to be used in root treatment it should be taken into account that (i) anaerobic bacteria play a decisive role in gangrenous processes of the root canal [1–3] and (ii) that neither the dentinal tubules nor the delta apicale of the gangrenous tooth can be dilated mechanically. Therefore, diffusible bactericidal drugs [4] active against anaerobic bacteria should be applied to the root canal. A number of procedures and drug combinations of variable effectivity have been developed for this purpose [5, 6].

In the present work attempts were made to check the assumed diffusion of metronidazole through the dentinal tubules and thus to explain the excellent clinical results that have been obtained with the drug when used in root treatment.

Materials and methods

Drug. Metronidazole (Richter, Budapest) was used throughout.

Preparation of teeth. Twenty-six freshly extracted gangrenous single-rooted teeth were instrumented to Kerr 60 *M* reamer and rinsed with hydrogen peroxide and chloramine-B. Then 200–400 mg of metronidazole wetted with sterile distilled water was introduced into the root canal. The foramen apicis dentis and the cavity were closed with amalgam. The teeth were sterilized in ethylene oxide and aerated for 72 h before the experiment. Teeth prepared identically, but without metronidazole, were used as control.

Checking of diffusion of metronidazole through the dentinal tubules. Blood agar plates containing Witte peptone, meat extract and 5% defibrinated bovine blood were used in the tests. A four-hour culture of *Clostridium perfringens* (120×10^6 viable cells per ml) was densely spread on the one half of each plate and that of *Bacteroides fragilis* (320×10^6 viable cells

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per ml) on the other half. Ten teeth pretreated with metranidazole and 10 control teeth were dipped in the medium. The plates were then kept at room temperature for 30 min and, subsequently, in an anaerobe jar at 37 °C for 24 h. The diameters of the inhibition zones were measured.

Quantitative assay of diffusion of metronidazole through the dentinal tubules. Three teeth pretreated with metronidazole and three control teeth were placed into small glass test tubes each containing 2 ml sterile distilled water. Samples were taken from the fluids after 30 min, 1 h, 2 h, 4 h, 8 h, 24 h and 72 h of incubation at room temperature. Sterile filter paper discs were impregnated with 0.02 ml aliquots and placed on plates inoculated with *C. perfringens*. After standing at room temperature for 30 min, the plates were incubated in an anaerobe jar for 24 h, then the inhibition zones were measured.

To obtain a reference regression line, a doubling dilution series representing 100, 50, 25, 12.5, 6, 3 and 1.5 μg was prepared from an aqueous solution of metronidazole. After incubation for 24 h anaerobically, the diameters of the inhibition zones were measured and plotted against the logarithms of the reference concentrations [7, 8]. The metronidazole concentrations in the samples under testing were calculated by use of the regression line.

Results

Wide inhibition zones developed around each of the 10 teeth that had been pretreated with metronidazole. The average diameter was 31.3 mm on *C. perfringens* plates and 40.8 mm on *B. fragilis* plates. One of the plates is shown in Fig. 1. There was no inhibition around the control teeth (Fig. 2).

Samples of distilled water used for soaking the metronidazole-treated teeth showed measurable antibacterial levels increasing with the time of soaking. Samples taken from the tubes containing the control teeth displayed no inhibition. The reference regression line and the scattergrams for the samples are shown in Fig. 3. Concentrations between 12 and 19 μg/ml had been reached by the 24th hour. At 48 h and 72 h, the metronidazole concentrations ranged between 22 and 42 μg/ml and between 51 and 100 μg/ml, respectively.

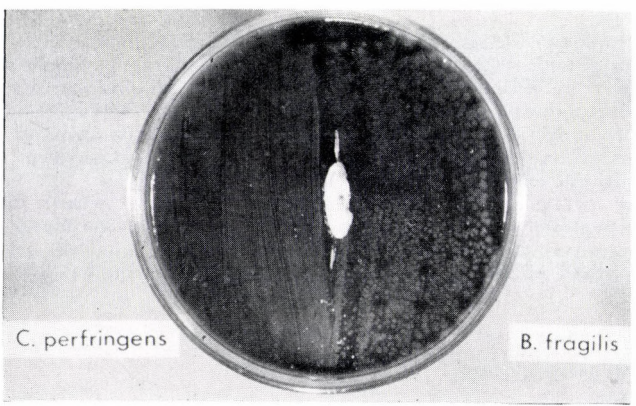

Fig. 1. Inhibition zone around a tooth filled up with metronidazole

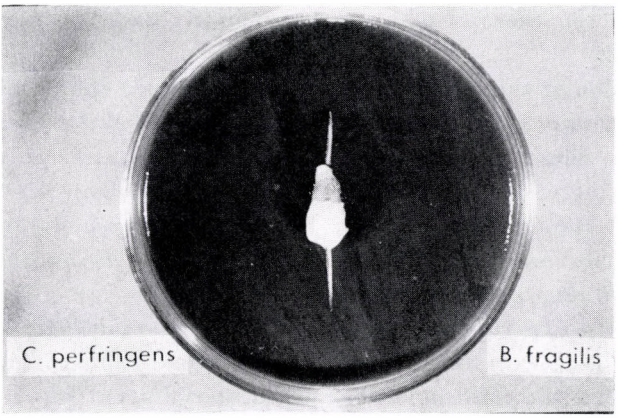

Fig. 2. Control for the plate in Fig. 1. The tooth had been filled up with a mass containing no metronidazole

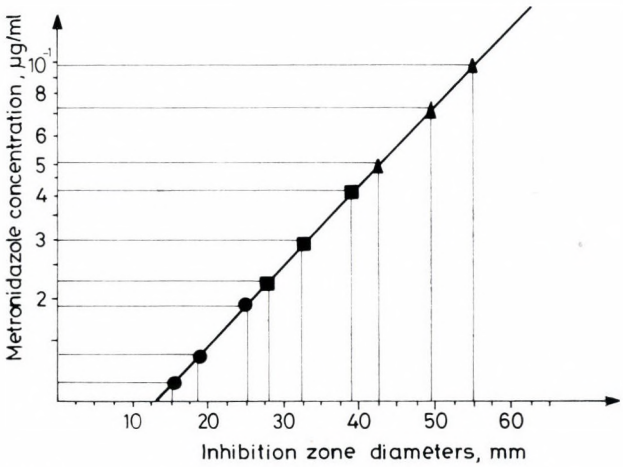

Fig. 3. Metronidazole concentrations in three samples of distilled water used for soaking metronidazole-filled teeth. Samples were taken after 24 h (●), 48 h (■) and 72 h (▲)

Discussion

Cosar and Julou [9] were the first, in 1959, to synthesize metronidazole and to describe the antiprotozoan effect of the drug. The discovery of its activity against anaerobic bacteria by Füzi and Csukás [10, 11] in 1969 attracted great interst of investigators. Gráber [12] demonstrated bactericidal effect with one-tenth, and even one-hundredth, of the blood level which can be reached therapeutically. Metronidazole is considered very useful in the therapy and reasonable prophylaxis in a wide variety of diseases, e.g., in gynaecology and surgery [13, 14]. In dentistry it has been applied with favour-

able results in the therapy of ulcerous gingivitis and Plaut–Vincent angina since 1962 [15].

Elimination of the anaerobic microflora has been shown of great importance in the therapy of gangrenous teeth. Ferenczi et al. [16] reported in 1975 that the viable count of Gram-negative anaerobes considerably decreased in 200 cases of gangrenous root canals treated successfully with metronidazole. More recently, Bánóczy et al. [17], Hess [18, 19] and Maurette [20] have used an ointment containing 10% metronidazole with favourable effect in the root therapy of gangrenous teeth.

The present experiments have proved that metronidazole introduced into the root canal is able to diffuse trough the dentinal tubules. The quantitative tests showed that the diffusion of the antibacterial agent was continuous. Ten to 100 μg/ml levels i.e. values 100 times higher than the minimum inhibitory concentrations for anaerobes (0.1–1.0 μg/ml) can be reached around a tooth pretreated with metronidazole.

Although Ferenczi et al. [16] reported as early as 1975 a decrease in the anaerobe count in the root canal after local metronidazole treatment, it has been confirmed in the present report that the drug applied in the root canal will diffuse from there through the dentinal tubules to the periapical space and the periosteum. There is no doubt that, besides reducing the viable count of anaerobes in the root canal, metronidazole kills these organisms, and thus suppresses the inflammatory processes in the dentinal tubules and periapical tissues as well.

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ANTIBIOTIC RESISTANCE AND PLASMID PROFILES OF *ESCHERICHIA COLI* AND *KLEBSIELLA* ISOLATED FROM IN-PATIENTS RECEIVING PROLONGED ANTIBIOTIC THERAPY

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Distribution by serogroup, phage type, colicin production, colicin type, sensitivity to antibiotics and plasmid characteristics of 74 *Escherichia coli* and 11 *Klebsiella* strains isolated from hospitalized patients receiving prolonged antibiotic therapy indicated that the infections were not associated with the hospital environment. Resistance was tested to 26 antibiotics, some of them being not generally used in therapy; 30 strains were resistant to 4 to 17 antibiotics. There was a significant difference in the antibiotic resistance of strains derived from patients with urinary-tract infections (UTI) and with leukaemia (LP). As compared to the UTI group, among *E. coli* strains in the LP group the frequency of multiple resistance was significantly higher, the MIC values were higher and R-plasmids were more frequent. Out of 30 multiple resistant *E. coli* strains 27 were R-plasmid carriers. Three different kinds of plasmid profile were shown in more than one strain (2 out of 10 UTI strains and 3 and 2 out of 10 LP strains). The rest of the isolates differed in plasmid profile from these and from one another; the presence of "epidemic plasmid" was not demonstrated. Plasmid epidemiological examinations may forecast the efficacy of an antibiotic or of a group of antibiotics.

Conditions brought about by broad-spectrum antibiotic or immune suppressive therapy predispose to the establishment and multiplication of nosocomial and multiresistant pathogenic and facultatively pathogenic bacteria, especially when there are faults in the aseptic technique [1]. Patients subjected to urological examinations often become infected with bacterial species belonging to *Enterobacteriaceae* [2–7]. Antibiotic or cytostatic treatment is frequently followed by infection with antibiotic resistant, Gram-negative bacteria [8]. The resistance of these bacteria may develop as a result of chromosomal mutation, but that determined by R-plasmids is more frequent and more significant. R-plasmid controlled resistance to the newly introduced antibiotics may develop through different mechanisms:

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— by the selection of bacteria carrying R-plasmids responsible for resistance to the new antibiotics;
— by the recombination of the new resistance determinant and the R-plasmid which is present in the bacterial population;
— by transposition of the new resistance determinant either to the chromosome or to the primarily present plasmid;
— by mutation in the gene coding inactivating enzymes of the primarily present R-plasmid [9].

Evidence has been brought to show that all four mechanisms may exist [10–14].

Sporadic or epidemic incidence and spread of bacterial plasmids controlling antibiotic resistance can be proved and traced by plasmid identification and characterization methods (plasmid profile analysis, [15–22]).

In the present work we determined the serogroup, phage type, colicin production, colicin type and sensitivity to antibiotics of *Escherichia coli* and *Klebsiella* strains derived from patients receiving prolonged antibiotic chemotherapeutic therapy in different hospital wards. The epidemiologically significant strains were examined for the presence of R and other plasmids and the plasmids were characterized. As regards the *E. coli* isolates, answers were sought to the following questions:

— did they derive from the same source or from different sources;
— what is the frequency of the plasmid-mediated resistance;
— are the plasmids spreading in the wards derivatives of identical, so called "epidemic" plasmids or do they represent different resistance plasmids selected as a consequence of antibiotic therapy;
— is there a difference in the frequency of resistant, mainly multiple resistant strains; and
— does the R-plasmid carrier state of the multiple resistant strains vary in the different wards. As a control, *E. coli* strains isolated from out-patients with urinary tract infections were examined.

Materials and methods

Bacterial strains. A total of 74 *E. coli* strains isolated from 60 persons and 11 *Klebsiella* strains isolated from 7 persons were examined. Table I shows the origin of the strains. The strains were derived from urinary tract infections (UTI) occurring in general medical wards and among out-patients of hospital "A", and from leukaemic patients (LP) in hospital "B". The examination lasted from January 1982 to October 1982.

Antibiotic sensitivity test. Ampicillin (AM), carbenicillin (CB), streptomycin (S), gentamicin (GM), oxytetracycline (OT), chloramphenicol (CM), sulphamethoxazole-trimethoprim (SXT), polymyxin-B (PB), nalidixic acid (NA), nitrofurantoin (FT) disks were purchased from the Human Institute for Serobacteriological Production and Research, Budapest. Azlocillin (AZL) (60 μg), mezlocillin (MZ), cloxacillin (CX), cephaloridine (CD), cephalotin (CF), cephacetril (CAC), cefazolin (CZ), cefamandole (MA), cefuroxime (CXM), cefoxitin (FOX) and cefoperazone (CFP) (30 μg each), tobramycin (TM), amikacin (AN) and sisomicin (SIS) (10 μg each), doxycycline (DO) and oxolinic acid (OS) (30 μg each) disks were prepared by us.

Determination of MIC values for ampicillin (BRL), carbenicillin (BRL), azlocillin (Bayer), mezlocillin (Bayer), cephalotin (Glaxo), cephaloridine (Glaxo), cefamandole (Eli Lilly) was carried out by agar-dilution method in three parallel experiments. One drop from a 3 h broth culture, inoculated from freshly streaked strains, was spotted by use of a Lidwell phage-typing machine. For determining the geometrical average of the MIC values the following formula was used:

$$\sqrt[N]{d_1^{n_1} \cdot d_2^{n_2} \ldots d_n^{n_n}},$$

where N = total number of strains; d_1, d_2, d_n = MIC values in dilutions; n_1, n_2, n_n = number of strains showing the corresponding MIC values. Two-fold dilution differences were regarded significant.

Determination of serological groups was performed as described previously [23].

Determination of phage type and colicin production was carried out by the method of Milch and Gyenes [24].

Colicin types were determined according to Lewis [25], using Frédéricq's indicator strains [26].

Demonstration of R-plasmids was done as described previously [27].

Antibiotics used for selection were rifampicin, 250 μg/ml; nalidixic acid, 50 μg/ml; chloramphenicol, oxytetracycline, streptomycin, carbenicillin, 30 μg/ml each.

Determination of incompatibility groups (Inc) was carried out as described by Datta [28], using Datta's and Chabbert's reference plasmids.

Determination of phage inhibition, phage restriction. Phage sensitivity of R^+ and R^- *E. coli* K12 J5-3 strains was examined by routine test dilution of an adequate phage set.

Phage set used for determination of phage inhibition. T1 T7; ∅2; *E. coli* type phages [17]; *Shigella flexneri* type-phages [29]; modified phages [30].

Isolation of plasmid DNA was carried out according to Kado and Liu [31].

Agarose gel electrophoresis and molecular size estimation was performed as described by Meyers et al. [32]. For electrophoresis TBE (89 mM Tris base–2.5 mM Na_2EDTA–89 mM boric acid) buffer and 0.7% Sigma agarose was used. Electrophoresis lasted at 125 V, 35 mA for about 3 h in a vertical slab gel, then the gel was stained with ethidium bromide (1 mg/l) for 30 min and washed for 15 min. Photographs were taken of gels placed over a short wave UV light source, using Fortepan film (18 DIN). For molecular size standards the following reference plasmids were used: V517 [33] (1.4; 1.8; 2.0; 2.6; 3.4; 3.7; 4.8; 35.8 Md); R27 (112 Md); RI (62 Md); Tp116 (143 Md).

Statistical analysis. The χ^2 test was used to compare antibiotic resistance of *E. coli* and *Klebsiella* strains isolated from the wards indicated above. The probability of the presence of multiple resistant and R-plasmid carrier strains in a ward was calculated by probability-estimation.

Results

Tables II/a and II/b show the distribution of *E. coli* strains derived from different hospital wards according to serogroup, phage pattern, colicin type, antibiotic resistance and R-plasmid carrier state. The sero- and phage typing examinations carried out on 52 *E. coli* strains, derived from 41 persons, mainly with UTI in hospital "A" revealed that these organisms did not originate from the hospital environment but were associated with community-acquired or endogeneous infections. They belonged to 16 different serogroups (O1, O2, O4, O6, O7, O8, O12, O18ac, O19, O21, O30, O57, O75, O86, O149 and not typable) and within the serogroups to different phage patterns. Out of the 52 strains 26 were sensitive to the 26 antibiotics tested, 15 were resistant to 1–3 antibiotics and 11 were multiple resistant (resistant to 4–10 antibiotics). R-plasmid was carried by 17 strains.

Table I

Source of E. coli and Klebsiella strains

Source	No. of persons*	No. of strains*	Diagnosis		Specimen		Antibiotic therapy
			Urinary tract infection	29+(4)	Urine	34+(3)	Cefoperazone Cephaloridine
Hospital "A", medical ward	32+(4)	43+(4)	Cholecystitis	2	Bile	2	Tobramycin
					Faeces	2	Doxycycline
			Leukaemia	1	Sputum	2	Nitrofurantoin
					Other	3+(1)	Cephalexin
Hospital "A", out-patient department	9+(7)	9+(7)	Urinary tract infection	9+(7)	Urine	9+(7)	Cefazolin Tobramycin
Hospital "B", haematological ward	19	22	Leukaemia	14	Urine	7	Cephalotin
					Faeces	11	Cefuroxime
			Other	5	Other	4	Tobramycin Co-trimoxazole
Total	60+(11)	74+(11)				74+(11)	

* Bracketed figures indicate *Klebsiella* strains

Table II/a

Type and antibiotic resistance of E. coli strains isolated from UTI in the medical ward of hospital "A"

No. of strains	Serogroup	No. of		Sensitive to	Resistant to		No. of R+ strains
		phage patterns	colicin types	antibiotics	1–3	4–10	
1	O1	1	—	1	—	—	x
6	2 : K1	3	—	6	—	—	x
3	O4	2	—	1	—	2	2
1	O6	1	—	1	—	—	x
1	O7	1	1	1	—	—	x
2	O8	2	1	1	1	—	1
2	O12	2	1	2	—	—	x
11	O18ac	2	2	—	10	1	6
1	O19	1	—	1	—	—	x
2	O21	2	—	2	—	—	x
1	O30	1	—	1	—	—	x
1	O57	1	—	1	—	—	x
2	O75	2	—	—	—	2	2
1	O86	1	—	—	—	1	1
1	O149	1	—	—	1	—	x
16	ONt	10	3	8	3	5	5
52				26	15	11	17

Table II/b

Type and antibiotic resistance of E. coli strains isolated from LP in the haematological ward of hospital "B"

No. of strains	Serogroup	No. of		Sensitive to	Resistant to		No. of R+ strains
		phage patterns	colicin types	antibiotics	1–3	4–16	
3	O2	1	—	—	—	3	3
2	O4	1	—	1	—	1	1
1	O5	1	—	—	1	—	.
1	O7	1	—	—	—	1	1
1	O9	1	1	—	—	1	1
1	O12	1	—	1	—	—	.
1	O15	1	1	—	1	—	1
1	O16	1	—	—	1	—	.
1	O22	1	1	1	—	—	.
1	O46	1	—	1	—	—	.
1	O83	1	1	1	—	—	.
8	ONt	4	2	—	—	8	3
22				5	3	14	10

. = Not examined

Table II/c

Phage type distribution and antibiotic sensitivity of Klebsiella strains

No. of strains	Phage type	Sensitive to	Resistant to	
		antibiotics	1–3	4–10
4*	I.B25	—	—	4
2**	II.A1	1	1	—
1*	III.B6	—	—	1
1*	IV.A1	—	1	—
2**	VII.A4	—	2	—
1**	Nt	1	—	—
11		2	4	5

* Hospital "A", out-patients department
** Hospital "A", medical ward

The 22 *E. coli* strains isolated mainly from LP in the haematological ward of hospital "B", belonged to 12 serogroups and 13 phage patterns. Epidemic type was not found. Out of the 22 *E. coli* strains 5 were sensitive to 26 antibiotics, 3 were resistant to 1–3 and 14 to 4–16 antibiotics; R-plasmid was carried by 10 multiple resistant strains. The *Klebsiella* strains belonged to 6 phage types; part of them were resistant to two and others to 14–17 anti-

biotics. Out of the 74 *E. coli* and 11 *Klebsiella* strains 22 were resistant to 1–3 and 30 were resistant to 4–17 antibiotics. The strains belonging to the latter group were derived mainly from LP.

Thirty *E. coli* strains were examined for R-, Col- and other plasmids; R-plasmid was demonstrated in 27 strains (Tables II/a, b).

Figure 1 shows the resistance to certain antibiotics in the different wards. With penicillin derivatives ampicillin and carbenicillin resistance was frequent among strains isolated from all the three hospital units, whereas azlocillin and mezlocillin resistance was frequent among LP strains; cloxacillin resistance occurred infrequently. As to the first generation cephalosporins, cephaloridine, cephacetrile, cephalothin resistance was frequent, but cefazolin resistance was rare. With second generation cephalosporins, cefamandole resistance occurred more frequently than cefuroxime resistance. Resistance to third generation cephalosporins was rare, and was recorded mainly among LP strains. In the aminoglycoside group: gentamicin and tobramycin resistance was found in 4 and 5 strains, respectively. One strain was resistant to amikacin and another to sisomicin. UTI and LP strains were resistant most frequently to tetracyclines, the incidence of chloramphenicol resistance was the same as azlocillin and mezlocillin resistance. Out of the 85 strains 12 were resistant to sulphamethoxazole trimethroprim (mostly LP strains); only 6 UTI strains were nitrofurantoin resistant (Fig. 1). Table III shows the spectrum of the 49 antibiotic resistant strains.

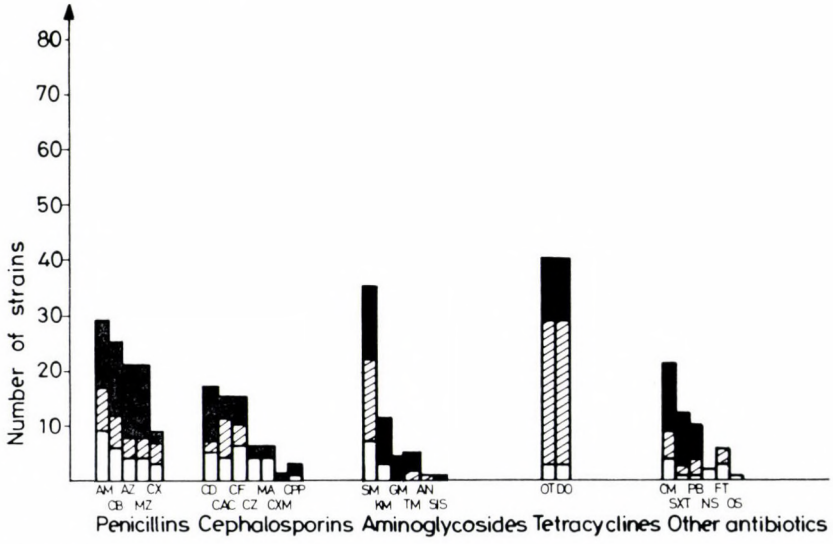

Fig. 1. Incidence of antibiotic resistance determinants of *E. coli* and *Klebsiella* strains according to source. Open columns, Medical Ward 1, Hospital "A" (16 strains); shaded columns, Medical Ward 2, Hospital "A" (47 strains); closed columns, Haematological Ward, Hospital "B" (22 strains)

Table III

Antibiotic resistance spectra of E. coli and Klebsiella strains

No. of resistance determinants	Resistance determinants	No. of strains*
1	SXT	1
2	OT DO	15
		(1)
2	AM CB	1
		(3)
3	SM OT Do	1
3	OT DO CM	1
4	OT DO CM KM	1
4	AM SM CM NA	1
4	OT DO CM SXT	1
5	AM CB AZL OT DO	1
5	CAC OT DO CM SXT	1
7	AM MZ CB AZL OT DO CM	1
7	AM CX CAC OT DO PB FT	1
8	AM MZ CB AZL SM KM OT DO	2
8	AM MP CB AZL CAC OT DO CM	1
9	AM MZ CB AZL KM OT DC CM SXT	1
9	AM MZ CB AZL CD SM OT DO CM	1
10	AM MZ CB AZL CX CD CF OT DO CM	1
10	AM MZ CB AZL CD OT DO CM SXT PB	1
11	AM MZ CB AZL CD SM KM OT DO CM SXT	1
11	AM MZ CB AZL OT DO CM KM GM TM SXT	1
12	AM MZ CB AZL CX CF CAC CZ MA CXM CFP SM	1
13	AM MZ CB AZL CD CF CAC SM KM OT DM CM SXT	1
13	AM MZ CB AZL CD CF SM KM TM OT DO CM SXT	1
14	AM MZ CB AZL CX CF CD CAC CZ MA OT DO CM SXT	(4)
15	AM MZ CB AZL CD CZ MA CFP KM GM TM OT DO CM PB	1
16	AM MZ CB AZL CX CD CF SM KM GM TM OT DO CM PB SXT	1
17	AM MZ CB AZL CD CF CZ MA CFP SM OT DO CM OS NA FT SXT	(1)
		49

* Bracketed figures indicate *Klebsiella* strains

Table IV

Geometric averages of MIC values for wild E. coli strains and their transconjugants isolated from hospitals "A" and "B"

Source of strains	No. of strains	Antibiotics μg/ml						
		AM	CB	AZL	MZ	CD	CF	MA
Hospital "A", medical ward, UTI	10 W	168.9	157.6	181.0	84.4	22.6	18.4	2.0
	10 T	78.8	97.0	68.6	42.2	6.1	7.5	1.3
Hospital "B" haematological ward, LP	10 W	477.7	415.9	337.8	168.9	19.7	9.2	2.1
	10 T	274.4	388.0	181.0	90.5	12.1	10.6	1.8

W = Wild
T = Transconjugant

MIC values of some broad spectrum penicillin derivatives and cephalosporins were determined for selected multiple resistant *E. coli* strains and their transconjugants. Geometric average of the MIC values was calculated from

MICs obtained for single strains. Table IV summarizes the results obtained for wild and transconjugant strains. *E. coli* J5-3 recipient strain was used as control. In the case of *E. coli* J5-3 strain the MICs of penicillins varied between 2–4 μg/ml and those of cephalosporins between 1 and 8 μg/ml. The lowest MICs were found with cefamandole. MICs of penicillins were high in all groups. MICs for both wild and transconjugant strains were significantly higher in the LP group than in the UTI group (Table IV).

Plasmid profile analysis of *E. coli* was carried out with 10 UTI (Table V/a) and with 10 LP strains (Table V/b). These Tables show the phenotypic characters of the strains (serogroup, phage pattern, colicin production, colicin type, antibiotic resistance pattern), the transferable resistance determinants and the number, molecular size, incompatibility group (Inc) and phage restrictive effect of plasmids. One plasmid mediating resistance to 6–9 antibiotics was carried by 7 strains. In case of UTI strains resistance to 4–7, in case of LP strains resistance to 8–9 antibiotics was demonstrated. Other strains carried 2 to 10 plasmids (see Tables V/a and V/b).

Among the UTI strains a plasmid of the same molecular size and Inc group (100 Md, Inc FI) was carried by two strains of the same serogroup and phage pattern. Plasmids in other strains possessed different plasmid profiles; "epidemic" plasmids were not found.

Among the LP strains 6 plasmids of the same molecular size were carried by two strains; a plasmid of 70 Md, belonging to Inc B and possessing phage restrictive effect was harboured by three strains; out of these three two belonged to the same serogroup and were isolated from the same person. The 10 strains examined were characterized by 7 kinds of plasmid profile.

Examining the resistance determinants of 20 plasmid-carrying strains it was demonstrated that in 17 out of 18 penicillin resistant strains, the resistance to penicillins was transferable. Transferable resistance was found only in 3 cephalosporin resistant strains. Out of the 17 tetracycline resistant strains 11 possessed transferable resistance; SXT resistance was transferable in the 6 SXT resistant strains.

The number of strains resistant to all antibiotics tested was significantly higher among the LP strains than among the UTI strains ($p < 0.01$, Table VI). The probability of resistance developing to 4–17 antibiotics was higher >99.9% for LP as compared to UTI strains. The probable occurrence of R^+ strains was higher by 89.8% for the former group of strains. The number of resistance determinants per one strain in the two groups was 1.6 and 6.8, respectively (4.25-fold difference). The plasmid mediated resistance per one strain also differed significantly in the two groups: 3.8 and 8.1, respectively (2.13-fold difference) (Table VII).

Table V/a

Complex typing of E. coli isolated from UTI in hospital "A"

Designation of strains/month	Serogroup	Phage pattern	Colicin type*	Antibiotic resistance*	No. of plas-mids	Plasmid profile, molecular size in Md	Inc group	Phage restric-tion
199/I	O75	4, 4a, 9 14	—	AM MZ *CB* AZL CAC OT DO CM SM	3	83; 63; 1.0	.	+
369/III	O8	2, 4ab, 6, 7, 13	*V*	*OT DO* SM FT	4	110; 70; 5.1; 1.2	I1	+
482/III	O86	22	—	*SM OT DO* CM KM	1	125	.	+
509/IV	O4	4ab, 20, 22	—	*AM MZ CB AZL* CX CD CF *OT DO CM* SM	1	100	FI	+
513/IV**	Nt	17	*Nc*	MZ *OT DO CM* SXT SM KM FT	3	90; 53; 43	FI, P	—
1095/X**	O4	4ab, 20, 22	—	*AM MZ CB AZL* SM KM *OT DO*	1	100	FI	+
631/IV	O75 : K5	4b, 15, 16	—	*AM CB MZ AZL* OT DO SM	10	75; 53; 34; 8.0; 6.0; 4.8; 4.2; 3.6; 2.7; 2.5	I1	+
296/II	O18ac : K5	4, 4ab, 15	*Nc*	*AM MZ CB AZL* CD CF CAC CZ MA CFP	8	65; 42; 31; 26; 5.6; 4.1; 3.3; 2.4	I2	+
629/IV	Nt	1, 2, 3, 4, 4ab, 6, 7, 12, 13, 15	*Nc*	*AM MZ CB AZL* CF *CAC* SM CM FT	2	90; 64	FI	+
512/IV	Nt	2, 3, 4ab, 6, 7, 17, 30	—	*AM* SM CM NA *OT*	5	60; 5.9; 4.5; 3.3; 2.0	K	—

* Letters printed in italics designate transferred Col plasmids and resistance
** Strains isolated from the same patient
Nc = not characteristic

Table V/b

Complex typing of E. coli isolated from LP in hospital "B"

Designation of strains/month	Serogroup	Phage pattern	Colicin type*	Antibiotic resistance*	No. of plasmids	Plasmid profile, molecular size in Md	Inc group	Phage restriction
253/II	Nt	2, 3, 4, 6, 7, 12, 15, 23, 24	*Nc*	*AM MZ CB AZL* CD CAC *SM KM OT DO CM SXT*	6	93; 83; 73; 18; 5.4; 1.0	I1	+
294/II	O9	2, 3, 6, 7, 12, 24	*Nc*	*AM MZ CB AZL* CD CF CAC *SM* KM *OT DO* CM *SXT*	6	93; 83; 73; 18; 5.4; 1.0	I1	+
371/III	O15	4ab, 12, 13, 23, 30	*Nc*	*AM OT DO*	3	58; 4.2; 3.3	FI	+
391/III	Nt	Nt	—	*AM MZ CB AZL CX CD* CF *CAC* CZ MA CXM CFP *SM KM OT DO*	3	94; 2.1; 1.4	FI	+
811/V	O4	4ab, 20, 22	—	*AM MZ CB AZL SM KM* OT *DO CM*	1	70	B	+
1059/V	O7	4a, 4b, 5	—	*AM MZ CB AZL* SM *KM OT DO CM SXT*	1	90	FI	+
1061/VI	O7	4ab, 13	—	*AM MZ CB AZL CD* CZ MA CFP *KM* GM PB	2	75; 63	FIV	—
1062/IX	ONt	Nt	*V*	*AM MZ CB AZL* CD SM *KM OT DO CM SXT PB*	2	78; 69	FI	+
1065/X**	O2	15	—	*AM MZ CB AZL* CD CF SM *KM* TM *OT DO CM SXT*	1	70	B	+
1068/X**	O2	15	—	*AM MZ CB AZL OT DO* CM SM *KM* GM TM *SXT*	1	70	B	+

* Letters printed in italics designate transferred Col plasmids and resistance
** Strains isolated from the same patient
Nc = not characteristic

Table VI

Antibiotic resistance and R plasmid carrier state of E. coli and Klebsiella strains in hospitals "A" and "B"

Source	Sensitive to	Resistance to		No. of R+ strains	No. of strains
		1–3	4–17		
	antibiotics				
Hospital "A", medical ward	23	17	8	14	48
Hospital "A", out-patients department	5	2	8	3	15
Hospital "B" haemato-logical ward	5	3	14	10	22
Total	33	*22*	*30*	27	85

Table VII

Resistance determinants of E. coli and Klebsiella strains in hospitals "A" and "B"

Source	No. of strains	No. of resistance determinants	No. of resistance determinants per one strain	No. of R+ strains	No. of transferable resistance determinants	No. of transferable resistance determinants per one strain
Hospital "A" medical ward	48	75	1.6	10	38	3.8
Hospital "B", haemato-logical ward	22	149	6.8	10	81	8.1
Total	70	224	3.2	20	119	5.9

Discussion

Although carried out with a limited number of strains, complex typing performed in the present study has given answers to several of the questions. *E. coli* and *Klebsiella* strains belonging to different serogroups and phage patterns were present in both hospital units examined, but epidemic strains were not found. There was a significant difference in the resistance of strains originating from the two groups of patients (UTI and LP). Resistance was significantly commoner and the R-plasmid mediated resistance was more frequent among the LP than among the UTI *E. coli* strains. The resistance determinants of different penicillin derivatives were, as a rule not transferred together.

Schaberg et al. [34] reported on R-plasmid transfer of multiple resistant strains in catheter-bag filled with urine. Hughes et al. [35] found differences in the plasmid carrier state of *E. coli* strains of different serogroups isolated from urinary tract infections. Multiple resistance was more frequent in serogroups O4, O9 and O18, which differed from the other serogroups in colicin production, or in colicin type. In our material, serogroup O2, O4, O7, O9 and O75 strains were multiple resistant, and, except two strains, were not colicinogenic.

Detection of plasmids coding resistance to different antibiotics, and the route of their spreading may forecast the efficacy of an antibiotic or of a group of antibiotics. Resistance to some cephalosporins (CD, CAC) was mediated by plasmids coding multiple resistance, whereas insusceptibility to other cephalosporins (CF, CZ, MA, CXM) was not plasmid-determined, and consequently their spread could not be expected. Krčméry et al. [37] described CXM and MA determining plasmids isolated from hospital *Klebsiella* strains. The MA resistance occurred together always with CF resistance and TEM-1 type β-lactamase (AM, CB, AZL) [36, 37].

Jacoby and Sutton [38] found that certain β-lactamase producing strains were resistant to MA in a high degree, but to other cephalosporins they exhibited a lower degree of resistance. For our UTI and LP strains the MIC values of CD varied between 4 and 256, of CF between 4 and 64, of MA between 1 and 32. SXT resistance was coded by transferable plasmid in LP strains and accordingly, in this group the spread of SXT resistance might be expected. Co-trimoxazole is often used to prevent infection of leukaemic patients. Wilson and Guiney [8] also found plasmid mediated SXT resistance in *E. coli* and *K. pneumoniae* strains isolated from leukaemic patients. Jacoby [39] directed attention to the danger caused by the prophylactically given SXT; the SXT given for prevention caused the selection of the resistant microorganisms. In his opinion, the best antibiotic therapy for this endangered disease-group is not sufficiently elaborated yet.

By one single plasmid was determined AM, CB, MZ, AZL, OT, DO resistance (and in one strain CM resistance, too) in two UTI *E. coli* strains of serogroup O4 of the same phage pattern; the size of the plasmid was 100 Md and belonged to Inc FI. Similarly, the AM, CB, AZL, MZ, TC, CM and SXT resistance in three LP strains were coded by one single plasmid too; the strains belonged to two serogroups and phage patterns, the molecular mass of this plasmid was 70 Md and belonged to Inc B. Mayer et al. [40] isolated a plasmid from *E. coli* and *K. pneumoniae*, which transferred β-lactam and SXT resistance together, its size was about 52.5. The SXT resistance is often linked to plasmid or transposon, so its spread may be frequent and rapid [18]. An endemic R plasmid of broad host range was reported from a hospital for veteran soldiers in the USA; the 89 Md plasmid determined resistance to 9

antibiotics. A molecular analysis for epidemiological purposes of multiple resistant, nosocomial *Serratia marcescens* strains was carried out by Tombkins et al. [20]. Schaberg et al. [41] recommend "agarose gel electrophoresis pattern" as a suitable tool for epidemiological examination of nosocomial infections; they mentioned that the method is applicable in "conventional" microbiological laboratories, too. Rubens et al. [6] carried out long-term examinations to characterize the plasmids of multiple resistant hospital strains; using gel electrophoresis, restriction enzyme analysis and DNA–DNA hybridization. The application of these "molecular epidemiology" methods is becoming more and more important in the examination of nosocomial infections [42].

Sero- and phage typing completed with plasmid profile analysis in our study allows a better marking of the infective bacteria on the one hand, and the characterization of the plasmids coding antibiotic resistance on the other. These investigations may contribute to developing an adequate antibiotic strategy.

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PROTECTIVE ACTIVITY OF TWO HUMAN INTRAVENOUS IMMUNOGLOBULIN PREPARATIONS IN EXPERIMENTAL INFECTION WITH AN ENCAPSULATED *STAPHYLOCOCCUS AUREUS* STRAIN

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The protective effect of two different human immunoglobulin preparations for intravenous use and one specific staphylococcal immunoglobulin for intramuscular application were compared in mice infected with the capsular *Staphylococcus aureus* Smith strain. Immunovenin is produced by partial fragmentation of IgG with plasmin; it contains about 60% intact IgG and 40% Fab and Fc fragments. Immunovenin-intact is produced by a polyethylene glycol (mol wt 6000) fractionation method followed by ion exchange chromatography and contains more than 95% intact IgG molecules. A specific staphylococcal immunoglobulin is obtained by a rivanol/ethanol fractionation method from selected sera with high anti-alpha toxin level. All three types of immunoglobulins induced similar degree of protection when their effect was determined as activity (ED_{50}) per gram immunoglobulin.

Infections caused by *Staphylococcus aureus* remain a serious problem despite the availability of increasing number of antibiotics. The therapeutical problems are caused by the antibiotic resistance of many of the isolated strains and by the variety of means by which this organism evades the defence mechanisms of the body. *S. aureus* produces a number of extracellular enzymes and toxins which cause destruction of cells including polymorphonuclear leukocytes. The surface protein A binds to the Fc portion of IgG and so prevents the efficient opsonization of the bacteria [1]. The newly described decomplementation factor [2] protects the bacterial cell from the action of complement by induction of abortive complement-consuming reactions in the surrounding fluid phase. The polysaccharide capsule, when present, protects the bacterial cell from phagocytosis and it is an important factor for invasiveness. Protein A and the decomplementation factor inhibit the action of humoral immune defences (antibodies and complement), while leukocidin and the capsule suppress directly the phagocytosis and killing of the bacteria by polymorphonuclear leukocytes and macrophages. About 70% of the *S. aureus* strains isolated from patients with sepsis possess capsules of type 5 or 8 [3].

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It is known that in the presence of anticapsular antibodies encapsulated bacteria are effectively opsonized and killed by phagocytes in vitro and in vivo [4]. The serum of adult blood donors contains anti-staphylococcal capsule antibodies [5]. It seems a rational approach to treat severe staphylococcal infections by the administration of immunoglobulin preparations. In the last years human immunoglobulin preparations for intravenous use have become available. Their main advantages, when compared to the human immunoglobulin solutions for intramuscular application, are obvious — they supply large doses of IgG, the antibodies enter directly the blood stream and the effect is immediate. Immunoglobulin preparations for intravenous use are produced by a wide variety of technological processes. Some of them result in alterations of the IgG molecule from minor ones to a complete elimination of the Fc fragment. These structural differences have inevitably an effect on the biological activity of the immunoglobulins [6, 7].

This report describes the protective activity of two types of human immunoglobulin preparations for intravenous use and of a specific staphylococcal immunoglobulin in mice infected with an encapsulated *S. aureus* strain.

Materials and methods

Bacterial strain. The encapsulated *S. aureus* Smith diffuse strain was kindly provided by the Human Institute for Serobacteriological Production and Research, Budapest. It formed diffuse colonies on semisolid agar with serum, had no detectable surface protein A and was not sensitive to the available group of phages. The organism was grown on blood agar overnight, collected from the surface, washed once and using an optical standard, suspended to contain 10^9 bacteria per ml.

Experimental animals and treatment schedule. Outbred ICR mice weighing 30–35 g were used throughout the experiments. They had free access to food and water. The LD_{50} of the *S. aureus* strain injected intraperitoneally as determined by the method of Kärber [8], was 2×10^8 bacterial cells. This dose did not change after four consecutive passages of the strain in ICR mice. Groups of five animals were injected intraperitoneally with different doses of immunoglobulins and 10–15 min later with 10^9 bacteria by the same route. Deaths were recorded daily for one week. The experiment with each dose was repeated at least twice.

Immunoglobulin preparations. All preparations listed below were produced in the Institute of Infectious and Parasitic Diseases in Sofia.

Immunovenin was obtained by partial fragmentation with plasmin of IgG, isolated from placental blood. It contained about 60% intact IgG molecules and up to 40% immunoglobulin fragments (Fab + Fc) [9].

Immunovenin-intact was produced by a polyethylene glycol (mol wt 6000) precipitation method and contained more than 95% intact IgG molecules [10]. The two preparations contained 5% immunoglobulins and have passed the quality and safety tests required for intravenous immunoglobulin G.

Specific staphylococcal human immunoglobulin for intramuscular use was produced by a modified rivanol/ethanol fractionation method as described in [11] and by Peeva et al. (manuscript in preparation) from selected sera of retroplacental blood obtained from obstetrical departments in regions in this country where staphylococcal alpha-toxoid vaccine has been widely applied for the prevention of staphylococcal infections in newborns.

The anti-alpha toxin and anti-leukocidin titres of the immunoglobulin preparations were determined as described [12, 13].

Statistical analysis. In order to test the significance in the difference of the protection conferred by different doses of the immunoglobulin preparations, the survival data were

entered into a computer and analysed using a software system for chi-square analysis with the Yates' correction. The protective dose 50 per cent and its 95 confidence limits were calculated by Finney probit analysis. The difference between the effects of the immunoglobulin preparations was considered significant if the 95% confidence limits did not overlap [4].

Results

The molecular composition of the three types of the immunoglobulin preparations — two for intravenous and one for intramuscular application — was compared by gel-chromatography on Sephadex G-200 (Fig. 1). The content of fragments in Immunovenin is about 22% (Fig. 1A). It is not known whether the fragments of IgG present in this preparation play any protective role. The protective action of Immunovenin is due to the large amount of IgG molecules present. The IgG molecules in Immunovenin-intact (Fig. 1B) are close to molecular homogeneity, only a small fraction IgG dimers is present. The presence of IgG aggregates with higher molecular weight in the staphylococcal human immunoglobulin (Fig. 1C) limits its application only to the intramuscular route.

Single lots of the immunoglobulin preparations are produced from plasma and serum from more than 1000 individual donors and their antibody content

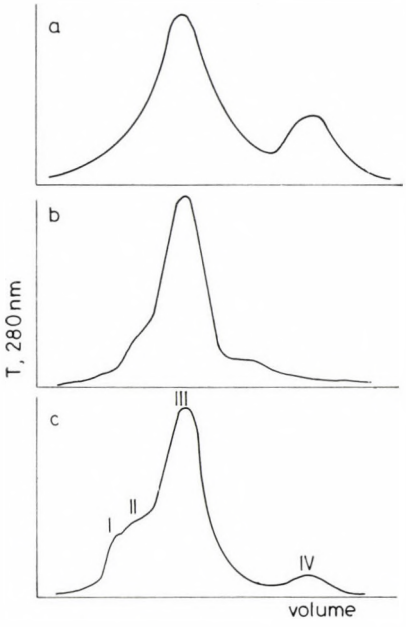

Fig. 1. Typical elution profiles of the immunoglobulin preparations. Gel-filtration, Sephadex G-200. A = Immunovenin; B = Immunovenin-intact; C = specific staphylococcal immunoglobulin for intramuscular application. I = aggregates of IgG; II = dimers; III = intact IgG molecules; IV = fragments of IgG

Table I

Levels of anti-alpha toxin and anti-leukocidin antibodies in the human immunoglobulin preparations investigated

Immunoglobulin preparation	Lot	Anti-alpha toxin antibodies (AU/ml)	Anti-leukocidin antibodies
Immunovenin	52	8 AU	1 : 24
	55	8 AU	1 : 192
	64	7 AU	1 : 6
Immunovenin-intact	10	3 AU	1 : 12
	11	7 AU	1 : 24
Specific staphylococcal immunoglobulin for intramuscular application	C-46	40 AU	1 : 12

AU = Antitoxic units

is expected to be similar because it represents the mean value of a large group of people. The data presented in Table I show considerable differences in the levels of anti-leukocidin and anti-alpha toxin antibodies. These differences are not reflected in the protective effect of the investigated human immunoglobulin solutions in experimental systemic infection in mice (Table II). The dosage range used for almost all preparations extends from doses with no effect on survival to those ensuring nearly complete protection. The 95% confidence limits of the protective dose 50% of the tested lots of Immunovenin and Immunovenin-intact overlap, no significant difference exists between them. The protective dose 50% of the specific staphylococcal immunoglobulin for intramuscular application is within the same limits when corrected for its higher immunoglobulin content (15% compared to 5% in the intravenous preparations).

The lack of effect of human serum albumin on the course of the infection points out that the observed activity of the immunoglobulin preparations is specific and not due to the plasma volume replacing action of the five per cent protein solution itself.

Discussion

Doses of the intravenous preparations higher than 0.1–0.2 ml per animal protect mice from death caused by a systemic infection with an encapsulated *S. aureus* strain. It is reasonable to expect their activity to be more potent in humans than in the heterologous mouse system. Although one could not use the mouse data as a firm basis for determination of the therapeutic dose in humans, it could be provisionally calculated to be of the order of 2–3 ml per kg body weight. This happens to be the recommended dose intravenous

immunoglobulin for treatment of severe bacterial infections. Such amounts of immunoglobulin solutions could not be applied with the preparations for intramuscular use.

It has been expected that the effect of Immunovenin-intact would be superior to that of Immunovenin because of its higher content of intact IgG molecules, but no difference in their effects was found. Immunovenin-intact

Table II

Comparison of the prophylactic activity of human immunoglobulin preparations in systemic S. aureus infection in mice

Treatment		Dose ml/mouse i.p.	Number survived[a] (number treated)	Protective dose 50% (95% confidence limits)
Saline		0.2	7 (49)	—
Human serum albumin 5%		0.2	2 (15)	—
Immunovenin	Lot 52	0.05	3 (10)	0.149 ml
		0.1	2 (10)	(0.078–0.285 ml)
		0.2	3 (10)	
		0.4	7 (10)**	
		0.8	9 (10)**	
	Lot 55	0.05	4 (15)	0.142 ml
		0.1	7 (15)*	(0.079–0.190 ml)
		0.2	6 (15)	
		0.4	14 (15)**	
		0.8	12 (15)**	
	Lot 64	0.05	2 (10)	0.100 ml
		0.1	5 (10)*	(0.060–0.165 ml)
		0.2	8 (10)**	
		0.4	10 (10)**	
		0.8	9 (10)**	
Immunovenin-intact	Lot 10	0.05	4 (10)	0.103 ml
		0.1	3 (10)	(0.046–0.203 ml)
		0.2	6 (10)**	
		0.4	10 (10)**	
		0.8	9 (10)**	
	Lot 11	0.05	4 (10)	0.131 ml
		0.1	3 (10)	(0.074–0.233 ml)
		0.2	5 (10)*	
		0.4	8 (10)**	
		0.8	10 (10)**	
Specific staphylococcal immunoglobulin for i.m. use	Lot C-46	0.006	2 (10)	0.025 ml
		0.012	3 (10)	(0.016–0.039 ml)
		0.025	3 (10)	
		0.05	15 (20)**	
		0.1	10 (12)**	
		0.2	10 (10)**	
		0.4	10 (10)**	
		0.8	10 (10)**	

[a] Protection induced relative to controls (saline and human serum albumin treated groups combined) determined by chi-square test

* $p = 0.05$

** $p = 0.01$

should at least theoretically be regarded as the better choice for treatment of infections where not only the antitoxic but primarily the opsonizing activity of antibodies is expected to benefit the patient. The protective activity of the different lots of the two preparations is very close. The low lot to lot variation points to the fact that the level of antibodies in the blood donor population, which protect the mice, is stable. The results presented here do not prove that the protective antibodies are anticapsule antibodies, but a number of indirect evidence lends support to this assumption. Freshly isolated from cases of severe local infections, strains of *S. aureus* which are not encapsulated but highly toxigenic, failed to kill the mice in doses up to 5×10^9 (not presented). Pathogenicity of *S. aureus* strains for mice is thought to be due mainly to their invasiveness associated with the polysaccharide capsule [14]. The preparations investigated have a well-expressed prophylactic effect. The protective action of the immunoglobulin preparations is obviously not solely determined by the levels of anti-alpha toxin and anti-leukocidin antibodies.

Many attempts have been made to immunize humans with purified capsular polysaccharides of *S. aureus* and other bacteria [3, 5]. Due to the weak antigenicity of the vaccines, the antibody response is weak. In an effort to make them potent immunogens, capsular polysaccharides have been coupled to immunogenic carriers [4]. Mouse monoclonal antibodies to *S. aureus* capsule types 5 and 8 have been obtained [14]. Human monoclonal anti-capsule antibodies may be used in the future for therapy of staphylococcal infections. It is hoped that the better understanding of the mechanisms of capsular synthesis would permit to find specific inhibitors of this synthetic pathway which could be active in vivo. Such agents could reduce the virulence of the invading microorganism and thus facilitate the defence against it [4]. These approaches in the treatment of infections caused by *S. aureus* and other encapsulated bacteria will be used in the near or more distant future. Meanwhile the human immunoglobulin preparations for intravenous use are already available. Their application would be of particular value in the treatment of patients with a compromized immune system (after splenectomy, major trauma and burns), in newborn and prematurely born children. The rational use of the intravenous immunoglobulins will permit their great immunotherapeutic potential to be fully exploited.

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PHASES OF THE SEROLOGICAL RESPONSE IN *PSEUDOMONAS AERUGINOSA* INFECTIONS

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Sixty-four infections due to *Pseudomonas aeruginosa* in patients of a respiratory intensive care unit were studied. The length of serologically latent period following the appearance of *P. aeruginosa* decreased with the growing severity of the infection, however, the longest latent period occurred in lethal cases. As the phase of development of peak anti-LPS antibody titres was constant in each group, in fatal infections the serological response developed too late when sepsis had already started. The duration of the persistence of peak titres was also stable. In consequence of a close negative correlation between these two latter parameters, the period from the onset of antibody increase till the beginning of decrease was especially constant, independent of the severity of infection, the duration of the antigenic stimulus and the intensity of the serological response. The rate of decrease was an another stable value. All these refer to an endogenous, time-dependent regulation which, despite existing clinical symptoms and the presence of *P. aeruginosa*, starts anti-LPS antibody level to decline. Pseudomonas carrier state in convalescence was observed in cases with prolonged persistence of peak titre and with a lower rate of titre decrease.

An analysis of the kinetics of the serological response in 59 *Pseudomonas aeruginosa* infections [1] made obvious that the anti-lipopolysaccharide (anti-LPS) antibody (Ab) titres decreased in many patients whose condition tended to improve before the stop of antigenic stimulus, i.e. when *P. aeruginosa* was still present. Therefore, it seemed desirable to study the length of the different phases of the serological response according to the severity of infection, the duration of the presence of *P. aeruginosa* and the intensity of the Ab response.

Patients and methods

Patients. In thirty patients treated at the respiratory intensive care unit of the László Hospital for Infectious Diseases, Budapest, infectious complications mainly due to *P. aeruginosa* were examined during their whole hospital stay. Their average age was 46.9 years. The underlying disease was tetanus in sixteen, polyradiculitis in five, myasthenia gravis in five, meningo-encephalomyelitis in two patients, cerebral embolism and subarachnoideal bleeding in one case each. Twenty-five of them were intubated and tracheotomized, and subjected to artificial respiration for at least 24 h. Four patients had mild (quick recovery, in some cases

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only transient respiratory insufficiency), six moderate (expressed clinical symptoms with respiratory insufficiency of short duration), seventeen severe (life-threatening situation, with several days of artificial respiration), and three lethal underlying disease.

Bacteriological and serological surveillance. Facultatively pathogenic bacteria growing from the skin, nose, ear, throat or trachea, urine, faeces, pus and blood on admission, before and during the acute phase of infections at 3–4, in the convalescence at 6–7 day intervals were registered, and the serogroups of *P. aeruginosa* were determined on the basis of the antigenic scheme by Lányi and Bergan [2]. Blood samples were taken at the beginning of hospitalization, then on every second day, under ongoing infections on every third or fourth day, during convalescence once a week and stored at −20 °C. All samples were tested in one series of experiment for Ab by passive haemagglutination carried out as described by Neter et al. [3] and adapted to Takátsy's microtitrator [4]. The method was performed with separate batches of sheep erythrocytes sensitized by purified LPS [5] of 9–13 different *P. aeruginosa* serogroups which occurred in the patient or in his environment. The Ab thus determined were considered as "total antibody" (TAb), while the IgG anti-pseudomonas Ab level was measured after the sera had been treated with 0.2 mol/litre 2-mercaptoethanol.

Infections. General and local clinical symptoms with a positive bacteriological result from the site of inflammation as well as a significant rise in the specific Ab titres confirmed that all the 59 infections were associated with pseudomonas [1]. Among these 7 tracheobronchitis, 23 pneumonia, 10 urinary tract infections, 7 thrombophlebitis, 8 rhinitis of nasogastric tube origin, 1 enteritis, 1 infected decubitus, 1 postinjection abscess and 1 peritonitis (as a consequence of septic parametritis) were observed.

The severity of the infections was categorized as follows: *mild:* good general condition without intensive local symptoms, temperature below 38 °C; *moderate:* more expressed clinical signs, temperature exceeding 38 °C; *severe:* septic state, i.e. intermittant fever, heavy prostration, erythrocyte sedimentation rate about 100 mm/h, rapidly developing anaemia with either pozitive or negative blood culture; *lethal:* fatal sepsis. Shock was stated when the systolic blood pressure was lower than 70 mmHg for more than one hour, with markedly reduced diuresis. Thus, 11 mild, 20 moderate, 12 severe and 16 fatal infections were recorded.

Together with these 59 manifest pseudomonas infections, when analysing the immunological reaction in connection with the duration of harbouring *P. aeruginosa* and the intensity of the immunological response, 5 latent pseudomonas infections accompanied by a serological response have been evaluated.

Besides of these 64 infections, 11 mild or moderate infections were associated with other facultatively pathogenic bacteria *(Klebsiella pneumoniae, Proteus hauseri, Escherichia coli, Acinetobacter calcoaceticus* or *Staphylococcus aureus)*. However, in every instance a simultaneous occurrence of a more severe pseudomonas infection determined the clinical picture. For more details about patients, infections and methods see previous papers [1, 6].

Parameters of the Ab response studied. Duration of latent phase: from the appearance of *P. aeruginosa* at the site of the later developing inflammation to the onset of increase in specific Ab titre in days. Blood samples were taken after admission till the onset of infection, every second day; later every third or fourth day.

Phase of development of Ab titres: days elapsing from the date of blood sample preceeding the onset of increase in Ab titre to the appearance of peak titre.

Phase of latency plus development: from the start of antigenic stimulus to the development of peak Ab titre.

Rate of development of Ab titres: total increase in Ab titre in dilution step per duration of development in days.

Persistence of peak Ab titre: period in days during the peak Ab titres remained unchanged. When only one sample yielded the peak titre, the persistence was considered 3 days, because blood samples had been taken during infection at 3–4 day intervals.

Phase of development of Ab level plus persistence of the titres: the sum of the two phases.

Phase of decrease of Ab titre: period of diminution from the last peak titre to the starting level, in days.

Rate of decrease in Ab titre: decrease in dilution step per duration of decrease from the last peak titre, in days.

Duration of the serological response: period of development + persistence + decrease.

Processing of data. Nine infections, followed by symptomless *P. aeruginosa* carrier state, were evaluated separately. Latent phase could not be determined and was missing in those cases in which the rise in anti-LPS natural Ab titres [1] had preceeded the first pseudomonas-positive bacteriological sample. In severe infections, after the development of septic shock, further parameters e.g. phase of persistence or decrease were omitted because they could have been shortened by the shock. As IgG values (phases according to titrations performed

in sera samples treated with 2-mercaptoethanol) were paralleled with those of "total antibodies" (TAb; i.e. passive haemagglutination titre in untreated serum) strictly and regularly, being only 1–3 days longer sometimes as those of TAb, for the sake of simplicity, IgG data are not shown separately in Tables I and II.

Statistical analysis. In comparing two groups of data, Student's *t*-test or Mann–Whitney's method was used, according to mathematical conditions.

In some cases, only TAb titres could be determined because of the small volume of serum samples [1]. The coefficient of correlation (r) between the available corresponding TAb (~IgM: 1) and IgG data in phases of latency, development, persistence of titres, etc. was calculated.

Results

Serological latent phase (Tables I–III). The latent phase was correlated only with the severity of the infection. Latency of TAb and IgG response both decreased in the following order: lethal > mild > moderate > severe infection.

Phase of development (Tables I–III). This period was constant and independent of all the three factors examined (severity of infection, persistence of *Pseudomonas* and intensity of Ab response). Development of IgG peak titres lasted one to three days longer than that of TAb titres.

Phase of latency plus development (Tables I–III). As the phase of development was stable, the period of latency + development varied according to the changes in latency. For survivors, the period between the appearance of *P. aeruginosa* and peak titre decreased with the growing severity of the infection; but this phase was the longest in lethal cases. IgG latent + development phase surpassed that of TAb by two to three days.

Rate of development (Tables I–III). In both TAb and IgG response the rate of development increased in the order mild < moderate < lethal < severe infections. It rose proportionally with the duration of the presence of *P. aeruginosa* and it was significantly greater in the cases with high Ab titres. As the development phase was constant in every group, the rate of development was determined by the titre of Ab produced. Significant differences in average titres were accompanied by significant changes in development rate.

Persistence of peak Ab titre (Tables I–III). In spite of some fluctuations, there were no significant differences among the groups examined.

Phase of development plus persistence of the titres (Tables I–III). In both TAb and IgG response the mean value of this parameter in each group was very constant. There was no difference between TAb and IgG values either. Close, significantly negative correlation existed between the corresponding development and persistence values (Table IV).

Phase of decrease of Ab titre (Tables I–III). Both TAb and IgG values decreased equally in the following order: severe > moderate > mild > lethal infections; C > B > A; $\bar{x} = 1/640 > \bar{x} = 1/320$. Most of the differences were significant. The duration of the phase of decrease was determined by the peak titre, as the rate of decrease proved to be stable (see below).

Table I

Parameters in days of TAb response according

Severity grade	Parameters	Latent phase	Phase of development	Latency plus development	Rate of development
Mild	n*	8	9	8	9
	$\bar{x}$**	4.25	12.33	16.13	0.21
		±1.75	±1.50	±1.73	±0.03
	R	0–13	7–20	9–22	0.19–0.43
Moderate	n	11	17	11	17
	$\bar{x}$	2.82	11.53	15.64	0.34
		±0.42	±1.05	±1.11	±0.05
	R	0–6	6–18	11–21	0.11–0.80
	p/Md	NS	NS	NS	=0.08
Severe	n	8	8	8	8
	$\bar{x}$	2.0	9.0	11.0	0.61
		±0.89	±1.41	±1.20	±0.18
	R	0–7	5–14	7–16	0.15–1.80
	p/Md	NS	NS	<0.05	<0.05
	p/Mt	NS	NS	<0.02	=0.07
Lethal	n	11	14	9	14
	$\bar{x}$	7.64	10.86	17.78	0.54
		±0.86	±1.86	±1.70	±0.09
	R	4–13	4–21	10–26	0.16–1.14
	p/Md	=0.08	NS	NS	<0.05
	p/Mt	<0.001	NS	NS	NS
	p/S	<0.001	NS	<0.01	NS

* No. of data
** Mean ± SEM
*** Variation coefficient in %

Table II

Parameters in days of TAb response in P. aeruginosa

Duration of harbouring *Pseudomonas*	Parameters	Latent phase	Phase of development	Latency plus development	Rate of development
A	n	2	6	2	6
3 days	$\bar{x}$	7.0	11.67	18.0	0.31
		...	±2.22	±4.0	±0.08
	R	7–7	3–16	14–22	0.15–0.67
B	n	19	23	19	23
4–10 days	$\bar{x}$	4.16	10.48	15.26	0.42
		±0.82	±1.0	±1.07	±0.06
	R	0–13	4–21	8–26	0.11–1.14
	p/A	...	NS	...	NS
C	n	19	23	17	23
⩾ 11 days	$\bar{x}$	4.58	9.65	14.47	0.47
		±0.92	±0.95	±1.23	±0.08
	R	0–13	3–20	7–24	0.10–1.80
	p/A	...	NS	...	NS
	p/B	NS	NS	NS	NS

p/A, B = Significance as compared to the groups A and B

to the severity of P. aeruginosa infections

Persistence of the peak titre	Development plus persistence	Phase of decrease	Rate of decrease	Duration of the serological response	Peak titre averages $1/\bar{x}$
7	7	7	7	7	9
7.86	21.14	26.71	0.10	47.86	160.0
±1.98	±1.06	±4.40	±0.01	±4.41	145.7***
3–16	18–25	15–50	0.04–0.13	37–73	40–640
14	14	9	9	9	17
8.14	20.29	31.11	0.12	48.11	408.7
±1.46	±1.06	±3.14	±0.01	±2.79	136.7
3–23	13–29	14–40	0.08–0.15	37–62	160–2560
NS	NS	NS	NS	NS	<0.02
6	6	6	6	6	8
8.80	21.83	49.0	0.10	70.83	1522.2
±2.76	±2.34	±6.82	±0.01	±5.24	144.5
6–21	12–27	25–66	0.08–0.15	51–86	320–10 240
NS	NS	<0.02	NS	<0.01	<0.001
NS	NS	<0.02	NS	<0.01	<0.01
7	7	8[a]	8[a]	16[a]	16[a]
8.29	20.29	16.38	0.28	23.75	668.3
±1.82	±1.02	±4.04	±0.10	±4.09	165.3
3–16	17–24	6–42	0.10–0.60	5–56	40–5120
NS	NS	...	=0.05	...	<0.01
NS	NS	...	<0.05	...	NS
NS	NS	...	<0.01	...	NS

[a] Till death; modified values by septic shock included
R = Range
p/Md, Mt, S = Significance as compared to the groups mild, moderate or severe

infections according to the duration of harbouring Pseudomonas

Persistence of the peak titre	Development plus persistence	Phase of decrease	Rate of decrease	Duration of the serological response	Peak titre averages 1/x
5	5	5	5	5	6
5.20	18.60	25.60	0.12	44.20	201.6
±1.56	±0.25	±1.83	±0.004	±1.69	157.3
3–11	18–19	19–30	0.11–0.13	38–48	40–640
14	14	10	10	10	23
8.50	21.07	33.50	0.10	52.70	518.3
±1.24	±0.91	±3.20	±0.009	±2.98	145.2
3–18	15–26	18–52	0.06–0.15	41–68	160–5120
NS	NS	=0.12	NS	=0.08	=0.06
18	18	9	9	9	26
10.28	20.61	41.22	0.10	61.89	560.1
±1.42	±1.03	±6.76	±0.012	±6.30	164.6
3–23	12–29	14–66	0.04–0.15	37–86	40–10240
=0.10	NS	=0.05	NS	=0.06	=0.08
NS	NS	NS	NS	NS	NS

For other explanations see legend to Table I

Table III

Parameters in days of total-antibody (TAb) and IgG response according to

Peak antibooy titre		Parameters	Latent phase	Phase of development	Latency plus development	Rate of development
TAb	$\leqslant 320$	n	20	25	18	25
		$\bar{x}$	5.35	10.04	15.44	0.31
			± 0.86	± 0.91	± 0.95	± 0.04
		R	0–13	5–20	9–22	0.10–0.80
	$\geqslant 640$	n	20	27	20	27
		$\bar{x}$	3.65	10.44	14.70	0.54
			± 0.77	± 0.95	± 1.23	± 0.07
		R	0–13	3–21	7–26	0.13–1.80
		p/320	NS	NS	NS	<0.01
IgG	TAb $\leqslant 320$	n	15	20	14	20
		$\bar{x}$	6.27	12.10	17.14	0.23
			± 1.14	± 1.28	± 1.20	± 0.023
		R	0–17	4–23	12–25	0.09–0.50
	TAb $\geqslant 640$	n	18	21	17	21
		$\bar{x}$	4.22	12.76	17.35	0.37
			± 0.91	± 0.89	± 1.02	± 0.042
		R	0–16	5–20	12–28	0.15–0.83
		p/320	NS	NS	NS	<0.01

p/320 = As compared to groups $\leqslant 320$

Table IV

Correlation of the development period and persistence of the peak anti-LPS total-antibody (TAb) and IgG titre in P. aeruginosa infections

Antibody	Parameters of the immune response		Development 3–10 days	Development 11–23 days	p	Development 3–23 days
TAb	Development	n	19	18		37
		$\bar{x}$	7.53	15.89	<0.001	11.60
			± 0.46	± 0.60		± 0.79
	Persistence	$\bar{x}$	12.53	5.11	<0.001	8.92
			± 1.11	± 0.64		± 0.89
		r	-0.205	-0.530	.	-0.719
	Development plus persistence	p/r	NS	<0.001	.	<0.001
		$\bar{x}$	20.06	21.0	NS	20.52
			± 1.05	± 0.61		± 0.62
IgG	Development	n	10	23		33
		$\bar{x}$	8.20	15.96	<0.001	13.61
			± 0.61	± 0.07		± 0.81
	Persistence	$\bar{x}$	10.80	5.48	<0.01	7.09
			± 1.83	± 0.75		± 0.86
		r	-0.534	-0.468	.	-0.630
	Development plus persistence	p/r	NS	<0.05	.	<0.001
		$\bar{x}$	19.0	21.44	NS	20.70
			± 1.59	± 0.74		± 0.72

p/r = Significance of correlation
For other explanations see legend to Table I

the intensity of the anti-LPS antibody response in P. aeruginosa infections

Persistence of the peak titre	Development plus persistence	Phase of decrease	Rate of decrease	Duration of the serological response	Peak titre averages 1/x
18	18	12	12	12	27
9.22	20.44	28.75	0.11	48.08	177.3
±1.31	±0.85	±3.02	±0.008	±2.79	132.8
3–23	13–29	14–50	0.04–0.14	37–73	40–320
19	19	12	12	12	28
8.63	20.58	41.08	0.10	60.67	1280.0
±1.24	±0.90	±4.76	±0.008	±4.60	130.5
3–21	12–27	18–66	0.06–0.15	38–86	640–10 240
NS	NS	<0.05	NS	<0.05	...
14	14	10	10	10	21
7.36	21.57	27.0	0.10	47.20	25.2
±1.70	±1.08	±3.45	±0.011	±3.21	140.9
2–21	16–29	18–50	0.04–0.14	34–73	10–80
19	19	12	12	12	23
6.90	19.79	41.25	0.12	61.58	114.9
±0.87	±0.90	±4.13	±0.008	±4.47	154.2
3–18	12–27	22–70	0.07–0.16	42–90	20–640
NS	NS	<0.02	NS	<0.05	.

For other explanations see legend to Table I

In one mild, two moderate and two severe infections titre decreases began at the end of the third week of infection, during still existing clinical symptoms, in three cases even before improvement. As a persisting antigenic stimulus, patients harboured *P. aeruginosa* at the site of infection a few weeks further. Parameters of these infections did not differ from those of the others, except that harbouring of *Pseudomonas* was longer in these cases (data not shown). In eight other lethal infections, under similar conditions, decrease in both TAb and IgG titres started also in the third or fourth week of the infection.

Rate of decrease in Ab titre (Tables I–III). The rate of decrease was constant, except for lethal infections, showing a 2.5-fold faster Ab titre decrease.

Duration of the serological response (Tables I–III). As phase of development + persistence and rate of decrease were constant, duration of the serological response depended only on the height of the peak titre. IgG response lasted only one to three days longer than that in TAb.

Correlation. In all groups, except the latent phase, the rate of development, the persistence and the rate of decrease of lethal infections, there was a significantly positive correlation between corresponding TAb and IgG parameters (data not shown).

Reconvalescent pseudomonas carrier state. In two mild, three moderate and four severe infections after improvement, a transient carriage of *P. aeruginosa* at the site of the healed inflammation developed during convalescence,

the average being 12.7 ± 1.1 days. The values of latency, of phase of development and of rate of development were the same as in other infections. However, the late parameters, as persistence and phase of decrease were prolonged significantly (TAb, 30.9 and 57.9; IgG, 28.6 and 56.6, respectively) and decrease rate was considerably less (TAb, 0.06; IgG, 0.07). In consequence, the serological response became significantly longer (TAb, 100.9; IgG, 100.3). Out of these infections in seven cases TAb and in eight cases IgG titres began to diminish though clinical symptoms and *P. aeruginosa* were still present.

Discussion

Comparing the present results with literary data, it should be kept in view that our knowledge on anti-LPS immune response is based mainly on not standardized animal experiments, and in natural infections the antigenic stimulus is continuous, first increasing, then decreasing in contrast with the repeated Ag doses in vaccination trials.

The two to four day intervals of bacteriological and blood sampling has made a precise determination of the different periods impossible; a pseudo-negative bacteriological sample could have resulted in 0 time for the latent phase, and the latent phase could not be determined at all when a rise in natural Ab started before the appearance of *P. aeruginosa*, as well as values of different parameters had to be omitted after septic shock. However, despite these difficulties, differences between averages of the groups are considered reliable, being not far from the exact values.

Many observations refer to that in both man and animal, anti-LPS Ab appear in 2–8 days after the onset of antigenic stimulus [7–13]. Our cases showed the same interval but the latent phase varied according to the later manifesting severity of the infection. The phenomenon may be in connection only with the relative intensity of the antigenic stimulus, which is related to the massiveness of the infection [1]: i.e. the grade of the antigenic stimulus is in accordance with the ongrowing number of the penetrating *P. aeruginosa* (infective dose) and approaches more and more the optimal intensity, resulting in more and more shortening latent phase (mild > moderate > severe infection). However, above the optimum dose, it becomes longer again (fatal infections), until immunoparalysis occurs [14–19], as happened in 6 out of our 39 patients [6].

The phase of development was constant; thus, latent phase plus development phase, i.e. the period from appearance of *P. aeruginosa* till the development of peak titre, changed as latency varied: it was the longest in case of fatal infections. This resulted not only in a depressed immune response but also in a delayed serological answer and in an insufficient Ab level in sepsis [1].

Accordingly, the point of time of the onset and of the maximum of the serological response depend beyond genetic conditions and the momentary reactivity of the immune system also on the massiveness of the infection; furthermore, the production of Ab peak titre, the rate of development and the severity grade of the infection are considerably influenced by the intensity of the exogenous, initial antigenic stimulus.

Besides the development period, the persistence of the peak titre was also constant in each group, and the period of development plus persistence was especially stable. Moreover, the significant, close negative correlation between these two parameters refers to an endogenously controlled and time dependent process which stops Ab production after a certain period. Thus, not an excess amount of Ab [20–24] was responsible for the feed-back. It may be rather supposed that this period is needed for the development of specific suppressor cells (T, B, macrophages), for the starting of lymphokine production and/or to the appearance of antiidiotypic Ab [25–37].

The most striking observation was that this regulation is to some extent independent of the presence of *P. aeruginosa* and the duration of the infectious process: ten days of antigenic stimulus was already sufficient for the highest TAb titres to develop [38]. The maximum IgG response required only a few days more. When the infectious process and the presence of *P. aeruginosa* lasted more than three or four weeks, Ab titres began to diminish, independently of the clinical symptoms. This phenomenon ("misteriously reduced Ab level" [39]), was observed in vaccination trials on animals [39–41], but was not connected with the regulation of Ab response. Only recent investigations in mice revealed a mechanism due to suppressor T cells that switches off the immune reaction automatically, as soon as it reached its maximum value [42]. The same phenomenon occurred in those cases in which *P. aeruginosa* was present longer than the signs of inflammatory process lasted, i.e. a pseudomonas carrier state developed later during convalescence.

The cause of the decrease rate stability is difficult to explain. Anyhow, it has been found that anti-LPS Ab level decreases in mice also at a constant rate [43]. It is doubtful whether the significantly lower rate of titre decrease observed only in pseudomonas carrier state was in connection with harbouring *P. aeruginosa*. Accordingly, variations in duration of the serological response are due to the differences in the phase of decrease, which is determined by the peak titre.

In accordance with animal experiments [9, 44, 45] IgG response was generally only a few days delayed as compared to TAb ($\sim$IgM) response. This may be common in all immunoserological reactions given on thymus-independent antigens [46]. The significant correlations between IgG and TAb parameters confirm that endogenous regulation of these two classes of Ab is closely related [1].

Anti-LPS Ab-s are protective against *P. aeruginosa* infection [7, 43, 45, 47, 48]; in our patients there was a good correlation between the Ab titre and the severity and outcome of the infection [1]. It may be assumed that under still existing infections, when anti-LPS Ab began to decrease, other mechanisms were already protecting in the five surviving patients and the eight other pseudomonas carriers. For the protective effect, first of all, anti-flagellar, anti-pilus or non-agglutinating Ab might have been responsible [41, 43, 49–51].

Finally, it has to be emphasized that the results presented are statistical averages. Great individual differences may occur as it is shown by the ranges of the different groups.

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IgG RESPONSE OF DYSENTERIC PATIENTS TO ANTIGENS CODED BY THE VIRULENCE PLASMID OF ENTEROINVASIVE PATHOGENS

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IgG antibody response to antigens coded by the virulence plasmid of enteroinvasive pathogens was studied in dysenteric patients by ELISA. A plasmidless *Escherichia coli* K-12 strain and its transconjugant harbouring the 140 megadalton plasmid of a virulent *E. coli* 0124 strain were used as antigens. Sera of 12 dysenteric patients taken on the first, third an twelfth week after *Shigella sonnei* infection were examined. For comparison sera of 23 non-dysenteric persons were also applied. Titres of the first week samples did not differ significantly from those of the controls. Patients' sera taken on the third week showed a fivefold rise in titres as compared to the first week ones. Antibody activity remained on a comparable high level till the twelfth week. The data prove that there is a considerable IgG response to plasmid coded antigens common in shigellae and enteroinvasive *E. coli*.

In dysentery caused by shigellae and enteroinvasive *Escherichia coli* (EIEC), epithelial cell penetration is a characteristic step of the pathomechanism [1]. A 140 Md plasmid is necessary for the capability of bacteria to invade the host cell [2, 3]. In case of *S. sonnei* this plasmid measures 120 Md in size and is responsible for the expression of the form I antigenic character, too [4].

Previously we could demonstrate an ELISA-reactive antigenic entity shared by all the virulent shigellae and EIEC strains tested [5]. Later it was proved that this antigenic entity is coded by the virulence plasmid [6]. Data published recently by Hale et al. [7] suggest that the molecular basis of such an antigenic relationship may be the similarity of plasmid-coded outer membrane proteins of these pathogens.

Little is known, however, about the antibody response of dysenteric patients to pasmid coded antigens. Recently Oaks et al. [8] demonstrated IgG, specific to some of the plasmid coded outer membrane proteins in sera of dysenteric Thai children. Here we describe a simple ELISA technique to measure such an antibody activity of dysenteric patients. At the same time evidence is provided that persons infected by *S. sonnei* exhibit a significant antibody response to the above antigens.

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Materials and methods

Sera. In October, 1985 a dysentery outbreak caused by *S. sonnei* took place in an old people's asylum in Keresztespuszta near Pécs. Altogether twelve patients (mean age 76.8 ± 12.7 years) showing clinical symptoms of dysentery and having at least one positive faecal culture for *S. sonnei* were involved in the study. The first serum samples were taken 1–3 days after the beginning of symptoms, the second one on the third week of the illness. In case of 11 patients a third sample, taken on the 12th week, was also tested.

Sera of 23 inpatients (mean age 73.5 ± 8.1) of the Department of Gerontology, Baranya County Hospital, Pécs, who had had no recent history of dysentery, were used as controls. Sera were kept at −20 °C in aliquots until used.

Bacteria. E. coli K-12 J53 was kindly provided by N. Datta (London). Its plasmid-harbouring counterpart J53(pSP1) contains the 140 Md virulence plasmid of an *E. coli* 0124 strain. J53(pSP1) is capable to penetrate epithelial cells and expresses plasmid coded antigens of enteroinvasive strains. The detailed description of plasmid transfer and the properties of clones are given elsewhere [6].

Absorption of sera. Aliquots of sera diluted 1 : 5 with phosphat buffered saline (PBS, pH 7.2) were absorbed with equal amounts (v/w) of living bacterial cells by incubating for 2 h at 37 °C then overnight at 4 °C. Then the whole procedure was repeated with boiled cells, too.

ELISA. Living bacterial cells grown on nutrient agar and suspended in 100 μl of bicarbonate buffer (pH 9.6) were used to sensitize the wells of Dynatech Microelisa plates (M 129 A) overnight at 4 °C. The optimal amount of the antigen giving maximal optical density (OD) was determined in a series of preliminary experiments using control and reconvalescent sera. According to these, 10^8 cells per well set under photometrical control at 690 nm were used throughout this study.

Sensitized plates were washed three times with PBS containing 0.05% Tween-20 (PBS-T). Washing was repeated between each subsequent step of ELISA.

Sera diluted serially in PBS-T containing 0.5% bovine serum albumin (fraction V, Serva, PBS-T-BSA) were applied in 100 μl volumes in duplicates. After 1 h incubation at 30 °C, the conjugate (100 μl of anti-human IgG-HRPO, Dako, diluted 1 : 1000 in PBS-T-BSA) was added and the incubation repeated for an additional hour. The reaction was developed with 150 μl of substrate (10 mg o-phenylenediamine-dihydrochloride, Fluka, in 30 ml citric acid buffer, pH 6.0) and stopped by adding 50 μl 4 N H_2SO_4 after incubating for 15 min.

OD was measured at 492 nm on a Titertek Uniskan (Flow) photometer. End-point titres were estimated graphically on log paper and defined as the reciprocal dilution of sera where the dose-response curve crossed the OD = 0.1 value.

In a series of experiments corrected OD values were used to estimate the end-point titres. They were calculated at a given serum dilution as follows: OD corrected = OD against J53/pSP1 − OD against J53.

Results

Antibody response to plasmidless and plasmid harbouring E. coli K-12 strains. The 120 Md plasmid of *S. sonnei* strains codes not only for virulence-related protein antigens but also for form I LPS antigen [4]. In order to avoid measuring of anti-LPS activity in *S. sonnei* infected patient sera, a K-12 strain harbouring the 140 Md plasmid of an *E. coli* 0124 strain was used as test antigen in ELISA.

First, sera of four dysenteric patients taken on the 1st, 3rd and 12th week after the infection were tested against the above clone and against its plasmidless counterpart. The results are summarized in Table I. Though antibody response against the plasmidless J53 strain could also be detected, the titres against J53(pSP1) were higher and increased considerably with the time.

Table I

End-point titres of four patients' sera to J53 and J53(pSP1)

Patients	Sera*	Antigens	
		J53	J53(pSP1)
I	1	2160	5 750
	3	1300	8 600
	12	2500	11 200
II	1	1320	22 400
	3	1000	51 200
	12	1350	51 200
III	1	400	8 500
	3	550	51 200
	12	700	51 200
IV	1	1350	9 000
	3	1320	41 200
	12	1120	41 000

* Sera taken on the 1st, 3rd and 12th week after the infection

Absorption of sera with isogenic plasmidless and plasmid harbouring clones. To examine whether high titres obtained to J53(pSP1) are due to plasmid-coded antigens rather than to the antigens of the original recipient, aliquots of one patient's sera were absorbed with strains J53 and J53(pSP1). These absorbed sera were tested with the transconjugant and the parent strains. As can be seen in Table II, absorption with J53(pSP1) practically abolished or strongly reduced the antibody activity against both clones. After absorption with J53 no antibody activity could be detected against the same strain. Though two of the three sera showed a decrease in titres against J53(pSP1), no considerable activity could be removed by absorption with J53.

Table II

Reactivity of absorbed serum samples with E. coli J53 and J53(pSP1)

Serum samples*	Absorbed with	Tested against	
		J53	J53(pSP1)
1	none	1350	9 000
	J53	<25	2 650
	J53(pSP1)	<25	<25
3	none	1320	41 200
	J53	<25	22 100
	J53(pSP1)	<25	240
12	none	1120	41 000
	J53	<25	41 000
	J53(pSP1)	<25	25

* Sera of patient "IV" (Table I) taken on the 1st, 3rd and 12th week after the infection

Investigation of sera of dysenteric and non-dysenteric patients. Sera of dysenteric patients taken on the 1st, 3rd and 12th week after the infection were examined and compared to samples of 23 non-dysenteric persons. To avoid the effect of antibody activity directed against non-plasmid coded antigens of J53(pSP1), corrected OD values were used in subsequent experiments to estimate end-point titres (see Materials and methods). Titres and mean of titres are shown in Fig. 1. Though the mean of titres of the first week sera was higher than that of the control group (4794 $\pm$ 4387 versus 3550 $\pm$ 4867) this difference was not significant as tested by unpaired t test ($p > 0.1$).

Sera taken at the third week showed significantly higher titres as compared to those of the first week ones (23 976 $\pm$ 21 977, $p < 0.01$, paired t test). The rise of titres varied from 1.61 to 9.72, the mean of tise was 5.01 $\pm$ 2.73. Only two of the 12 patients' samples showed less than a twofold increase in titres.

In case of 11 patients the 12th week samples were also tested. Though a moderate decrease in titres could be found in 7 of the 11 cases (mean of change between 3rd and 12th week samples: 0.96 $\pm$ 0.31) the difference between these two series of samples was not significant (mean of 12th week titres was 21 275 $\pm$ 20 906, $p > 0.1$, paired t test).

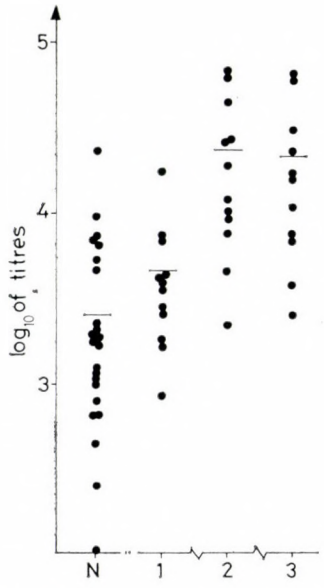

Fig. 1. IgG titres of dysenteric and non-dysenteric patients' sera against plasmid-coded antigens of enteroinvasive pathogens. N = non-dysenteric persons (n = 23); 1, 2, 3 = first, second and third samples from dysenteric patients taken on the 1st (n = 12), 3rd (n = 12) and 12th week (n = 11) after the infection. The horizontal lines represent the mean of titres

Discussion

Until now little has been known about the components of bacterial cell involved in the pathomechanism of dysentery. Recently, several plasmid-coded antigens have been described as common factors among enteroinvasive pathogens [5, 7]. However, they were detected in vitro only [5, 7] and their exact role remained to be discovered.

Our results show that in dysentery the plasmid coded antigens are expressed in vivo, and they do even stimulate the immune system of the host. Though the data presented do not allow to draw a final conclusion as to the dynamics of the antibody response, it is clear that a significant increase of titres occurred in most of the patients. Since first week titres showed no remarkable difference as compared to the controls, and no samples were available between the first and third weeks, we can state only that a significant increase takes place in titres by the time of reconvalescence. These findings are in good agreement with data of Oaks et al. [8]. In addition our results demonstrate that the level of antibodies keeps at a comparably high level at least up to three months.

It shold also be noted, however, that our patients represented a high age group with a reactivity probably not equal to younger individuals.

Further studies are needed to clarify whether the antibodies detected have any protectivity. These investigations may prove helpful in constructing an effective vaccine against dysentery.

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PROTECTIVE VALUE OF THE PLASMID-CODED OUTER MEMBRANE PROTEIN OF ENTEROINVASIVE *ESCHERICHIA COLI*

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O-antigenically not related enteroinvasive *Escherichia coli* strains and rabbit sera prepared with them were used to study the role of plasmid-coded outer membrane proteins in protective immunity. Active immunization experiments were performed using a mouse model based on a long-lasting symptomless carriership after elimination of the bowel flora by streptomycin. Preliminary histological studies showed adhesion, penetration, intraepithelial multiplication, and epithelial desquamation after infection. In active immunization experiments only massive oral doses evoked protective immunity. Seroconversion against the plasmid-coded antigens was not observed in mice. Passive immunization was carried out in chick embryos with unabsorbed sera. A high level of protectivity was reached by serogroup-specific sera and a very low but significant protection was yielded by antibodies against the plasmid-coded protein antigens.

The 120–140 Mdal plasmid carried by virulent *Shigella* and enteroinvasive *Escherichia coli* (EIEC) strains [1–3] encodes seven or more outer membrane proteins [4]. Using ELISA, Pál et al. [5] elaborated a highly specific method for showing virulent strains of these organisms. The antigen(s) involved in the test (designated: VMA = Virulence Marker Antigens) were shown by Hale et al. [6] to represent a few polypeptides of the plasmid-coded outer membrane proteins. Since these proteins are of a paramount importance in pathomechanism, and immunogenicity, and are identical in all *Shigella* and EIEC strains, it might be assumed that they are of protective value.

Lacking the purified form of these antigens, we had to carry out active and passive immunization experiments in heterologous systems, i.e. with strains having no lipopolysaccharide (LPS)-directed 0-antigenic relationships.

Materials and methods

Strains are listed in Table I. The transfer of the 140 Mdal plasmid into *E. coli* K-12 strain J53 was carried out as described [7] by Tnl transposon-mediated conduction according to the method of Watanabe and Nakamura [8]. Before immunization or challenge, virulent strains were checked for congo red absorption (CR^+) character and by VMA–ELISA (Table I).

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Sera. Immune sera were produced in rabbits by strains *E. coli* 0143 Nos 2, 2/33, 31, and by *E. coli* K-12 strain J53 and its plasmid carrying derivative J53(pSP1). The sera were preserved at −20 °C.

VMA–ELISA was carried out as described previously [5].

Determination of congo red character and selection were made on congo red agar plates [9].

The Serény test was performed using the classical method [10].

The "Mouse Shigellosis Model" was developed earlier and published in 1966 by us [11–13]. CFLP outbred mice (LATI, Gödöllő, Hungary) were used. Their bowel flora was eliminated by 50 mg doses of streptomycin given orally on two consecutive days. The animals were checked for the absence of *E. coli*, each placed in a sterile jar, haused in a sterile box and fed autoclaved food. Our early experience showed that selected virulent clones of shigellae were able to cause a long-lasting asymptomatic carriership ($ID_{50} < 10$ germs), while selected avirulent clones were unable to cause infection ($ID_{50} > 10^8$ germs). This model was suitable for active or passive immunization experiments.

Chick embryo test. Ten days old chick embryos (a broiler strain of Baksa, Hungary) were infected with graded doses by injecting 0.1 ml culture into the allantoic cavity. In virulence tests the LD_{50} values, in passive protection tests the ED_{50} values were determined after 48 h of incubation at 37 °C. In our earlier experiments by this route of inoculation the LD_{50} value was in the order of 10^1 germs for virulent *Shigella* strains and 10^3 for avirulent ones [14].

For active immunization group of mice were injected in a two-week period 5 times subcutaneously, or treated orally at 3-day intervals with living bacteria. A week after the last dose the mice were separated, treated with streptomycin and handled as described under "Mouse Shigellosis Model".

In some cases at the end of immunization mice from each group were bled from the retrobulbar plexus, the sera were pooled and preserved at −20°.

Passive immunization using the mouse model was performed by graded doses of sera given twice daily two days before, on the day and two days after challenge. Passive immunization of chick embryos was made by serum dilutions mixed with a minimal dose of challenge, incubated in water bath at 37 °C for 30 min and administered into the allantoic cavity at aliquots of 0.1 ml.

Statistical analysis. LD_{50}, ED_{50} and ID_{50} values (50% end point of Infective Dose which is sufficient to cause the symptomless carriership) were calculated by the method of Kärber [15]. Statistical significance was estimated by the χ^2 method.

Results

1. *Determination of virulence of VMA^+ and VMA^- EIEC strains using the Mouse Shigellosis Model and in chick embryo allantoic test.* On the basis of the identical pathomechanism of shigella and EIEC infections, it was supposed that the behaviour of the latter will be analogous to that of shigellae in our early animal models. The results of these studies proved this preliminary hypothesis as it is summarized in Table II.

2. *Preliminary observations on the histological background of the "Mouse Shigellosis Model".* Preliminary investigations were made by J. Fischer (Institute of Pathology, University Medical School, Pécs) into the symptomless carriership observed in this model. The studies were made on mice at the 13th day of carrying strain J53(pSP1). Two labelling methods were applied: aldehyde–bisulphite–toluidine blue reaction specific for bacterial polysaccharides and immuno-peroxidase method using absorbed anti-VMA serum. The bacteria seemed to aggregate, forming patches on the mucus at the opening of the mucus-producing cells. Many slides showed evidence of intraepithelial multiplication of bacteria, moreover, a well-pronounced epithelial destruction or a

Table I

Strains used

Groups and serogroups of *E. coli*		Designation	Plasmid, 140 Mdal	VMA–ELISA[1]	Virulence in Serény test
EIEC	O143	No. 2	+	+	+++
	O143	No. 2/33[2]	+	−	−
	O143	No. 31	−	−	−
	O124	No. 34	+	+	+++
K-12		J53	−	−	−
		J53(pSP1)[3]	+	+	−
		J53(pSP1)StrR[4]	+	+	−

[1] VMA–ELISA = specific ELISA [5] for virulent strains based on polypeptides encoded by 140 Mdal plasmid

[2] No. 2/33 = isogenic avirulent derivative of strain No. 2 carrying a defective 140 Mdal plasmid

[3] pSP1 = designation of 140 Mdal plasmid originating from strain O124 No. 34

[4] StrR = streptomycin resistant (1000 μg/ml) derivative

Table II

Virulence of the isogenic virulent and avirulent strains of EIEC O143 and the plasmid carrier K-12 and its parent strain in Serény test, "Mouse Shigellosis Model" and intraallantoic chick embryo tests

Strain and designation		Virulence in tests		
		Serény	chick embryo LD_{50} (counts)	mouse model ID_{50}* (counts)
EIEC O143	No. 2	+++	$<1.2\times10^1$	$<10^1$
EIEC O143	No. 2/33	−	7.0×10^3	$>10^6$
E. coli K-12	J53	−	2.0×10^4	$>10^6$
E. coli K-12	J53(pSP1)	−	1.6×10^1	$<10^1$

* ID_{50} = 50% end point of infective doses leading to long lasting symptomless carriership

very rapid, intensive epithelial renewing. The above-described phenomena were observed only in the colonic mucosa, the small bowel was free of bacteria.

These observations need further investigations, but it seems already clear that in the Mouse Shigellosis Model there is a penetration into the colonic epithelial cells followed by intracellular multiplication. There are further questions as to the exact nature of adhesion and the epithelial desquamation, in contrast to the symptomless character of the process.

3. *Active immunization experiments using the mouse model.* In the lack of purified outer membrane protein antigens, or rather plasmid-coded ones, the experiments were carried out with whole, living bacteria considering that the effect of VMA on the base of heterogenous vaccination, can be estimated by

immunizing with strains which have no LPS relationship to the challenging strain.

Very high subcutaneous doses elicited no significant VMA-directed protectivity. Therefore, series of oral vaccination were performed. Using massive doses for immunization and a minimal dose for challenge, we obtained protection only with the VMA$^+$ EIEC strain, in which an intact plasmid of 140 Mdal was present and, consequently, in which the plasmid-coded outer membrane proteins responsible for virulence were expressed. An example is presented in Table III.

In the case of the experiment presented, the challenging agent was *E. coli* K-12 strain J53(pSP1) in a minimal dose of about 10 ID_{50}. Significant protection was afforded only by the virulent *E. coli* O143 strain No. 2, but not by its derivatives of VMA$^-$ character, carrying defective plasmid (No. 2/33), or missing it (No. 31). The difference between No. 2 and No. 2/33 strains was significant in protectivity: $\chi^2 = 13.2$, $P < 0.001$, or between No. 2 and No. 31: $\chi^2 = 14.6$, $P < 0.001$. There was no significant difference between the VMA$^-$ avirulent strains: $\chi^2 = 0.018$, showing a statistical homology of about 90% (Table III).

Passive immunization experiments on the mouse model using repeatedly even massive serum doses did not give significant protection.

4. *Passive immunization of chick embryos.* Injecting non-absorbed sera into the allantoic cavity allowed the estimation of protective effect of antibodies directed against plasmid-coded outer membrane proteins (VMA) if no LPS relationship existed between the strains used for serum production and for challenging. If such relationship did exist, the results also reflected the potency of anti-LPS antibodies.

Table IV summaries the experiments. In the case of heterologous challenges using strain J53(pSP1) and *E. coli* O124 strain No. 34, it is clear that avirulent, VMA$^-$ strains produced sera not exhibiting any protective effect.

Table III

Heterologous protection in mice immunized massively by virulent and avirulent strains of EIEC against K-12 J53(pSP1) using the "Mouse Shigellosis Model"

Strains and designation	Plasmid, 140 Mdal	$ED_{50} \pm SD^*$ values	RP**	Probability (5%) (χ^2 test)	
EIEC O143 No. 2	present	$10^{9.06} \pm^{0.5}$	1	<0.001	significant
EIEC O143 No. 2/33	defective	$10^{10.31} \pm^{0.32}$	0.013	~0.90	
EIEC O143 No. 31	missing	$10^{10.26} \pm^{0.28}$	0.004	not significant	

* SD = standard deviation

** RP = relative potency

Oral immunization, in five doses at 3-day intervals, challenged by about 10 ID_{50} one week after the last immunization dose

Table IV

Passive immunization of chick embryos with homologous and heterologous sera (intraallantoic route)

Sera	Strains used for challenge in minimal infective doses					
	J53(pSP1)*		O143 No. 2**		O124 No. 34***	
	ED_{50}	RP	ED_{50}	RP	ED_{50}	RP
K-12 J53	>0.05	1	>0.05	1	>0.05	1
K-12 J53(pSP1)	0.028	1.8	0.028	1.8	0.028	1.8
O143 No. 2	0.016	3.1	<0.0005	>100	0.028	1.8
O143 No. 2/33	>0.05	1	0.0028	18	>0.05	1
O143 No. 31	>0.05	1	0.0009	55	>0.05	1

* 3.9×10^1 germs
** 2.2×10^1 germs
*** 4.9×10^1 germs
Challenge doses between 1 and 10 LD_{50}

Sera produced with VMA^+ strains gave a low but significant protection. For J53(pSP1) challenge the statistical difference between sera with and without anti-VMA antibodies was $\chi^2 = 11.886$, $P < 0.001$. The same was true for challenge with O124 No. 34: $\chi^2 = 4.711$, $0.05 > P > 0.02$. Challenge with virulent strain O143 No. 2 allows a comparison between LPS-directed homologous protection and protection reached by VMA antibodies only. The sera for the VMA^+ K-12 strain and for the VMA^- O143 strain differ in relative potency about 30 times ($\chi^2 = 5.865$, $P < 0.02$). Sera for heterologous K-12 and homologous O143 show a marked difference: $\chi^2 = 22.525$, $P < 0.001$ (Table IV).

5. *Antibodies in vaccinated mice detected by VMA-ELISA.* Pooled sera from mice vaccinated orally with massive, gradued doses, and normal mouse sera were tested by VMA–ELISA. No seroconversion was observed.

Discussion

The findings [4, 5] that outer membrane proteins encoded by 120–140 Mdal plasmids are responsible for the basic steps of pathomechanism of shigella–EIEC infections, and the fact that the antigenicity of these proteins seems to be identical according to the highly specific ELISA [5], led us to perform experiments on their protective value.

It has never been easy to choose a convenient animal model. One of the candidates might be the guinea pig eye model, but only when the immunization process does not evoke keratoconjunctivitis because of its long lasting and severe damage of the corneal epithelium. This model was promising also in our case when in the lack of purified antigens we were forced to use living

bacteria for immunization. Testing of the plasmid carrier strain K-12 J53(pSP1) was considered worthwhile, because it failed to give the Serény test, however, the very rapid clearance (data not presented) of this strain made the model unsuitable for us. Therefore, we chose our "Mouse Shigellosis Model". Twenty years ago it was suitable for both active and passive immunization studies on *Shigella* strains. The fact that this test is very sensitive, can be perhaps attributed to the fact that it does not estimate the usual survivor/lethal rate but the success of infection expressed in carriership. That time we were not concerned with the histological, cellular background of *Shigella* infection. Our preliminary investigations summarized in this paper showed the probability of mucus (?) adhesiveness, demonstrating clearly penetration and intraepithelial multiplication, followed by epithelial desquamation. It is especially interesting that the causative agent in the above observations is a K-12 derivative carrying the 140 Mdal plasmid of EIEC origin.

Active immunization shows that extremely high doses and oral administration evoked only a low protective immunity in the mouse model using living bacteria without LPS relationship. This low level immunity may be associated with the lack of seroconversion against plasmid-coded outer membrane proteins (VMA). The degree of coproantibody response remains to be investigated.

Passive immunization of chick embryos served not only to measure anti-VMA antibodies but also to compare their protectivity with that of homologous LPS-directed antibodies. The data clearly show a significant but low level protection by anti-VMA antibodies compared to the high level of protection based on anti-O antibodies.

The slight, perhaps insignificant immunity to plasmid-coded outer membrane proteins was shown by active immunization with living whole cell vaccines and by passive immunization with non-absorbed sera. These results do not forecast that purified, concentrated outer membrane proteins, or some fractions of it are equally unsuitable for producing a higher level of protective immunity. Adamus et al. [16] using an outer membrane fraction of shigellae showed protective immunity in guinea pig eye model and a serum produced with this protein fraction led in rabbits to passive immunity — both homologous and heterologous. An adequate answer of the question needs further experiments with purified, concentrated protein antigens.

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PAPER-DISC METHOD FOR CAMPYLOBACTER HIPPURATE-HYDROLYSIS TEST A NOTE

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Paper discs were impregnated with sodium hippurate dissolved in 1/15 phosphate buffer pH 7.2. The descs, each containing 2 mg substrate, were stored in dry state at room temperature. After adding a disc to bacteria suspended in distilled water, and incubated at 37 °C for 2 h, one drop of Skirrow and Benjamin's ninhydrin reagent was added and the reaction was read after re-incubation at 37 °C for 10 min. 169 campylobacter strains were tested. The results for a total of 169 strains were identical with those obtained with the modified rapid method of Skirrow and Benjamin. Of 92 campylobacter strains isolated from human faecal samples 59.8%, of 36 strains isolated from slaughtered poultry 41.7% proved to be *Campylobacter jejuni*.

Thermotolerant campylobacters are important enteric pathogens all over the world. As human enteric pathogen *Campylobacter jejuni* occurs the most frequently, whereas *Campylobacter coli* was isolated mainly from the intestines of domestic and breeding animals [1]. Interspecies differentiation between strains causing diarrhoea is of epidermiological importance. Due to the ever increasing demand, it is an actual task to develop and introduce a simple, rapid and reliable method for this purpose. For differentiation of the two most important species *(C. jejuni, C. coli)* Skirrow and Benjamin [1] applied the hippurate hydrolysis test, which is a simple method giving results consistent with the results of the laborious procedures of genetic differentiation [2, 3]. The procedure reported in the following is still more simple.

Materials and methods

Bacterial strains. As reference strains, *C. jejuni* (hippurate-positive) HNCMB 200001 and *C. coli* (hippurate-negative) HNCMB 200004 were used. Part of further strains (169 altogether) were received from the collection of H. Lior [4] and of S. Lauwers [5], part of them were isolated in laboratories of Hungarian Public Health Stations in the years 1980–1983. The strains were stored freeze-dried. Distribution by origin of the strains is shown in Tables I and II.

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Cultivation. Rehydrated bacteria were streaked on Skirrow's modified nonselective (Ca_0) campylobacter agar [6] and incubated at 42 °C for 48 h in a nitrogen atmosphere containing about 7% CO_2 and 7% O_2. For the tests 48 h cultures were used.

Substrate. For the modified Skirrow–Benjamin test 1 g of sodium-hippurate (Merck) and 1.19 g of $Na_2HPO_4 \cdot 2\ H_2O$ were dissolved in 100 ml distilled water. To adjust pH to 7.2, crystalline KH_2PO_4 was added under continuous stirring. The substrate solution was stored in frozen state either in bulk or distributed in tubes.

For the disc method, 0.119 g $Na_2HPO_4 \cdot 2\ H_2O$ and 2 g sodium-hippurate (Merck) were dissolved in 10 ml distilled water. Crystalline Na_2HPO_4 was added under continuous stirring to adjust the pH to 7.2. Ten-μl volumes of this solution were pipetted onto Whatman-200 filter paper discs 7 mm in diameter. The discs were dried at room temperature and stored in glass vessels until used.

Ninhydrin reagent. In a mixture of 50 ml acetone and 50 ml n-butanol 3.5 g ninhydrin was dissolved.

The tests. Skirrow and Benjamin's modified (SBM) method. A small loopful amount of 48 h bacterial culture was suspended in 0.2 ml hippurate solution to give a density corresponding approximately to 10^9 bacteria per ml, and the suspension was incubated at 37 °C for 2 h. One drop of ninhydrin reagent was added. The tubes were shaken and re-incubated at 37 °C for 10 min. Colourless solution indicated a negative reaction. Colouration from bluish lilac (+) to deep violet (++++) indicated positive reaction.

Disc method. The culture was suspended in 0.2 ml distilled water to give a density similar to that described above and an impregnated paper disc was added to the suspension. The final concentration of hippurate was 1% and the pH was 7.2. After a thorough shaking the reaction mixture was incubated at 37 °C for 2 h. Ninhydrin reagent was added and, after re-incubation for 10 min, the result was read and judged as above. For comparison, the two reference strains were tested simultaneously. For comparison, the SBM method and the disc method were performed simultaneously, in three parallels each.

Results

A hundred and sixty-nine strains were collected from several counties of Hungary and from institutes abroad. The results obtained for the serotype strains of Lior's scheme (19 *C. jejuni* and 5 *C. coli*) agreed in both tests and with literary data.

The results of the tests carried out with the Belgian and Hungarian isolates are shown in Table III. The agreement is complete, except for those few strains which grew poorly on Ca_0 agar and the colonies of which diffused into the culture medium. Thus, in these few cases, the inocula were too small to cause a distinct coloration in the SBM test (see isolates from Pest and Csongrád counties). The test was repeated in three parallels with each of these strains.

In summary, out of 92 isolates from human faeces 55 (58.8%), out of 36 isolates of animal origin 15 (41.7%) were positive, i.e. were identified as *C. jejuni*.

Table I

Serotype and other reference strains of Campylobacter

Serotype strains of *C. jejuni*	Designation of strain	Supplied by	Source
LIO 1	134	H. Lior	man
LIO 2	195	National Enteric Reference	man
LIO 4	1/NTCC11168	Center, Bureau of	man
LIO 5	170	Bacteriology, Laboratory	man
LIO 6	6	Center for Disease	man
LIO 7	35	Control, Ottawa, Canada	man
LIO 9	88		man
LIO 10	142		man
LIO 11	244		man
LIO 13	343/TO15		man
LIO 16	728		man
LIO 17	556		hen
LIO 18	563		hen
LIO 19	544		hen
LIO 22	918/TO40		man
LIO 26	913/TO35		nonhuman
LIO 27	919/TO41		nonhuman
LIO 28	1180		man
Serotype strains of *C. coli*			
LIO 8	52	H. Lior	man
LIO 14	348/TO20		man
LIO 21	699		hen
LIO 25	1228		duck
LIO 29	1982/BR11		
Serotype strains LA 1, 3, 5, 6, 7, 11, 30, 45		S. Lauwers Akad. Ziekenhuis Vrije Univ., Brussels, Belgium	man
Reference strains			
C. jejuni	HNCMB* 200001 IP (E9)	Akad. Ziekenhuis Vrije Univ., Brussels, Belgium	man
C. coli	HNCMB* 200004	Univ. of Lund, Malmö, Sweden	man

* Hungarian National Collection of Medical Bacteria, Budapest

Table II

Hungarian isolates of Campylobacter

Designation of strains		Supplied by	Source
CS	1, 2, 4, 5, 11, 13, 18, 21, 35, 36, 41, 45, 52, 53, 56, 65, 66, 67, 68, 70–74	Medical Bacteriology Lab., Public Health Station, Szeged, Hungary	man
CSÉ	109, 110, 112–115, 118, 120–135, 139, 141, 142	Food Bacteriology Lab., Public Health Station, Szeged, Hungary	slaughtered poultry
N	7, 11–18, 37, 38, 40–47, 52–55, 57–61, 63, 64, 66, 67, 69–76, 78–80, 84, 85, 87–91, 93–98, 99/a, 99/b, 99/b, 100/a, 100/b, 101/a, 101/b, 102–105	Medical Bacteriology Lab., Public Health Station, Salgótarján, Hungary	man
Pm	965–968	Food Bacteriology Lab., Pest County Public Health Station, Budapest, Hungary	slaughtered poultry
	969–974		poultry-processing environment
V	2	Medical Bacteriology Lab., Public Health Station, Veszprém, Hungary	man

Table III

Hippurate hydrolysis by Camplyobacter isolates received from different laboratories

Laboratory	No. of isolates tested	SBM method		Disc method
		1st testing positive	2nd and 3rd testings positive	positive
S. Lauwers, Brussels	8	4	n.t.	4
Public Health Stations				
Pest county, Budapest*	10	3	5	5
Csongrád county, Szeged	25	10	15	15
Csongrád county, Szeged*	26	10	n.t.	10
Nógrád county, Salgótarján	66	33	39	39
Veszprém county, Veszprém	1	1	n.t.	1
Totals	136	62	74**	74

n.t. = not tested repeatedly

* Isolates from food

** This figure includes the 15 isolates that were obviously positive in the first testing and were not retested

Discussion

The present results have shown that the disc method developed for the hippurate test gives the same results as the SBM test. Although the principle of the two procedures is the same, the disc method has advantages.

(i) The paper discs impregnated with sodium-hippurate (similarly to the discs used in antibiotic-sensitivity tests) can be produced commercially, thus, a method providing comparable results is available for the laboratories. Shortage in facilities necessary for the original SBM method was the main reason why the disc method had to be developed.

(ii) Unlike the test solution for the SBM method, the discs do not need refrigerator or its freezing compartment to be stored in; they can be stored in dry state at room temperature without any risk of decomposition, and require little room.

(iii) Laboratories having large numbers of isolates may supply themselves with discs economically.

(iv) In contrast to the SBM reagent, the disc method provides a buffered reaction mixture. A study presented at the Second International Workshop on Campylobacter Infections demonstrated the importance of the pH in the hippurate-hydrolysis test. The optimum pH is around 4.9 for streptococci and around 7.2 when campylobacters are examined. J. Heinzer (Bern, Switzerland) demonstrated the role of pH in enzymatic hydrolysis spectacularly [7].

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THE EFFECT OF VARYING ILLUMINATION ON IMPRINTING OF *TETRAHYMENA* BY INSULIN

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(Received September 9, 1986)

Tetrahymena pyriformis cultures were imprinted with insulin. Hormone binding was reduced in the dark, but alternation of dark and light periods were in this respect more effective than the dark itself. The deviations observed may be attributed, besides the reduced insulin binding by the imprinted cells in the dark, to an enhanced binding by the non-imprinted control cells kept in the dark. It is suggested that the dark-induced structural transformation of the membrane, manifesting among others in a changed hormone binding, may be caused by alterations in haem synthesis due to varying illumination.

Environmental effects on cells are of decisive importance for the development of cell function in both unicellular and multicellular organisms. The extraneous parameters, including ambient temperature, pH and ion saturation, may take up values varying between wide limits; their variation from the optimum may cause in cells changes manifesting themselves biochemically and functionally.

The above changes apply to unicellular organisms with a special accentuation, for these organisms must develop an ability to adapt, often very rapidly, to the outworld varying frequently. In the present experiments we used as model cell the unicellular organism *Tetrahymena pyriformis*, which had been the object of our previous investigations into hormonal effects [1].

Hormonal imprinting has been demonstrated in both unicellular and multicellular organisms. It indicates an enhanced responsiveness — memory — which is acquired by cells in an early phase of their development, when they meet the "imprinting" substance, usually a hormone.

The imprinted cells and their progeny will remember the cell-hormone meeting through generations and will respond with an enhanced reaction to repeated hormonal effects [2, 3].

To be imprintable, the cell needs an intact membrane [4], and changes at other levels, viz. the cytoplasm and the nucleus, are also essential for imprint-

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ing. The evolution of imprinting may be disturbed by environmental changes perturbing the membrane structure [5, 6].

Changing illumination is one of the extrinsic factors compelling the cell to respond and adapt to its environment. In *Tetrahymena*, haem is synthesized by a photosensitive reaction. An intermediary metabolite of haem synthesis is protoporphyrin IX, a substance that, integrating in the cell membrane, may change membrane structure and fluidity [7].

In the present work we examined the effects of changing illumination, effects supposedly mediated by changes in haem synthesis, on the hormonal imprinting attainable with insulin, a hormone of polypeptide nature.

Materials and methods

The GL strain of *Tetrahymena pyriformis* was cultured at 28 °C in a medium containing 1% Bacto Tryptone (Difco) and 0.1% yeast extract; 24 h cultures were used.

Three main stages of experiment were distinguished: (i) adaptation period, i.e. the period preceding hormonal treatment and aimed at adapting to the prescribed environment (24 h); (ii) period of imprinting, i.e. treatment with insulin (Semilente Novo, Copenhagen, Denmark) for 60 min; (iii) transfer into fresh medium (24 h).

The effect of changes in illumination on imprinting was examined by changing the illumination of cultures in consecutive periods of time. A group with unchanged illumination (without dark period) served as control and another group of cultures growing in the dark throughout displayed the effect of uninterrupted lack of illumination.

The scheme of experiment is set out in Table I.

The cells left to rest for 24 h were fixed in 4% formalin diluted in PBS (PBS = 0.05 M phosphate buffer of pH 7.2 containing 0.9% NaCl) and washed with PBS. The cells were then incubated for 1 h in the presence of insulin labelled with FITC (fluorescein isocyanate BDH, London, England).

The degree of insulin binding was estimated by using a cytofluorimeter built together with Hewlett–Packard 41C minicomputer. The fluorescence of 20 cells was measured in each group, and each experiment was performed five times. Thus, each column in Figs 1 and 2 indicates the mean value for the fluorescence of 100 cells.

Results

In Experiments 1, 2 and 3, cells had different times for adaptation to the dark (Table I). In Experiment 1, the cells kept in the dark constantly during adaptation, insulin treatment and the subsequent 24 h period displayed a reduced hormone binding after the three periods, in contrast to the control groups which were kept in natural illumination throughout. In the latter group the hormone binding was enhanced (Fig. 1), owing to a successful imprinting.

In Experiment 2, in which illumination was withdrawn only during the period of imprinting, the reduction in hormone binding was well-defined, although the cells were kept in the dark for a considerably shorter time than

Table I

Scheme of experiment

Experiment No.	Periods of experiment		
	adaptation	imprinting	culturing (24 h)
1	dark	dark	light
2	light	dark	light
3	dark	light	light

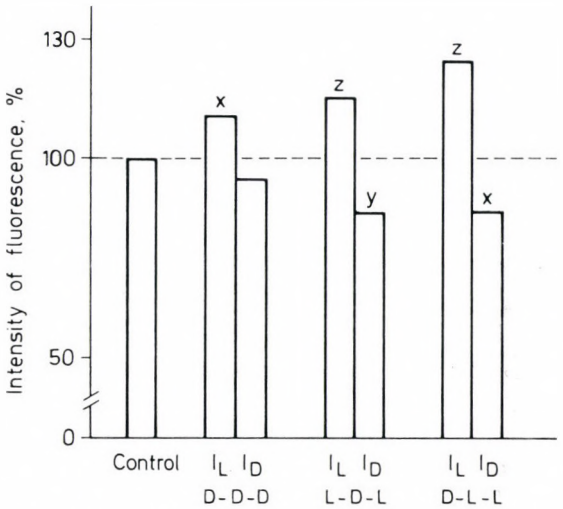

Fig. 1. Binding of FITC-labelled insulin to *Tetrahymena* cells kept in the dark for various periods of time. Binding to the control cells kept illuminated throughout is taken as 100%. C = Control; I = insulin-treated; L = light; D = dark; x, $P < 0.05$; y, $P < 0.01$; z, $P < 0.001$

the cells in Experiment 1. The insulin binding was significantly less than in the control groups, the illumination of which was not interrupted.

In Experiment 3 we wished to obtain new knowledge on the time dependence and the dynamics of the effect under study. We examined whether it is enough to keep the cells in the dark during the period of adaptation to reduce imprinting and the consequent increased hormone binding. The results showed that in this experiment, too, the withdrawal of illumination did exert its imprinting-reducing effect. The difference from the control values was significant.

Discussion

Environmental factors are of decisive importance for living organisms. Even complex biological processes, like the evolution, are, to some extent, directed by environmental factors, which owe their leading role to the fact

that they act at the cellular level by ensuring the dynamic stability of the cell.

The decisive character of environmental factors applies particularly to unicellular organisms, for the survival of these organisms depends on the environmental parameters. Rapid adaptation to environmental changes is a prerequisite for the survival of both the individual and the species.

During hormonal imprinting the cell acquires a special "memory". When it meets the signal molecule again, it will remember the first meeting and usually respond with an altered, usually enhanced, reaction. The enhanced reaction is first of all an increased binding of the given hormone [8], and further parameters may also show alterations [9, 10]. The mechanism of imprinting is only partially known; effects of the given hormone at the membrane level may play a role [4], but modifications in the intracellular space and at the nuclear level may contribute to the phenomenon [6].

The hormonal action, the undisturbed imprinting among them, is a membrane-bound process, therefore, it needs a "physiological" membrane structure, which is indispensable for hormone binding [11]. Every effect that changes the composition and structure of the membrane (effects of temperature, chemicals, ect.) causes a well-demonstrable disturbance in the process of imprinting [4, 5].

Considering that according to literary data *Tetrahymena* is sensitive to illumination [12, 13], we searched in the present work for an answer to the question whether a change in its illumination is capable of causing a structural change of the membrane observable as a change of hormonal imprinting.

The imprinting observed in illuminated *Tetrahymena* supports our earlier experimental results [2, 3]. The insulin binding by the imprinted organism during the second meeting was considerably higher than the binding by the nonimprinted control. On the other hand, imprinting in the dark was followed by a well-defined decrease in insulin-binding capacity, which was independent of the length of the period of imprinting. (The results were approximately the same either 24 h or 1 h was the duration of keeping in the dark.) Therefore, it must be the light-adapted membrane of *Tetrahymena* that is capable of accepting the positive imprinting effect of insulin.

In the experiment in which imprinting was performed in the dark and the cells were kept in the dark for 24 h both before and after the imprinting period, the negative change in insulin binding was less than in the experiments in which cells were kept in the dark only in the 1 h period of imprinting or during the 24 h period of adaptation (Fig. 1). This variation of response suggests that, though, adaptation to the dark is the primary factor causing an alteration in imprinting, *Tetrahymena* adapts to the uninterrupted dark and compensates its effects. Alternating of illumination and dark, on the other hand, seems to induce a considerably more intense effect than keeping in the dark itself.

The deviation between dark-adapted and light-adapted non-imprinted controls proved that a change had happened in the membranes of the cells kept in the dark (Fig. 2). The change in hormone binding was similar in tendency of extent and opposite in direction, compared to the change demonstrated in the case of imprinted cultures. The insulin binding by *Tetrahymena* cells kept in the dark continuously was approximately the same as that by the cells illuminated without interruption. The hormone bound by non-imprinted cells illuminated for only 1 h and kept in the dark before and after imprinting, on the other hand, considerably exceeded the amount of hormone bound by the non-imprinted cells kept illuminated throughout. Furthermore, the hormone binding of the *Tetrahymena* cells kept in the dark for 24 h was still more.

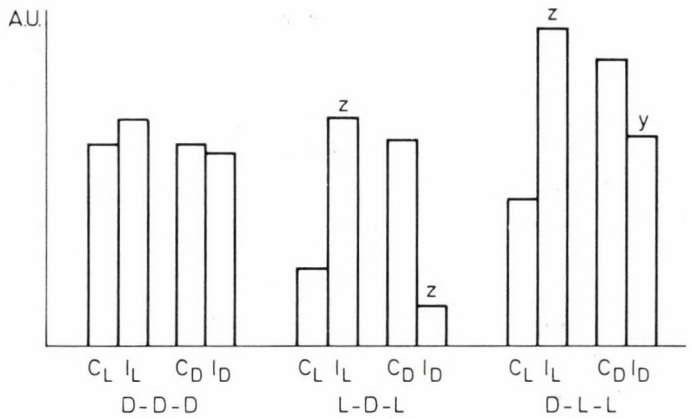

Fig. 2. Comparison of the absolute values of FITC-labelled insulin binding. For abbreviations and explanations, see Fig. 1

Our previous experiments [4, 14] and experiments reported by others [15, 16] have shown that *Tetrahymena* adapts to environmental changes well. This means that a disturbance in its membrane is followed by a steady state, which tends to compensate the changes and, just like the earlier steady state, ensures the life conditions for the unicellular, unless the membrane is impaired again. The changes to and fro, on the other hand, are poorly tolerated by the *Tetrahymena* membrane.

We observed the effect of the dark (in Experiments 1 and 3) and that of imprinting (Experiments 1, 2 and 3) after an interval of 24 h or more. During that time 5 or 6 new generations of *Tetrahymena* came about. Thus, the indi-

viduals that were examined for hormone binding were not the same as those that had undergone the environmental change. This means that, in accordance with our earlier conclusions [17], the changes caused in *Tetrahymena* remained detectable in the progeny through a number of generations. It seems that the periodicity of illumination, i.e. a stimulus regarded as not too strong, was strong enough to induce a change in the responsiveness of the unicellular, a change, which may persist over generations.

The changes discussed above are only selections from changes induced by environmental stimuli. It is not easy to explain the mechanism changing the responsiveness of cells for generations. The synchronizing effect of light and dark [18] must not be disregarded in this respect. Synchronization might favour the selection of a population which is characterized by a special membrane structure. Possible affections at the nuclear level should also be taken into consideration. Nevertheless, a change in the activity of haem synthesis, a photosensitive process, seems to be the most probable influencing factor. Changes in haem synthesis may disturb the membrane structure by integrating into the membrane protoporphyrin IX, an intermediary metabolite of haem synthesis. Protoporphyrin integration is an illumination-dependent process [12], which modifies the membrane structure, viz. impairs its structural stability, on which the undisturbed hormonal imprinting relies.

In conclusion, effects on hormonal imprinting induced by changes in the illumination of *Tetrahymena* cells were examined in the present work. Imprinting by insulin failed to develop in cells kept in the dark and illuminated alternatively while developed but weakly in those kept in the dark constantly. It seems that the dark-induced change in membrane structure is the more pronounced the more striking is the stress acting on the membrane. Furthermore, membrane restauration leads to a steady state, which follows the stimulus rapidly. Besides the reduced insulin binding by imprinted cells, a simultaneous increase in the insulin binding by non-imprinted cells is a characteristic phenomenon following changes in the illumination of *Tetrahymena* cells.

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COMPLEMENTABILITY OF TEMPERATURE-SENSITIVE ADENOVIRUS MUTANTS WITH EXTRACTS OF UROGENITAL TUMOUR CELLS*

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(Received November 4, 1986)

Complementation of two temperature-sensitive *(ts)* mutants of adenovirus type 5 was attempted with tumour extract in HEp-2 cell cultures at permissive and restrictive temperature. Both *ts* mutants were successfully complemented with adenovirus gene products extracted from bladder and kidney carcinoma cells. The percentage incidence of complementation appeared to be higher with extracts obtained from female patients than with those obtained from males. The same gene sequences may occur in penile carcinoma cells and in bladder carcinoma cells showing the early signs of malignization. Extracts of seminoma cells and of cells from prostatic hypertrophies contained adenovirus genes less frequently; infectious virus was never obtained from them. The same applies to cells from the non-tumourous ("control") patients tested. The old age of the patients and some properties of the tumours examined suggest that hormonal changes may contribute to adenovirus gene expression and, indirectly, to the malignant process and its continuity.

The uropoietic and conveying system and the phylogenetically and morphologically closely related genital system are highly exposed to haematogenous and exogenous viral infections [1, 2], which may be accompanied by acute or chronic illness, or may remain clinically inapparent [3, 4]. Furthermore, viruses may integrate in cells of the urogenital system, [5, 6] and reactivate there, due to other cocarcinogenic (chemical) factors [7] or pathophysiological changes as hormonal alterations [6, 8]. The activated virus may cause clinically apparent tumours. Among a number of viruses [1, 4, 8, 9] adenovirus (Ad) has been proposed to have an aetiological role in oncogenesis. The affinity of Ads for the urogenital system [4] on the one hand and their cell-transforming ability under experimental conditions [10] on the other, prompted us to examine the possible role of Ads in tumours of the urogenital system. In the first step, we searched for Ad antibodies in serum samples from patients bearing urogenital tumour. Of the 253 patients suffering from carcinoma of the bladder (53%), while of 263 non-tumourous patients only 4 to 18% had antibody to the early antigen of Ad-12.

* This work was rewarded with the 2nd prize of the competition advertized for young researchers by the Hungarian Society of Microbiology in 1986.

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In the present work, 145 persons were involved. Most of them were suffering from urogenital tumour. We attempted to isolate infectious virus from structural cells of tumours and, if failed, searched for Ad-specific gene products. For the latter purpose we used two temperature-sensitive *(ts)* mutants of Ad-5, which, in themselves, failed to replicate at restrictive temperature, i.e. 39 °C. We assumed that the tumour-cell protein and DNA added to the cultured cells simultaneously with mutant might contain Ad gene products which might complement the mutant virus and thus enable it to replicate.

Materials and methods

Patients and tissue samples. Tissue samples excised from malignant or benign tumours of the urogenital system or from removed organs of non-tumour-bearing patients were examined. The latter group of patients was taken as control, for tissue samples from healthy donors were not available. The operated "control" patients suffered from lithiasis or fibrosis of the urogenital tract, some of them from bacterial pyelonephritis or cyctitis. Distribution of the patients by histological diagnosis, age and sex is shown in Table I.

Processing of samples. Excised tumour samples 0.1 to 0.2 g in mass were kept in sterile test tubes for 1 to 2 h at 4 °C before transported to the laboratory. There, a few samples were stored at −20 °C for one or two days, the others were processed immediately. They were ground with quartz sand, one by one in porcelain mortars kept in a water bath of 0 °C. The minced tissue was suspended in 2 ml Hanks' solution and the suspension was centrifuged in a Janetzki K23 centrifuge at 2000 rpm at 4 °C. The supernatants were stored at −20 °C until used. The protein content of the cell extracts was assayed as described by Lowry et al. [11].

In vitro cell cultures and nutrient media. HEp-2 cell monolayers were used throughout. The cultures were prepared as described in detail elsewhere [12]. Briefly, the growth medium was Eagle's MEM enriched with 10% fetal calf serum (Human, Budapest), 50 U/ml penicillin and 50 μg/ml streptomycin. The cells were maintained in serial passage with trypsin treatment and distributed in tubes as usual. The maintenance fluid was Eagle's MEM containing 2% fetal calf serum and the above-mentioned antibiotics.

Virus strains. The Huie strain of human Ad-12 as well as the type-5 wild strain (G. Pereira, London) have been maintained in this Institute for years. The virus suspensions were produced in HEp-2 cell cultures, centrifuged [12], and the supernatants were titrated in HEp-2 cells at 37 °C on the basis of the viral cytopathic (CP) effects. The two *ts* mutants of Ad type 5, viz. *ts18* and *ts19*, had been isolated by J. Williams [13, 14] and some of their characteristics were determined by B. Taródi, Szeged [15]. The mutants were kindly supplied by the latter author. Both mutants are of the late type. Although some of the virus constituents are synthesized even at restrictive temperature in infected cells, virus replication is interrupted because virus assembly does not take place at 39 °C. In mutant *ts18*, because of a change in its steric structure, the precursor of structural polypeptide VI is not phosphorylated by protein kinase, consequently, assembly fails to come about [16]. In mutant *ts19* polypeptide X, a polypeptide originating from polypeptide VI, is not phosphorylated, whereby, supposedly, tethering of the viral DNA to the virion's capsid does not take place [3]. The loci of mutations in the gene map are known with fair approximation [17, 18].

The mutants were produced in HEp-2 cell cultures at permissive temperature (32 °C) and titrated at both 32 °C and 39 °C. From the titration at 32 °C the minimal, still obviously infectious, value, viz. $TCID_1$, was calculated by interpolation [19]. Taking into account that very little amounts of virus were titrated, we preferred the $TCID_1$ unit to the usual $TCID_{50}$. At 39 °C no signs of multiplication of either of the mutants were observable with the inocula used by us.

Complementation of mutants with cell extracts. Twenty four hour HEp-2 cell cultures were treated with extracts of tissues taken from patients. The calcium-phosphate transfection technique published by Graham and van der Eb [20] was followed. Briefly, tissue extract containing 50 to 100 μg/ml protein and DNA and mixed to calcium phosphate was added to HEp-2 cell cultures. After an incubation for 2 h at 37 °C, the superfluous calcium phosphate and the unadsorbed tissue extract were removed by washing two or three times with main-

tenance medium containing 20 mM HEPES. Then the same HEp-2 cell cultures were inoculated (superinfected) with 1 $TCID_1$ of the wild strain or of a *ts* mutant. The cultures pretreated with tissue extract and (super)infected with virus were halved. The one half was incubated at 32 °C, the other at 39 °C, both for 7 days. In the experiment in which mutant was used, CP changes appearing at 39 °C indicated complementation by the tissue extract under study. Superinfection with the wild strain at both temperatures and with mutant at 32 °C informed us on a possible enhancement or inhibition of virus replication by the tissue extract at permissive temperature, compared to the replication without tissue extract. The virus yield produced from the 2nd till the 7th day of incubation at 39 °C was titrated to show if recombination had occurred.

Demonstration of infectious virus in tissue extracts. Materials to be tested were added to HEp-2 cell cultures with and without calcium phosphate. Superinfection was performed neither with the wild strains nor with the mutants. Otherwise, the procedure was the same as described above. Incubation lasted for 21 days. The cases showing any kind of CP effect in this test were omitted in the evaluation of complementation experiments.

Complementation in vitro between Ad-12 and the ts mutants of Ad-5. To show if complementation like that described elsewhere [14] took place between different Ad types in the system applied by us, we performed experiments, with and without the calcium-phosphate technique, in which HEp-2 cell cultures were inoculated by the above-described method with Ad-12 and a type-5 mutant simultaneously. Two $TCID_1$ and 1 $TCID_1$ inocula were used for single and double infections, respectively.

In certain preliminary experiments the order of inoculations of cell cultures were reversed. The results were the same.

Results

Complementation of ts mutants with tissue extracts. In the HEp-2 cells treated with both tissue extract and mutant and incubated for 24 h at 39 °C striking CP changes were observed in a number of cases. The results show considerable intergroup differences between clinical groups of patients (Table II). A high frequency of complementation occurred with extracts of tumours removed from patients suffering from renal adenocarcinoma or carcinoma of the bladder. Complementation was especially frequent with extracts of tumours removed from female patients. The fact that most of the female patients were 60 to 70 years old, i.e. one to two decades after menopause, is suggestive of hormonal effects. The complementation rate was also high for patients suffering from papilloma of the bladder, mainly from papillomas already showing atypia, a sign of malignization. In these cases often both mutants, sometimes only one of them, were complemented by the same extract. It should be noted that the tumours originating in the kidney or the peripheral parts of the trigonum vesicae are of mesodermal origin [7]. The complementation rate was relatively high in cases of penis carcinoma, too, a malignancy containing ectodermal cells. In hypertrophia prostatae (adenoma prostatae), on the other hand, complementation occurred with the testicular carcinoma (seminoma), extracts as frequently as with extracts of tissues removed from non-tumourous ("control") urological patients.

Viruses obtained by complementation at restrictive temperature. At 39 °C complementation was rapid and of high degree, and the complementation index showed little variation (Table III). In the cases judged negative, no

Table I

Distribution by diagnosis, sex and age of patients examined in the complementation experiments

Diagnosis	Sex	No. of patients	Age (years) $\bar{x} \pm SD$
Adenocarcinoma renis	M	17	54.3 ± 12.0
	F	7	57.2 ± 14.2
	Total	24	55.2 ± 12.7
Carcinoma vesicae urinariae	M	32	63.3 ± 16.6
	F	12	67.5 ± 5.1
	Total	44	64.5 ± 13.4
Papilloma vesicae urinariae	M	9	60.6 ± 10.7
Adenoma prostatae	M	36	67.7 ± 14.2
Carcinoma planocellulare penis	M	5	64.2 ± 14.2
Seminoma	M	14	36.3 ± 13.9
Non-tumourous urological disease	M	11	47.9 ± 13.2
	F	2	18.0 ± 0
	Total	13	43.3 ± 15.9

Table II

The complementing activity of urogenital tissue extracts (in per cent of the patients)

Diagnosis	Sex	Complementation with			Total
		both mutants	only *ts18*	only *ts19*	
Adenocarcinoma renis	M	17.6	11.7	17.6	47.1
	F	42.8	14.3	28.6	85.7
	Total	25.0	12.5	20.8	58.3
Carcinoma vesicae urinariae	M	31.2	9.4	12.5	53.1
	F	50.0	25.0	—	75.0
	Total	36.3	13.6	9.1	59.1
Papilloma vesicae urinariae	M	—	33.3	33.3	66.7
Adenoma prostatae	M	—	5.6	13.9	19.5
Carcinoma planocellulare penis	M	—	60.0	20.0	80.0
Seminoma	M	—	7.1	7.1	14.2
Non-tumourous urological diseases	M	—	18.2	9.1	27.3
	F	—	—	—	—
	Total	—	15.4	7.7	23.1

signs of complementation could be observed. The "all or none law" seemed to apply to the process.

In the following, we had to examine the two possible mechanisms of CP effect appearing at restrictive temperature: (i) viral products originating from tumours can complement our mutants, (ii) defective viruses and/or products of integrated virus genes were complemented by the utilization of other non-damaged polypeptides of mutant viruses to produce infective viruses. Since both of these mechanisms might have taken place simultaneously, it seemed to be justified to investigate the possibility of recombination.

For this purpose, virus suspensions obtained from maintenance media of doubly-inoculated cell cultures that had been incubated at 39 °C were titrated at both 32 °C and 39 °C. At 32 °C, the titres indicated by CP changes attained 10^{-3} in a great majority, and even 10^{-4} in two-third of the cases. The CP changes were characterized by clusters of rounded cells. The supernatants from cultures that had displayed complementation, i.e. from those containing virus, were inoculated into fresh HEp-2 cultures, which were then incubated at 39 °C. No viral action was demonstrable in the cells, except for a very weak CP effect after a 10-day incubation. It may therefore be concluded that the mutants were complemented by Ad gene products that, having originated in the tumour cells, were introduced by the calcium phosphate method into HEp-2 cells, where they were expressed.

Complementation by tissue extracts of ts mutants at 32 °C and of the homotypic wild virus at both temperatures. In HEp-2 cells inoculated with both tissue extract and Ad mutant it was observed that at permissive temperature the development of the CP effect of the *ts18* mutant was slightly accelerated, compared to the control cultures inoculated with mutant alone. No similar acceleration was noted in experiments with the mutant *ts19*. There were tissue extracts which accelerated virus multiplication when mixed to the wild strain and incubated in the culture at 32 °C. Interestingly, one, or both, of the mutants was complemented by these extracts in experiments carried out at restrictive temperature. On the other hand, the replication of the wild strain was not enhanced by tissue extracts at 39 °C. In certain cases, in which complementation did not take place otherwise (e.g., extracts of prostatic adenoma or of tissues removed from non-tumourous patients), the CP effects of all the three strains were slightly slowed down, but similar phenomenon could not be observed when the supernatants of the HEp-2 cultures were subcultured at 32 °C.

The virus strains recovered at 32 °C from mixtures of tumour extract and mutant could be titrated at this temperature, but not at restrictive temperature, except for a few cases, in which placing of maintenance medium of HEp-2 cell cultures pretreated with tumour extract on fresh HEp-2 cultures resulted in a minimal CP effect at 39 °C. This phenomenon corresponds to the

successful complementation in the original experiment at the same temperature and might be attributed to traces of tissue extract in the transferred medium. It seems that several μg amount of tumour protein and tumour DNA may have caused complementation, for this phenomenon was never observed with extract that failed to complement both of the mutants. In conclusion, these observations speak against recombination and support the complementability of the mutants (Table III).

Table III

Complementation and recombination

	Between			
	tumour extracts and		Ad-H12 and	
	ts18	*ts19*	*ts18*	*ts19*
Complementation index	1.14×10^3	1.28×10^3	7.5×10^2	1.25×10^2
Recombination frequency	0	0	0	0

Complementability of the mutants with Ad-12. Experiments were performed to show whether our system is suitable for demonstrating complementation between different Ad types. Single and double infections were carried out with the Ad mutant *ts18* or *ts19* on the one hand and type 12 Ad on the other. When the infected HEp-2 cells were incubated at 32 °C, the CP changes appeared earlier in the doubly-infected cultures than in the controls, however the difference began to fade out after 1 to 2 days of incubation. The small heaps consisting of rounded cells, a picture characteristic of Ad-5, tended to break, the cells elongated and displayed a polymorphous character. If doubly-inoculated HEp-2 cells were incubated at 39 °C, the CP changes described above developed sooner than in cultures infected with Ad-12 alone. Using variable amounts from the two viruses in the same experiment (e.g., 1 $TCID_1$ of Ad-12 and 0.5 $TCID_1$ of mutant), we observed a consistent dominance of the type-5 mutant. The same had been described for other Ad mutants [21]. The relatively low index value in double infection is due to the fact that Ad-12, a virus also replicating in singly-inoculated cultures, has a considerably high value in the denominator of the index. Nevertheless, the index is high enough to prove the fact of complementation (Table III).

Infectious viruses in tumour cell extracts. To detect infectious virus in tissue extracts, we added 0.1 ml extract to HEp-2 cell cultures and incubated the cultures without superinfection at 32 °C, 37 °C or 39 °C for 21 days. Of the extracts obtained from 150 patients only those from 5 patients induced CP changes. The CP agents could be subcultured in fresh cell cultures. Either syncytia were formed (herpesviruses?) or cells became granulated and rounded

up (Ad or parvovirus?). Identification of the infectious agents is in progress. The five patients who yielded infectious agent were excluded from the above-described evaluation of complementation.

Discussion

Results of the present experiments convinced us that *ts* mutants were complemented by tissue extracts and recombination could be excluded. The same applied to intertypic complementation [14]. Cell extracts of papillomas of the bladder behaved similarly. The cells of the tumours mentioned above derive from mesodermal cells of the wolffian duct [7]. It is of interest, on the other hand, that Ad genes were often detected also in penile carcinoma cells, which are of ectodermal origin. As to the other urogenital tumours tested in the present work, the cells of seminomas originate in germinal cells, whereas benign prostate adenomas consist of cells deriving from the müllerian duct under special hormonal influence. Ad genes were detected in such tumour cells but rarely, approximately as rarely as in cells obtained from various cells of the "control" urogenital patients. Considering the low rate of detectability of functioning Ad genes in control cells, it may be assumed that not more than about 20% of the population carry such genes in their cells; infectious virus can be recovered from such cells but extremely rarely. The subjects positive in the complementation experiment may be regarded as members of a risk group.

Ads may play some, perhaps indirect, role in malignancies of ectodermal origin as well as in certain types of mesodermal cells being under the influence of androgens [4]. In mesodermal cells influenced by female hormones and in entodermal cells, on the other hand, herpesviruses [22–25], cytomegalovirus [1, 2, 26, 27] and papovaviruses [28, 29] might be incriminated. Herpesviruses and cytomegalovirus have been detected in prostate carcinoma as well [22, 26, 27]. To explain this fact, it should be considered that the carcinomas of the prostate, a gland consisting of a mixed cell population, are under the control of androgens [7]. Latent Ads may persist in the organism over decades [12], therefore, their aetiological role in prostate cancer would not be surprising. Prostate tumours grow very slowly [8, 27], even two decades may elapse until their clinical manifestation. Malignancies of müllerian duct origin appear usually in females during the reproductive age [6–8], whereas those of ectodermal or wolffian duct origin occur more frequently in males and aged females than in women in the reproductive age, e.g., the male/female ratio for bladder carcinoma is about 3 : 1 [7]. It should be noted that not only the tissues are hormone-sensitive but also the DNA viruses are sensitive to steroid hormones even in a direct way [6, 8, 12].

Besides DNA viruses, retroviruses have been detected in the sorts of tumours mentioned above [5, 30]. In the light of recent oncogene theories, retroviruses may play a very important role in oncogenesis [31]. None of the analyses of antiviral antibodies [32] and detection of viral genes and gene products [10, 32] or of viral particles [25] in tumours have brought a convincing evidence in favour of direct aetiological role of viruses. We undertook complementation experiments to show whether structural cells in urogenital tumours may contain Ad genes capable of continual functioning. The structural polypeptides damaged in mutants *ts18* and *ts19* (polypeptides VI and X, respectively) cannot be replaced in the process of virion assembly by any other protein except the respective polypeptides of other Ads closely similar in structure [14, 17]. For this reason, it is obvious that these were polypeptides which were expressed in tumour cells during the complementation experiments. Furthermore, since several hundreds of such polypeptide molecules are necessary in the assembly of a single virion [16], complementation of such a high degree as demonstrated by us, must need a continuous expression of the genes. The mutations affecting polypeptide VI are located in the middle part of the genome in the L3 region, which, like the L2 region, may be transcribed together with the early regions [9]. The phosphorylation of the polypeptides mentioned above has been attributed to a polypeptide kinase of virus origin [15], which, deriving from the mutant during complementation and ready for normal function, can use the nonphosphorylated precursor of polypeptide VI as substrate. Although it is still unknown which of the virus-coded proteins exert a kinase activity, and exactly which kind of kinase activity [16], it is justified to suppose that the coding for kinases may be disturbed, or even lost, during integration. Abnormal protein kinases and virus proteins may have some further role in the development of tumourous processes [33].

Based on our own results we can imagine that in the tumour cells mentioned above the genome of certain Ad types is partially transcribed without yielding infectious particles. Integration of viral nucleic acid is a prerequisite of such a phenomenon [10, 33]. We did no attempt to recognize the type of Ad involved, nevertheless, there is evidence enough to exclude C and D and further Ad groups. Most of the members of these groups fail to transform cells at all or are capable of transforming in vitro, but not in vivo, for the genes participating in in vivo carcinogenesis do not integrate from these types [10]. The role of group B Ads in similar tumours has been excluded by hybridization experiments [4]. In previous experiments we detected antibodies to early antigens of Ad-12 in blood sera collected from tumour-bearing patients [9]. Ad-12 transforms ectodermal cells [34] and its almost entire genome can be detected in the transformated cells [10]. The demonstration of viral particles is made easier by the fact that there were, besides infectious virions, great many defective, i.e. noninfective, particles similar to those coming about during

complementation (!). The defective genomes of these particles are capable of integrating. The L3 region at the middle part of the genome as well as the early regions E1A and A1B at its left and, together with the intermediary regions E5, L1 and L2, integrate most probably [3, 10]. The hexon-coding sequence right of the L3 region, i.e. that coding for polypeptide VI, may become defective between coordinates 53 and 63 during integration. Since the hexon gene is not expressed, antibody to the entire virus cannot be detected in the majority of cases [9]. Right of the defect all of the regions E2-L4-E3-L5-E4 can integrate, though, the late genes may be defective. Integration of the two larger segments that include a continuous series of the genes mentioned above, except the middle part of the genome, occurs more probably. This is because deletion and integration in eukaryotic cells prefers AT-rich loci like those occurring in the above-mentioned ones [17]. The regularities of the integration of Ad-12 and other oncogenic Ads are still unknown [10]. Nevertheless, integration in a bizarre form like that observed with Ad-2 and Ad-5 in rodent cells cannot be excluded [3, 10].

Statistical data suggest that at least one of the aetiological factors independent of one another in the similar tumours examined so far was an infectious agent [1, 8, 26–28]. The noninfectious factor, in the conventional sense of the expression, is the cell's protooncogene and/or oncogenes of retroviruses [31, 33, 35] together, whereas the factor that should be considered infectious is Ad in the present case and other DNA viruses, e.g. herpesvirus or parvovirus, in others. Integration of these viruses leads to activation of the protooncogene and/or retrovirus oncogenes [31, 33] by the mechanism of overexpression or translocation [36]. Since these DNA viruses are sensitive to hormones in themselves [6, 8, 12], hormonal changes in the organism may participate in their activation. Thus, the clinical signs of carcinoma may become noticeable as late as years after the start of the cascade system of malignization.

Theoretically it cannot be excluded that we introduced into cultured HEp-2 cells a gene coding for a protein kinase of wide specificity, or that we induced a new protein kinase. Both of these assumptions are highly improbable and our above-outlined conception is supported by the presence of anti-Ad antibodies in the blood serum of patients. Nevertheless, we have initiated further experiments to answer the question unequivocally.

Virus mutants are widely used to reveal biochemical-genetical markers of viruses [21]. It seems likely that in the future the great many well-characterized mutants available will be utilized in further investigations, viz. analysis of the pathomechanism of infections and diseases, so much more that expensive procedure will be replaced by use of mutants, and a great number of patients can be tested in this way easily.

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Note added in proof. Polypeptide X is cleaved from the precursor of protein VII, as it has been published recently (Weber, J. M., Anderson, C. W.: 7th Intern. Congress of Virology. Edmonton, 1987. Abstracts p. 202.). The gene coding for polypeptide VII is located in region L2, and this fact explains the difference found in complementation with two mutants.

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EFFECT OF POLY I:C-ACTIVATED PERITONEAL CELLS ON THE TAKE OF TRANSPLANTABLE MURINE TUMOURS

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The effect of syngeneic mouse peritoneal cells (PC) on the growth of four different transplantable tumours was studied in adoptive transfer experiments (Winn's test). PC from unstimulated mice did not influence the growth of a benzpyrene induced fibrosarcoma (BaF1) and a methylcholantrene induced mastocytoma (P815), but significantly enhanced the growth of a spontaneous adenocarcinoma (Sp4) and Lewis lung carcinoma (LL). PC induced by a single injection of thioglycollate did not influence, whereas PC elicited by proteose peptone markedly enhanced the growth of BaF1 fibrosarcoma. The enhancing effect of peptone induced PC was diminished by a single intraperitoneal dose (100 μg/mouse) of polyinosinic-polycytidylic acid (poly I:C) given after peptone injection. Transferring PC obtained after a single injection of poly I:C (100 μg intraperitoneally) resulted in retardation of growth of BaF1 fibrosarcoma and Sp4 adenocarcinoma or in a marked decrease in their take depending on the PC/tumour cell ratio. The effector cells involved in the protective effect proved to be different, using these two tumour models. Lewis lung carcinoma and P815 mastocytoma proved to be insensitive to poly I:C-stimulated PC.

A role for lymphoid cells and macrophages in controlling the growth of neoplastic cells has been suggested in the last decades [1–10].

Natural killer (NK) cells, natural cytotoxic (NC) cells, antibody dependent cytotoxic cells (ADCC) and spontaneous cytotoxic macrophages, having the capacity to destroy specific tumour cells, have been demonstrated in the peritoneal cavity of untreated mice [11–14].

This cytotoxic activity of NK cells and macrophages can be increased by several agents including the products of activated lymphocytes (lymphokines) [15–20], interferons or interferon inducers [21–25].

As the synthetic double stranded RNA (poly I:C) — in addition to other biological effects [26–30] — can boost NK activity in vivo and in vitro [31–33] and activates peritoneal macrophages even in experimental conditions when lymphokines fail to induce cytotoxicity [34], we have tested in four experimental tumour models, whether poly I:C-stimulated PC transferred to syngeneic mice have any effect on the take and growth of four different transplantable tumours.

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We have found that poly I:C-stimulated PC inhibit the take of a chemically induced fibrosarcoma and a spontaneous adenocarcinoma with a remarkable efficiency but do not suppress the take of Lewis lung tumour and P815 mastocytoma.

Materials and methods

Animals. Seven to 8 weeks old Balb/c mice of both sexes and female C57BL/6 and DBA/2 inbred mice (LATI, Gödöllő, Hungary) were used.

Tumours. 1. BaF1, a benzpyrene induced transplantable fibrosarcoma, established in a Balb/c male mouse was kindly provided by J. Fachet (Institute of Pathophysiology, University Medical School, Debrecen, Hungary). It was maintained by in vivo transplantations. (Intramuscular injection of 10^6 BaF1 cells results in a 100% tumour take). 2. A transplantable spontaneous adenocarcinoma (Sp4) originated from a Balb/c female mouse in our laboratory. It was maintained in syngeneic female mice by injecting 2×10^5 cells intramuscularly. 3. Lewis lung carcinoma (LL) was maintained by serial in vivo passages inoculating 10^5 LL cells intramuscularly into C57BL/6 females. 4. The methylcholantrene induced P815 mastocytoma was maintained in ascites form by weekly passage of 5×10^5 tumour cells into the peritoneal cavity of DBA/2 females. Intramuscular injection of 5×10^3 P815 cells resulted in solid tumours with a 100% take.

Peritoneal cells (PC). PC were obtained by washing the peritoneal cavities with 2.0 ml of medium 199 containing 10 mM 4-(2-hydroxyethyl)-1-piperazidine-ethanesulfonic acid (HEPES) and 7 U/ml heparin. Induction of exudate cells was carried out by giving of 1.5 ml of 3% proteose peptone (Oxoid) intraperitoneally, or 1.5 ml of Brewer's thioglycollate broth (prepared in our laboratory) 3 or 4 days before cell harvesting, respectively.

Poly I:C (Calbiochem, sodium salt A grade Lot 702045) was dissolved in sterile saline at 4 °C overnight and administered intraperitoneally (100 μg/mouse) 18 h before cell collection.

The collected cells were washed with medium 199 containing 10 mM HEPES at 4 °C and counted. Differential cell counts were determined on Giemsa smears in all experiments. Washed PC were resuspended in saline. Viability of the cells was controlled by the trypan blue exclusion test in all experiments. Only PC with more than 90% viability were used.

Fractionation of cells. Adherent and nonadherent cells were prepared by plastic adherence. Briefly: PC were cultured for 2 h in plastic tissue culture dishes in medium 199 containing 10 mM HEPES and 10% fetal calf serum at 37 °C in a 5% CO_2 atmosphere. Nonadherent cells and cells detached were removed by a careful repeated washing with warm culture medium. The remaining adherent cells were collected by a rubber policeman, washed twice in medium 199 and resuspended in saline. More than 80% of adherent cells resembled macrophages morphologically and ingested latex particles. Nonadherent cells were treated with carbonyl iron+magnet [35]. More than 90% of the remaining cells proved to be lymphocytes.

Experimental design. The effect of PC on tumour growth was tested by means of the Winn's assay [36]. Suspensions of PC and tumour cells were prepared in concentrations indicated and mixed immediately before intramuscular injection into the right thigh of mice. They were monitored three times weekly for tumour appearance and growth. Tumours could be detected at the site of inoculation by palpation when they were 2–3 mm in diameter. The two dimension diameters of the growing tumours were measured subsequently.

Statistical analysis. Student's *t*-test was used. Values of $p < 0.05$ were considered significant.

Results

Resident peritoneal cells (rPC) from mice did not influence the growth of BaFl fibrosarcoma when injected simultaneously with tumour cells at a 10:1 rPC/tumour cell ratio (Fig. 1/a). In contrast, macrophage enriched PC induced by proteose peptone (ppPC) three days before cells harvest given in

the same ratio resulted in a marked enhancement of tumour growth. The enhancing effect of ppPC was diminished when a single dose of 100 μg poly I:C was given after peptone injection (18 h before cell collection) (Fig. 1/b).

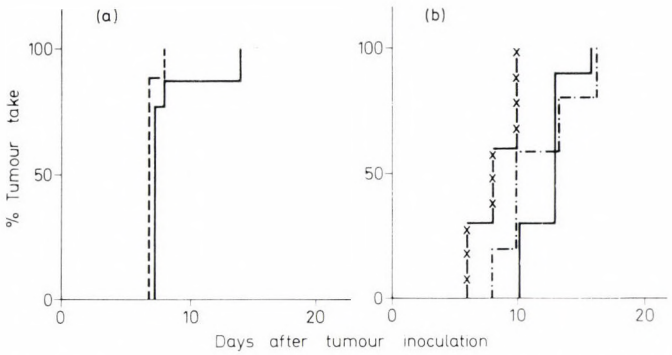

Fig. 1. Effect of resident, proteose peptone, and proteose peptone+poly I:C-induced PC on the take of BaFl fibrosarcoma. Recipient mice were inoculated i.m. with 10^6 BaFl cells (——) (a and b) and with 10^6 BaFl cells mixed with 10^7 PC from untreated mice (----) (a) and with 10^7 PC from mice treated with proteose peptone ($\times - \times$) or proteose peptone+poly (I:C ($- \cdot -$) (b). Each group consisted of 9–10 mice

Macrophage enriched PC induced by an other inflammatory agent, Brewer's thioglycollate (tgPC) (1.5 ml intraperitoneally) four days before cell harvest had no effect on the growth of BaFl tumour injected with tumour cells at 10:1 (data not shown) or 40:1 tgPC/tumour cell ratio. The same ineffectivity was observed, when poly I:C (100 μg intraperitoneally) was administered after tg injection (18 h prior cell collection; Table I).

PC from mice not pretreated with irritants but injected with a single dose (100 μg i.p.) of poly I:C 18 h before cell collection resulted in a marked

Table I

Take and growth of BaFl fibrasarcoma in mice after adoptive transfer of peritoneal cells from syngeneic mice treated with thioglycollate and thioglycollate+poly I: C

Cells inoculated to recipient mice on day 0	Tumour take on day 9	Mean diameter (mm) of tumours ±SE on day 16
10^6 BaFl i.m.	10/10	19.25 ± 0.84
10^6 BaFl + 4×10^7 tgPC i.m.	10/10	20.10 ± 0.69
10^6 BaFl + 4×10^7 tg + poly I:C[a]-induced PC i.m.	10/10	18.20 ± 0.78

[a] Poly I:C (100 μg i.p.) administered after thioglycollate injection, 18 h before cell harvesting

retardation of tumour take and growth when transferred to the recipients simultaneously with fibrosarcoma cells at a 10:1 PC/tumour cell ratio. Using this PC/tumour cell ratio, the tumour was taken, however, by all of the mice on the 22nd day after transplantation (Fig. 2).

In similar experiments, when PC/tumour cell ratio was elevated to 28:1, not only a retardation of tumour take and growth was observed, but the tumour was not taken at all by ~50% of mice even until the 180th day after transplantation (Fig. 3/a).

Figure 3 also shows that by decreasing the number of transplanted tumour cells and further elevating the PC/tumour cell ratio, an even more dramatic effect can be observed (Fig. 3/b).

The effector cell(s) involved in the tumour inhibitory effect of poly I:C-induced PC were analyzed by testing the effect of separated adherent and non-adherent cell populations.

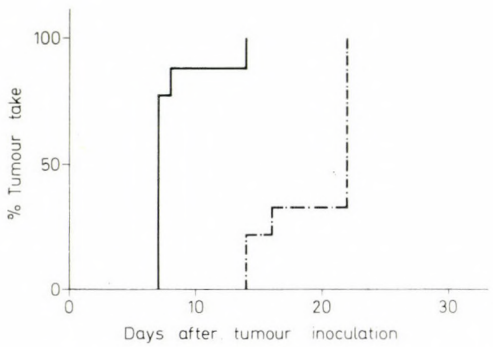

Fig. 2. Retardation of BaFl tumor development in recipient mice by PC from poly I:C treated animals. Mice were inoculated i.m. with 10^6 BaFl cells (——, n = 9) and with 10^6 BaFl cells mixed with 10^7 PC from poly I:C treated mice (— · — , n = 9)

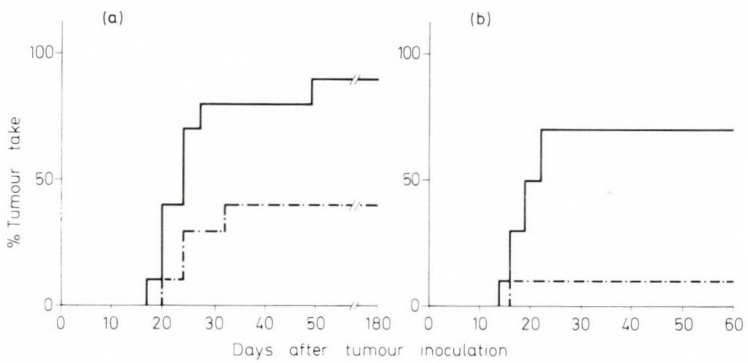

Fig. 3. Inhibition of the take of BaFl tumour by PC from poly I:C treated mice. Recipient mice were inoculated i.m. with 10^6 BaFl cells (——, n = 10) (a) or 10^5 BaFl cells alone (——, n = 10) (b) and mixed with PC from poly I:C treated mice (— · — , n = 10) in a 28:1 (a) or 50:1 PC: tumour cell ratio (b)

Figure 4 shows that both adherent and nonadherent cells separated from PC of poly I:C treated mice inhibited the take of fibrosarcoma. Thus, both cell populations are associated with the tumour inhibitory effect observed (Fig. 4). These results prompted us to study the effect of PC from poly I:C-stimulated mice also in other experimental tumour-models.

Figure 5 demonstrates that resident PC markedly enhanced the take of Sp4 adenocarcinoma when injected at a 10:1 PC/tumour cell ratio (Fig. 5/a). PC from poly I:C stimulated mice had a moderate inhibitory effect on tumour take when given in the same ratio (Fig. 5/b).

Increasing the PC/tumour cell ratio to 50:1 resulted in a significant decrease of tumour take. This is demonstrated in two independent experiments (Fig. 6).

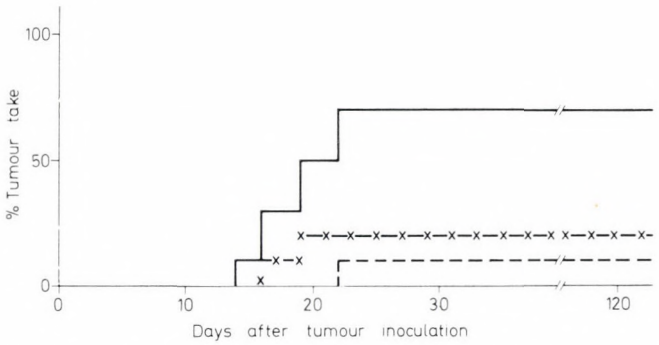

Fig. 4. Effect of poly I:C-stimulated adherent and nonadherent PC on the take of BaFl fibrosarcoma. Mice were inoculated i.m. with 10^6 BaFl cells alone (——, n = 10) and mixed with adherent (---, n = 10) or nonadherent cells (× — ×, n = 10) separated from PC of poly I:C-treated mice. PC: tumour cell ratio = 30:1

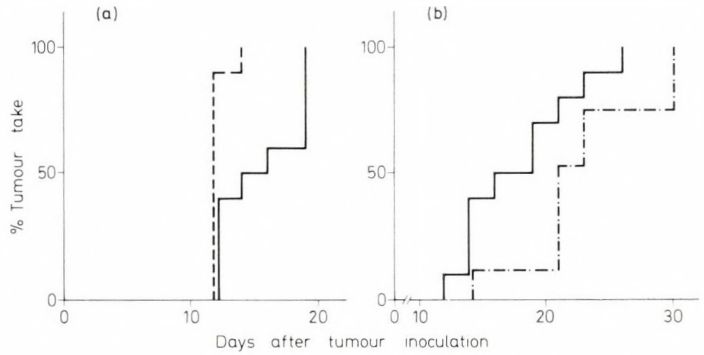

Fig. 5. Effect of resident and poly I:C-stimulated PC on the take of Sp4 adenocarcinoma. Mice were inoculated i.m. with 2×10^5 Sp4 cells alone (——) and mixed with PC from untreated mice (---) (a) or from mice treated with poly I:C (— · —) (b). Each group consisted of 9–10 mice

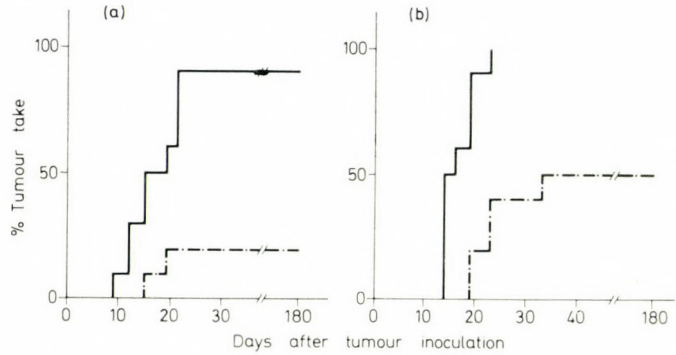

Fig. 6. Inhibition of the take of Sp4 adenocarcinoma by poly I:C-stimulated PC. Mice were inoculated i.m. with 2×10^5 Sp4 cells either alone (——, n = 10) or mixed with PC from poly I:C-treated mice (—·—, n = 10) (a and b). PC:tumour cell ratio = 50:1

We have studied the role of adherent and nonadherent cells also in this tumour system. Adherent cells did not suppress the take of Sp4 tumour, while nonadherent cells markedly decreased the tumour take when administered at a 25:1 PC/tumour cell ratio (Fig. 7).

The effect of PC from untreated and poly I:C stimulated mice on the development of Lewis lung carcinoma and P815 mastocytoma was also studied. Resident PC enhanced the growth of Lewis lung carcinoma (Fig. 8) but had no effect on the growth of P815 mastocytoma (Table II).

Table II also demonstrates that transferring PC from poly I:C-stimulated mice resulted in no significant decrease in the mean diameter of growing Lewis lung tumours in the group of recipient mice injected with tumour cells at a 50:1 PC/tumour cell ratio and no protection against tumour take could be observed. The growth of P815 mastocytoma was also uneffected by poly I:C induced PC.

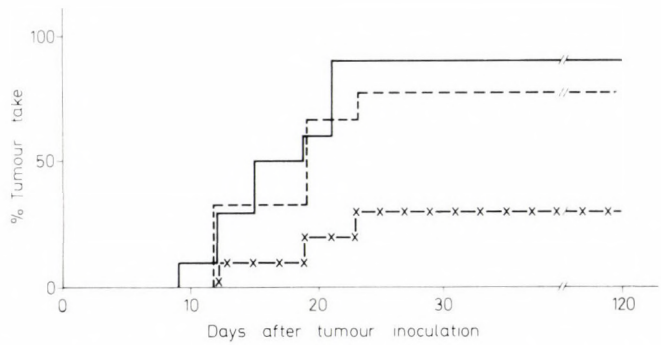

Fig. 7. Effect of poly I:C-stimulated adherent and nonadherent cells on the take of Sp4 adenocarcinoma. Recipient mice were inoculated with 2×10^5 Sp4 cells (——) and with 2×10^5 Sp4 cells mixed with adherent (---) or nonadherent cells (× — ×) separated from PC of poly I:C-treated mice. PC:tumour cell ratio = 30:1. Each group consisted of 9–10 mice

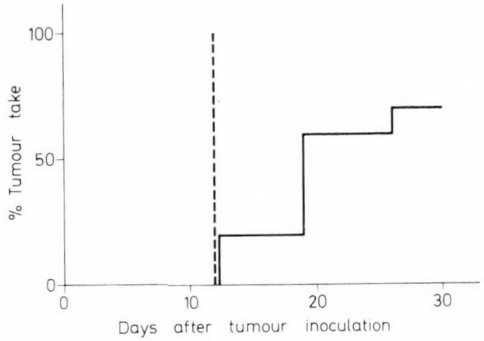

Fig. 8. Enhancing effect of PC from untreated mice on the take of Lewis lung (LL) carcinoma. Mice were inoculated i.m. with 10^5 LL cells either alone (——, n = 10) or mixed with PC from untreated mice (---, n = 10). PC:tumour cell ratio = 50:1

Table II

Inability of PC from poly I:C*-treated mice to inhibit development of Lewis lung carcinoma (LL) and P815 mastocytoma*

Cells inoculated intramuscularly on day 0	Tumour take on day 14	Mean diameter (mm) of tumours ± SE on day 14
10^5 LL	8/10	6.93 ± 0.70
10^5 LL + 5×10^6 poly I:C[a]-stimulated PC	8/10	6.31 ± 0.76
5×10^3 P815	10/10	13.70 ± 0.51
5×10^3 P815 + 10^6 resident PC	7/7	13.88 ± 0.65
5×10^3 P815 + 10^6 poly I:C-stimulated PC	9/9	14.17 ± 0.54

[a] Poly I:C (100 μg i.p.) was injected 18 h before cell collection

Discussion

In vivo enhancement of tumour growth by resident peritoneal macrophages as well as promotion of tumour cell proliferation in vitro by products of these cells have been documented [37–41].

Results of the present study demonstrate that PC (containing 25—30% macrophages and 70—75% lymphocytes) obtained from untreated mice did not influence (fibrosarcoma and mastocytoma) or markedly enhanced (spontaneous adenocarcinoma and Lewis lung carcinoma) the growth of syngeneic murine tumours in a Winn-type neutralization assay. Thus, the effect of resident PC is dependent on the tumour model used.

Macrophage-enriched PC populations from mice injected previously with irritants (thioglycollate or proteose peptone) resulted in different effects on the growth of fibrosarcoma depending on the kind of inflammatory agents used. Our results showing that thioglycollate-induced peritoneal cells (containing more than 80% macrophages) did not influence, but peptone-elicited cells (containing about 70% macrophages) markedly enhanced the tumour growth are in good accordance with the findings of Currie [41], who reported that supernatant of macrophages from thioglycollate injected mice did not promote the proliferation of FS6 fibrosarcoma cells, whereas macrophages elicited by proteose peptone provided supernatants with an active growth-promoting effect in vitro. The ineffectiveness of thioglycollate-elicited macrophages to influence the tumour growth was, however, not observed by Gabizon et al. [40], who demonstrated a significant enhancement of tumour development by thioglycollate-induced macrophages in Winn-type experiments. The difference observed is probably due to the different chemical composition of the eliciting agents and experimental tumour models used. In contrast to resident PC and PC obtained from mice treated with thioglycollate or proteose peptone, PC from poly I:C-treated mice resulted in a significant decrease in tumour take using BaFl fibrosarcoma and Sp4 adenocarcinoma models. Interestingly, however, adherent and nonadherent cells isolated from poly I:C-stimulated PC populations had different effects in the two tumour systems. A similar phenomenon was observed by Olstad et al. [42] using PC from BCG-stimulated mice. As to the potential use of poly I:C-stimulated nonadherent cells in cancer therapy, Morales and Pang [43] observed a suppression of growth rate of a murine bladder tumour after intratumoural inoculation of nonadherent spleen cells stimulated by poly I:C in vivo.

If we take also in consideration that PC from poly I:C injected mice had no effect on the take of Lewis lung carcinoma and P815 mastocytoma, we have to draw the conclusion that the effect of transferring PC to syngeneic mice depends on the tumour model used. In this respect, not only tumour cells as targets but participating effector cells (adherent and nonadherent) can vary. Thus, unfortunately, an individual analysis of these relationships is necessary in each type of tumours.

Activated PC, however, are potential candidates in immunotherapy, since they contain more than one effector cell populations and combination of poly I:C with other drugs may result in activated PC capable to inhibit the growth of a tumour resistant to the effect of PC activated by poly I:C per se (manuscript in preparation).

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MOUSE PERITONEAL CELLS ACTIVATED WITH A COMBINATION OF INDOMETHACIN, POLY I : C AND SYNCUMAR INHIBIT THE TAKE OF LEWIS LUNG CARCINOMA IN ADOPTIVE TRANSFER ASSAY

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Effect of peritoneal cells (PC) from mice treated with a combination of drugs (indomethacin, poly I:C and Syncumar) on the take of Lewis lung (LL) carcinoma was studied in Winn-type adoptive transfer experiments. Transfer of PC from mice given a single intraperitoneal injection of polyinosinic-polycytidylic acid (poly I:C) or indomethacine or Syncumar (100 μg of each) per se did not suppress the take of Lewis lung carcinoma in the recipient mice. PC obtained from mice treated with a combination of indomethacin and poly I:C or poly I:C and Syncumar also failed to inhibit the take of the tumour. In contrast, PC collected from mice after a combined treatment with the three drugs (indomethacin + poly I:C + Syncumar) resulted in a 30–60% decrease in tumour take depending on the tumour cell/PC ratio. This effect could not be observed when a single intraperitoneal dose of cyclophosphamide was administered three days before starting of the combined treatment of the donor mice. The effector cells contributing to the tumour inhibitory effect proved to be nonadherent cells, probably large granular lymphocytes (LGL), as their suppressive effect was abrogated after treatment with the lysosomotrop vital dye neutral red.

Enhancement of NK-activity in murine spleen cells by interferon (IFN) and the IFN-inducer poly I:C, as well as a dramatic increase in NK activity of PC after inoculation of bacillus Calmette-Guérin (BCG) has been documented [1–4]. Using an in vitro cytotoxicity assay Morales and Pang [5] showed that intraperitoneally administered poly I:C augmented mainly the NK-like cytotoxic activity of splenic cells, while BCG increased first of all that of the peritoneal cells.

In a previous study we have shown that PC and nonadherent cells separated from PC of poly I:C injected mice markedly inhibited the take of a chemically induced fibrosarcoma and a spontaneous adenocarcinoma in Winn-type adoptive transfer experiments (manuscript submitted for publication).

By using four different tumour models we have found, however, differences in the susceptibility of the experimental tumours used, as Lewis lung

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carcinoma [LL] and P815 mastocytoma proved to be resistant to PC transferred from poly I:C treated mice. This observation prompted us to study whether treatment of the donor mice with drugs in addition to poly I:C may result in PC capable to inhibit the take of a tumour uneffected by PC obtained from mice treated with poly I:C per se.

We report here that adoptive transfer of PC from syngeneic mice treated with indomethacin, poly I:C and Syncumar (referred to as "combined treatment" later) inhibit the take of Lewis lung carcinoma in the recipient mice. The effector cells responsible for this effect proved to be neutral red sensitive nonadherent cells, presumable large granular lymphocytes (LGL).

Materials and methods

Animals. Seven to eight weeks old inbred C57B1/6 female mice (LATI Gödöllő, Hungary) were used.

Tumour. Lewis lung (LL) carcinoma was kindly provided by Dr. L. Kopper (First Institute of Pathology and Experimental Cancer Research, Semmelweis University Medical School, Budapest). The tumour was maintained by serial in vivo passages inoculating 10^5 tumour cells intramuscularly into C57B1/6 females. Intramuscular injection of 10^5 LL cells resulted in a 70–100% tumour take.

Chemicals. Poly I:C sodium salt A grade Lot 702045 (Calbiochem La Jolla, CA, USA) was dissolved in sterile saline and kept at 4 °C overnight before use. Indomethacin (Chinoin, Budapest) was dissolved in 96% ethanol to a concentration of 10 mg/ml and further diluted in saline to the appropriate final concentration immediately before administration. It was then injected in a fine suspension form. The final concentration of ethanol was 9.6%. Ethanol in the same concentration was administered to control animals. Syncumar (3-[α(4-nitrophenyl)-β-acetylethyl]-4-hydroxy-coumarin), a gift of Alkaloida, Tiszavasvári, Hungary, was dissolved in sterile saline containing 0.1 N NaOH and neutralized with 0.1 N HCl. Syncumar solutions were prepared freshly before administration. Cyclophosphamide (VEB Jenapharm, Rudolstadt, GDR) was dissolved and diluted in sterile saline immediately before use. Neutral red was purchased from Sigma Chemical Co., St. Louis, MO, USA. A 10^{-2} M stock solution of the dye was prepared and diluted in culture medium to 10^{-4} M end concentration.

Treatment of animals. Mice were injected with 100 μg indomethacin, 100 μg poly I:C and 100 μg Syncumar i.p. 42, 18 and 3 h before cell collecting, respectively (Table I).

Peritoneal cells (PC). Cells were obtained by peritoneal lavage with 2.0 ml of medium 199 containing 10 mM HEPES. The collected PC were washed two times with the same medium at 4 °C and then counted. Washed PC were resuspended in sterile saline. Viability of cells was controlled by the trypan blue exclusion test. Only PC with more than 90% viability were used. Differential cell counts were determined on Giemsa smears in all experiments.

Separation of adherent and nonadherent cells. Adherent and nonadherent cells were separated by plastic adherence. Briefly, PC were maintained for 2 h in plastic tissue culture dishes in medium 199 containing 10 mM HEPES and 10% fetal calf serum at 37 °C in a 5% CO_2 atmosphere. Nonadherent cells and cells detached were removed by a careful repeated washing with warm culture medium. The remaining adherent cells were collected carefully with a rubber policeman, washed twice in medium 199 and resuspended in sterile saline. More than 80% of the adherent cells had a typical macrophage morphology and phagocytized latex particles. Nonadherent cells were treated with carbonyl iron + magnet [6] to remove the remaining phagocytic cells, and washed two times with medium 199 at 4 °C, then resuspended in sterile saline. Ninety per cent of the remaining cells proved to be lymphocytes by morphologic examination.

Treatment of nonadherent cells with neutral red. Treatment of nonadherent cells was carried out according to the method of Shau and Dawson [7]. Briefly, separated nonadherent cells were incubated in medium 199 containing 10 mM HEPES, 10% fetal calf serum in the presence of 10^{-4} M neutral red for 30 min at 37 °C in a 5% CO_2 atmosphere. Then the cells were washed three times with medium 199 and resuspended in sterile saline.

Experimental procedure. The effect of PC on tumour take was tested by means of the Winn's assay [8]. PC and single cell suspensions of the tumour cells were prepared in concentrations indicated and mixed immediately before intramuscular injection into the right thigh of the recipient mice. They were monitored three times weekly for tumour appearance and growth. Tumours could be detected by palpation when they were 2–3 mm in diameter. The two dimension diameters of growing tumours were measured subsequently.

Results

Treatment of groups of donor mice is shown in Table I. Transfer of PC collected from mice treated with indomethacin or Syncumar (group 1 and 2) per se did not influence the tumour take in recipient mice inoculated at a 50:1 PC:tumour cell ratio (Fig. 1). PC from mice treated with indomethacin and Syncumar (group 3) also failed to influence the tumour take in the recipient

Table I

Treatment of donor mice before collection of PC

Groups of mice	Treatment	Hours before cell collection
1	Indomethacin[a]	42
2	Syncumar	3
3	Indomethacin +	42
	Syncumar	3
4	Poly I:C	18
5	Poly I:C +	18
	Syncumar	3
6	Indomethacin +	42
	poly I:C	18
7	Indomethacin +	42
	poly I:C +	18
	Syncumar	3
8	Cyclophosphamide +	114
	indomethacin +	42
	poly I:C +	18
	Syncumar	3

[a] Indomethacin, poly I:C and Syncumar were administered i.p. (100 μg per mouse). Cyclophosphamide was injected i.p. (2.5 mg/kg body weight)

mice using the same PC:tumour cell ratio (data not shown). PC collected from poly I:C treated mice (group 4) did not suppress the tumour take, though a delay in tumour appearance could be observed (PC:tumour cell ratio = = 50:1) (Fig. 2).

PC from mice treated with poly I:C + Syncumar or indomethacin + poly I:C (groups 5 and 6) had similar effect as PC from poly I:C treated mice (Figs 2

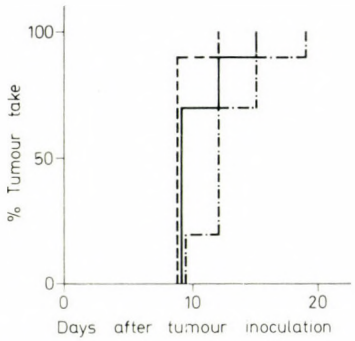

Fig. 1. Effect of PC from mice treated with indomethacin or Syncumar on the take of LL carcinoma. Mice were injected i.m. on day 0 with 10^5 LL cells (———, n = 10) alone or mixed with PC from indomethacin (– – –, n = 10) and Syncumar (—·—, n = 10) treated mice. PC: tumour cell ratio = 50:1)

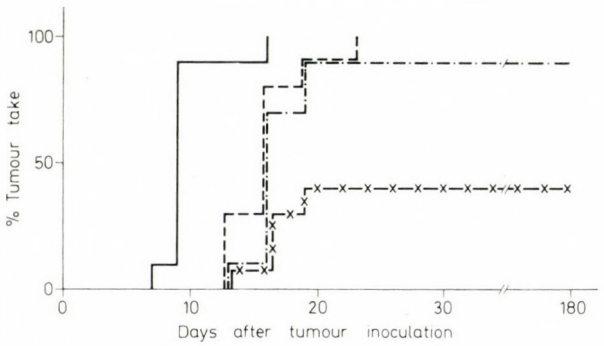

Fig. 2. Effect of PC from mice treated with poly I:C, poly I:C + Syncumar and indomethacin + poly I:C + Syncumar on the take of LL tumour. Groups of the recipient mice were inoculated. i.m. on day 0 with 10^5 LL cells (———, n = 10) alone or mixed with PC from mice treated with poly I:C, (—·—, n = 10), poly I:C + Syncumar (– – –, n = 10) and with indomethacin + poly I:C + Syncumar (× — ×, n = 10). PC: tumour cell ratio = 50:1

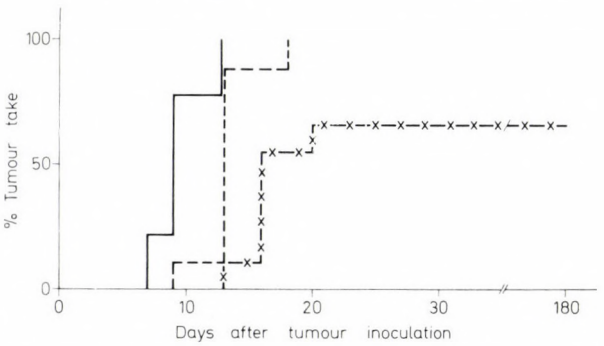

Fig. 3. Effect of PC from mice treated with indomethacin + poly I:C and indomethacin + poly I:C + Syncumar on the take of LL tumour. Groups of the recipient mice were inoculated i.m. on day 0 with 10^5 LL cells (———, n = 9) or with 10^5 LL cells mixed with PC from mice treated with indomethacin + poly I:C (– – –, n = 9) and indomethacin + poly I:C + Syncumar (× — ×, n = 9). PC: tumour cell ratio = 35:1

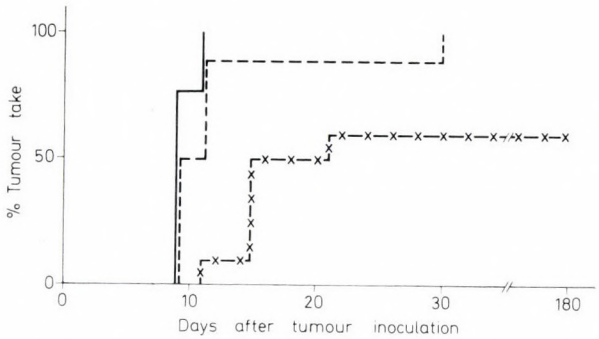

Fig. 4. Effect of cyclophosphamide-treatment of donor mice on the antitumour activity of PC activated by "combined" treatment. Recipient mice were inoculated i.m. on day O with 10^5 LL cells (———, n = 9) or 10^5 LL cells mixed with PC from mice underwent "combined" treatment. – – – group of recipient mice getting 10^5 LL cells mixed with PC from mice injected with 2.5 mg/kg cyclophosphamide i.p. and underwent "combined" treatment 3 days thereafter (n = 10); × — × group of recipient mice obtaining 10^5 LL cells mixed with PC from mice underwent "combined" treatment (n = 10). PC: tumour cell ratio = 50:1

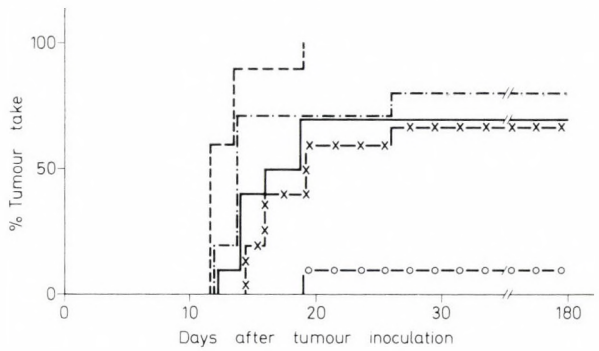

Fig. 5. Effect of adherent and nonadherent cells from untreated mice and from mice underwent "combined" treatment on the take of LL tumour. ——— injected with 10^5 LL cells; – – – injected with 10^5 LL cells mixed with adherent PC from untreated donors; —·— injected with 10^5 LL cells mixed with nonadherent PC from untreated donors; × — × injected with 10^5 LL cells mixed with adherent PC from "combined" treated donors; ○ — ○ injected with 10^5 LL cells mixed with nonadherent PC from "combined" treated donors. PC: tumour cell ratio = 20:1. Ten mice were used in each group

and 3). Transfer of PC collected from mice underwent "combined treatment" (group 7) markedly inhibited the take of tumour in the recipients (PC:tumour cell ratio = 50:1; Fig. 2). A smaller decrease in tumour take was observed using a lower (35:1) PC:tumour cell ratio (Fig. 3).

Administering cyclophosphamide to the donor mice 3 days before starting of their "combined treatment" (group 8), no suppressive activity of the collected PC could be observed (Fig. 4).

Figure 5 demonstrates the results of cell transfer experiments in which the effects of adherent and nonadherent cells separated from PC of mice

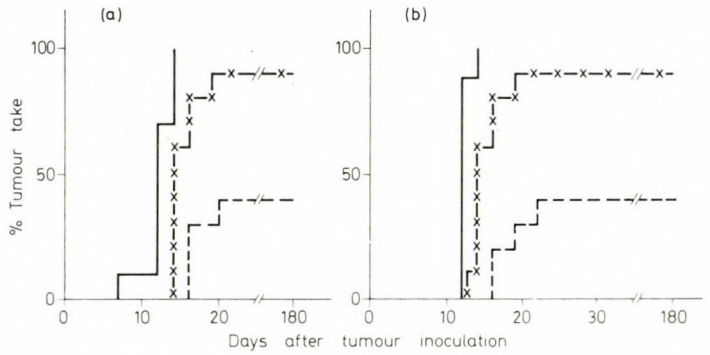

Fig. 6. Effect of neutral red (10^{-4} M) on the tumour take inhibitory action of nonadherent cells from mice underwent "combined" treatment. ——— injected with 10^5 LL cells; – – – injected with 10^5 LL cells mixed with nonadherent PC from "combined" treated donors; × — × injected with 10^5 LL cells mixed with nonadherent PC from "combined" treated donors. Before mixing the two cell types, the nonadherent PC were incubated with 10^{-4} M neutral red for 30 min at 37 °C. PC: tumor cell ratio = 50:1 in experiment (a) and 40:1 in experiment (b). Ten mice were used in each group

treated with indomethacin+poly I:C+Syncumar and of control mice were tested. Adherent cells from untreated mice enhanced the take of tumour (adherent cell:tumour cell ratio = 20:1), while adherent cells from treated mice did not influence the tumour take when administered at the same adherent cell:tumour cell ratio. Nonadherent cells from untreated mice slightly increased the tumour take; in contrast, nonadherent cells from mice underwent "combined treatment" markedly decreased the tumour take using the same (20:1) nonadherent cell/tumour cell ratio.

We examined the influence of neutral red (a lysosomotrop vital dye, a functional inhibitor of large granular lymphocytes (LGL)) on this inhibitory effect. Figure 6 shows the results of two independent experiments. Nonadherent cells from "combined treated" mice inhibited the take of tumour in more than 50% of the recipients using a 50:1 or 40:1 nonadherent cell:tumour cell ratio, respectively, but no effect was observed when the cells were transferred after incubation with 10^{-4} M neutral red for 30 min at 37 °C.

Discussion

In a previous study we could not influence the take of LL carcinoma by PC transferred from poly I:C treated mice.

The present study demonstrates that supplementing poly I:C treatment of donor mice with two other drugs (indomethacin and Syncumar) results in PC capable to inhibit the take of LL tumour.

These supplementary drugs alone or combined with poly I:C could not induce the generation of PC with tumour-take-inhibitory activity. The induction of PC responsible for suppression of LL tumour take proved to be cyclophosphamide sensitive [9].

The effect was mediated by nonadherent cells, probable large granular lymphocytes (LGL), as their effect could be abrogated by neutral red [7, 10]. This finding, in accordance with previous data [5, 11–16], suggests a potential role for NK and/or NK-like cells in controlling the growth of certain neoplastic cells.

Prostaglandins (A and E series) have a negative regulatory effect on NK-activity [17–19]. Thus, intraperitoneal injection of indomethacin, known to have an inhibitory action on prostaglandin synthesis, may support the action of poly I:C by decreasing the level of prostaglandins.

The question why administration of Syncumar following indomethacin+poly I:C treatment was also necessary to induce the generation of PC suppressing the take of LL carcinoma can not be answered yet. A systemic anticoagulant effect (via the inhibition of production of vitamin K dependent factors of coagulation in the liver) of Syncumar is unlikely in our experimental circumstances, as its presence in the donors was very short (3 hours). Syncumar, however, may affect the function of LGLs directly, or by influencing their interactions with other peritoneal cells. Peritoneal macrophages are known to produce vitamin K dependent factors of the coagulation system [20] and to express procoagulant and fibrinolytic activities [21], thus they are potential candidates as targets for Syncumar action.

Our data, however, are comparable with that of Gorelik et al. [22] who found that the antimetastatic effect of anticoagulants was dependent on NK cell activity and poly I:C could potentiate the suppression of metastasis by anticoagulants.

One has to consider also the possibility that Syncumar exerts its action in a way unrelated to its anticoagulant activity.

In conclusion, our data show that by the use of a combination of drugs with different mechanisms of action it is possible to induce the generation of PC capable to suppress the take of a highly malignant tumour uneffected by PC activated by poly I:C per se.

Experiments are in progress to study the passive immunotherapeutic value of PC activated by "combined treatment".

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CERVICAL AND VAGINAL MICROFLORA OF WOMEN UNDER CANCER SCREENING

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Gynaecological cancer screening was supplemented with examination of the vaginal microflora and pH in women suffering from vaginal discharge and/or colpitis. In alkaline samples *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* and, in cervical epithelial cells, herpes simplex virus antigen were of common occurrence, while in samples with acid reaction *Trichomonas* and, in cervical cells mainly from pregnant women, adenovirus antigen were often detected. Since vaginal pH may be informative of the pathogenic agent(s), its estimation by a rapid, simple and painless procedure, like litmus paper reaction, is recommended.

Gynaecological screenings are primarily aimed at detecting tumours in due time. In the town of Szentes, where females between 20 and 65 years of age are being screened, we have taken the opportunity to search for gynaecological diseases other than tumours. In this way we made attempts to approach the aetiology of symptoms manifesting among others, mostly in vaginal discharge.

Many authors [1–14] have suggested that protozoan, fungal, bacterial and viral colonization of the femal genitals may be aetiological cofactors in various disorders including fluor, colpitis and even tumour formation. Based on this consideration, we examined the relationships between the acidity or alkalinity of vaginal discharge and the microflora of the vagina.

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Materials and methods

Sevenhundred women 20 to 65 years of age, suffering from gynecological complaints, mostly from vaginal discharge, were examined. Of them, 500 were tested for bacteria, fungi and protozoa, further 200 for adenovirus and herpes simplex virus (HSV) antigens. Gram stains were examined for bacteria and fungi and Giemsa stains for protozoa. Bacteria were cultured on nutrient media of routine use. Acid or alkaline reaction was tested with litmus paper.

For detecting viral antigens, the portio vaginalis uteri was sampled with a cotton swab and each sample was spread on a slide. The preparations were left to dry at room temperature, fixed in acetone and treated with rabbit antiserum conjugates. The anti-adenovirus serum was conjugated with fluorescein isothiocyanate, whereas the anti-HSV serum with rhodamine. Immunofluorescence was examined with a Zeiss–Fluoval microscope as described earlier [15, 16].

Results

The incidence and frequency of bacteria, fungi and protozoa and their distribution in samples of acid and alkaline reaction are presented in Table I. Pathogens and opportunistic pathogens were detected in 170 of the 500 samples tested. Most of them, most frequently *Escherichia coli*, occurred in samples with alkaline reaction.

Among the 200 samples examined for viral antigens 100 had acid, the other 100 alkaline reaction. Of the 200 women, 50 were pregnant. The incidence and percentage of adenovirus and HSV antigen is shown in Fig. 1. HSV antigen was detected in 11% only in alkaline samples taken from the 150 non-pregnant women. In samples with acid reaction only adenovirus antigen was detectable, significantly more frequently in pregnant women (33%) than in non-pregnant ones (12%).

Table I

*Opportunistic pathogens found in acid and alkaline vaginal samples**

	Secretions of	
	acid pH	alkaline pH
Escherichia coli	9	114
Proteus	3	28
Staphylococcus	4	73
Candida albicans	2	64
Trichomonas	33	29
Totals	51	308

* Total no of samples tested = 500

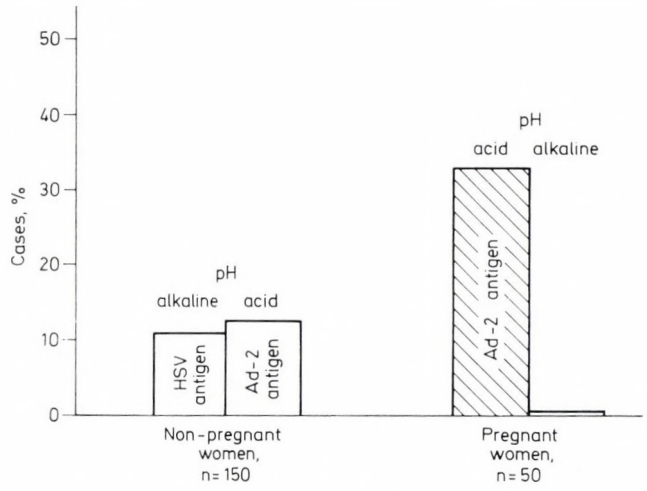

Fig. 1. Incidence of viral antigens in acid and alkaline vaginal secretions of non-pregnant and pregnant women

Discussion

Literary data and our observations suggest that certain disorders of the female genitals, as well as the alkaline shift of vaginal pH, show some relationship to the vaginal microflora. Vaginal pH may be influenced by microbes, hormonal changes, contraceptives, pregnancy, menopause, etc. Alkaline pH favours the colonization of opportunistic pathogens. The consequent signs of disease may include benign lesions of the os uteri, cervicitis, vaginal discharge, cellular atypia, etc.

Pathological processes of the female genitals have been increasing recently in both incidence and importance. Mainly inflammatory phenomena and vaginal discharge are caused by microbial pathogens [17–19]; viruses may play a role in malignant changes among others [20–23]. HSV prefers alkaline pH for replication, it soon loses its infectivity in acid medium. Interestingly, the same has been observed in cervical cells in which HSV was persisting [6].

It also deserves attention that we could not detect HSV antigen but in alkaline samples. In contrast, adenovirus antigen was only detectable in acid samples, with relatively high frequency, but not exclusively, in samples from pregnant women.

Microbes, *E. coli* among others, may contribute to the alkaline shift of the vagina, and opportunistic pathogens may play a role in the aetiology of, e.g., infections and purulent inflammations, or may persist without causing any symptom [23, 24].

Our findings agree well with the view that the presence of pathogenic (or opportunistic pathogenic) microbes in the female genitals is unfavourable and, to select an effective antimicrobial drug, the pathogenic agent(s) schould be identified. Since the vaginal reaction may be informative of the method to be used in searching for the pathogen, i.e. an alkaline reaction may be suggestive of the presence of bacteria, testing with litmus paper, a simple, rapid and painless method, is recommended for use in the gynaecological practice.

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ADSORPTION TO Al(OH)$_3$ GEL OF *ESCHERICHIA COLI* IS CORRELATED WITH O AND K ANTIGENS AND WITH TYPE OF EXTRAINTESTINAL INFECTION

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Living suspensions of 89 *Escherichia coli* strains were tested for adsorption to Al(OH)$_3$ gel in the presence of phosphate ions. On the basis of AC_{50} (phosphate molarity inhibiting 50% adsorption of the strain examined), *E. coli* strains could be classified into two main groups. Forty-three strains belonged to group 1 (AC_{50}, 0.01–0.04), and 42 of them fell into serogroups O1, O2, O5, O7, O18ac, O83 or were spontaneously agglutinable. One strain in group 1 was exceptional as it had antigen O4. Of these 43 strains 33 had K antigen K1. Serogroup distribution of 46 group 2 strains (AC_{50}, 0.001–0.009) was O2, O4, O6, O18ac, O75 and O78; 20 out of 46 possessed antigen K5. No correlation existed between H antigens or haemagglutinating capacity and AC_{50} of the strains. A close correlation was shown between AC_{50} pattern and the two main pathogenecity groups (i.e. "newborns' meningitis" and "sepsis and organotropic diseases") on one hand and between AC_{50} pattern and O, K serotypes on the other. The findings indicate that these *E. coli* strains with identical markers had a clonal connection.

The possibility that well-defined serotypes might represent bacterial clones was suggested by Ørskov et al. [1]. Data of Achtman et al. [2] supported the clone conception as well. On the basis of our previous study [3], showing a close connection among certain serogroups and certain markers of pathogenicity it was also suggested that certain *Escherichia coli* strains had a clonal connection. Beside, classification of *E. coli* with different virulence factors into different pathogenicity groups: "meningitis in the newborns" and "septicaemia with different organotropic diseases in adults" [4] gave further evidence of the existence of special clones.

Szöllősy [5] published a procedure, the principle of which was that bacterial adsorption to Al(OH)$_3$ gel was inhibited by secondary phosphate ions. Bacteria were characterized in terms of the molarity of phosphate which inhibited adsorption of 50% of the cells to Al(OH)$_3$(AC_{50}). Moreover — as

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the AC_{50} value is elevated in the presence of antibodies — the gel adsorption test offers a rapid and sensitive method for determination of the presence and quantity of antibodies [6].

The aim of this study was to examine the correlation between adsorption to $Al(OH)_3$ gel and different markers (i.e. O, K, H antigens, haemagglutination, site of infection, origin etc.) of *E. coli* strains.

Materials and methods

Bacterial strains were isolated from faeces (patients with enteritis, 6; healthy individuals, 5), urine (pyelonephritis, 12; cystitis, 5; asymptomatic bacteriuria, 5; urosepsis, 1; unknown, 1), blood (sepsis, 10), cerebrospinal fluid (meningitis, 11), and other extraintestinal sources (wound abscess, 8; autopsy material, 3; umbilicus, 2; vagina, 7; upper respiratory tract, 8; miscellaneous, 5). Basic differentiation of strains was carried out by determining their O and K antigens.

Classification according to clinical diagnosis of patients, serological examination of O and H antigens, haemagglutination, detection of K1 and K5 capsular antigens, morphological examination of fimbrial structures were carried out as described previously [3, 7, 8].

Gel adsorption. Aliquots of 0.25 mCi $Na_2H^{32}PO_4$ were measured into Petri dishes containing Colonization factor antigen agar (CFA) medium [9, 10] and inoculated with the *E. coli* strains chosen for study. The dishes were incubated at 37 °C overnight. The bacterial cells were suspended in Tris-buffered saline (TBS, 0.1 M, pH 7.5), centrifuged and the pellet was washed 4–5 times with TBS of the initial volume. Aliquots of 1 ml $Al(OH)_3$ gel were measured into 8 tubes (100 × 15 mm), while one test tube contained 1 ml of deionized water. Then 2 ml of TBS were measured into the first tube and 2 ml phosphate buffer from each dilution of a $\log_2$ series were added to the rest of the tubes. The highest and the lowest dilutions of the buffer were 0.00125 M and 0.32 M, respectively. Finally, aliquots of 1 ml of the labelled bacterial suspension (diluted to 10 000 cpm/ml) were added to the tubes. The tubes were then shaken and incubated at room temperature for 15 min. After centrifugation at 1000–2000 rpm for 1 min the supernatants' radioactivity was determined. The activity found at 0.32 M phosphate buffer corresponded to that of the control without gel since at this concentration bacterial cells failed to adsorb to the gel. Thus the activity measured in the supernatant at 0.32 M phosphate buffer was considered 100% and the activities found at other molarities were expressed in the percentage of the former. To characterize the bacterial strains, the molarity of the phosphate buffer at which — compared to the final concentration — 50% of the cells remained in the supernatant (AC_{50} value) was given.

Results

Altogether 89 *E. coli* strains were examined. *E. coli* isolates could be classified into two main groups on the basis of the AC_{50}. Properties of strains with AC_{50} 0.01–0.04 are presented in Table I. Forty-two out of 43 strains belonged to serogroups O1, O2, O5, O7, O18ac and O83 or were spontaneously agglutinable, and 33 out of 42 possessed antigen K1. One further strain (No. 892) was exceptional in this respect, as its O antigen (O4) failed to fit into the former pattern. AC_{50} of the strains was not correlated with H antigens, site of infection, mannose-resistant haemagglutination with human erythrocytes (MRHA), mannose-sensitive haemagglutination with guinea pig erythrocytes

Table I

Classification of E. coli strains according to AC_{50} on $Al(OH)_3$ gel
Common property: AC_{50} 0.01–0.04 (AC_{50} group 1)

Designation of strains	Antigenic structure			Source	Diagnosis or state	Haemagglutination with eryhtrocytes		AC_{50} on $Al(OH)_3$ gel	Note
	O	K	H			human	guinea pig		
005	O1	..[1]	H—	N	H	—	—	0.02	fimbria+
363	O1	..	H7	B	S	—	+[2]	0.01	
380	O1	..	H7	B	S	—	+	0.01	
491	O1	K1	H7	U	Pn	+[3]	+	0.01	
558	O1	K1	H7	E	H	+	+	0.02	
847	O1	K1	H—	tissue	..[4]	+	—	0.01	fimbria—
989	O1	K1	H7	U	Pn	+	+	0.02	
990	O1	K1	H7	U	Pn	+	+	0.01	
490	O2	K1	H4	U	Pn	+	+	0.02	
505	O2	..	H—	U	C	—	+	0.01	fimbria+
840	O2	..	H—	lochia	..	—	—	0.02	fimbria+
862	O2	..	H—	U	Pn	—	+	0.04	fimbria+
999	O2	K1	H7	U	Pn	+	+	0.02	
1142	O2	K1	H5	bowel	..(D)	+	+	0.03	
892	O4	..	H—	F	E	+	+	0.04	
49974	O5	K1	H—	umbilicus	H	—	+	0.01	
293	O7	K1	H—	cervix	H	+(MS)	+	0.01	
498	O7	..	H6	U	Pn	—	+	0.04	
540	O7	K1	H—	..	..	+(MS)	+	0.01	
573	O7	..	H—	brain abscess	S	—	+	0.01	
638	O7	K1	H—	B	S	+(MS)	+	0.01	
072	O18ac	K1	H7	cervix	H	—	+	0.04	fimbria+
218	O18ac	K1	H7	U	Pn	—	+	0.03	fimbria+
321	O18ac	K1	H7	CSF	M	—	+	0.03	fimbria+
364	O18ac	..	H7	U	ABU	—	+	0.04	fimbria+
561	O18ac	K1	H7	cervix	H	—	+	0.04	fimbria+
633	O18ac	K1	H7	cervix	H	—	+	0.02	fimbria+
893	O18ac	K1	H—	CSF	M	—	+	0.02	fimbria+
895	O18ac	K1	H—	CSF	M	—	—	0.01	fimbria+
896	O18ac	K1	H—	CSF	M	—	—	0.01	fimbria+
54740	O18ac	K1	H7	umbilicus	H	—	+	0.02	fimbria+
54742	O18ac	K1	H—	fomite	..	—	+	0.02	fimbria+
57257	O18ac	K1	H7	CSF	M	—	+	0.03	fimbria+
57261	O18ac	K1	H—	vagina	H	—	+	0.01	fimbria+
002	O83	K1	HNT[5]	ear	H	—	—	0.02	fimbria+
292	O83	K1	HNT	cervix	H	—	+	0.02	
574	O83	K1	HNT	pus	..	—	+	0.04	
16247	O83	K1	HNT	U	C	—	+	0.02	
87244	O83	K1	HNT	U	Pn	—	—	0.01	fimbria+
328	Sp.aggl.[6]	K1	H21	B	S	—	+	0.01	fimbria+
894	Sp.aggl.	K1	H7	CSF	M	—	+	0.02	fimbria+
897	Sp.aggl.	K1	H7	CSF	M	—	+	0.02	fimbria+
922	Sp.aggl.	K1	H—	B	S	—	+	0.02	fimbria+

[1] K antigen not determined; it is neither K1 nor K5; [2] mannose sensitive haemagglutination; [3] mannose resistant haemagglutination unless otherwise signed; [4] diagnosis unknown; [5] H antigen not typable; [6] spontaneously agglutinable; N = nose swab; B = blood; E = ear swab; U = urine; CSF = cerebrospinal fluid; F = faeces; H = healthy; S = sepsis; Pn = pyelonephritis; M = meningitis; E = enteritis; C = cystitis; ABU = asymptomatic bacteriuria; D = died

Table II

Classification of E. coli strains according to AC_{50} on $Al(OH)_3$ gel
Common property: AC_{50} 0.001–0.009 (AC_{50} group 2)

Designation of strain	Antigenic structure			Source	Diagnosis or state	Haemagglutination with erythrocytes		AC_{50} on $Al(OH)_3$ gel	Note
	O	K	H			human	guinea pig		
96476	O2	K5	H—	F	E	—	—	0.008	fimbria+
074/1	O4	..	H—	wound	S	+	+	0.003	
074/2	O4	..	H—	wound	S	+	+	0.003	
302	O4	..	H5	CSF	M	+	—	0.004	fimbria+
575	O4	..	H—	abscess	S	+	+	0.003	
686	O4	..	H5	U	Pn	+	+	0.002	
1111	O4	..	H1	B	S	+	—	0.004	fimbria+
1189	O4	..	H5	throat	S	—	+	0.003	
451	O6	..	HNT	CSF	M	+	—	0.004	fimbria+
452	O6	..	HNT	CSF	M	—	—	0.005	fimbria+
495	O6	K5	H1	U	Pn	±	±	0.004	fimbria+
613	O6	..	H1	cervix	H	+	+	0.003	
623	O6	..	H—	lochia	H	—	+	0.005	
837	O6	..	H—	lochia	H	+	+	0.005	
842	O6	K5	H—	N	H	+	+	0.006	
846	O6	K5	H—	ear	H	+	+	0.003	
865	O6	..	H1	wound	S	+	+	0.002	
119	O18ac	K5	H—	U	..	+	—	0.001	fimbria—
249	O18ac	K5	H—	brain abscess	M	+	—	0.004	fimbria+
376	O18ac	..	H7	U	ABU	—	—	0.001	fimbria+
380	O18ac	..	H—	U	ABU	—	+	0.001	
443	O18ac	..	H—	wound	S	+	+	0.001	
443	O18ac	..	H—	wound	S	+	+	0.001	
774	O18ac	..	H—	F	E	+	+	0.001	
1002	O18ac	..	H7	U	C	—	—	0.006	fimbria+
15752	O18ac	K5	H—	U	ABU	+	+	0.004	
15755	O18ac	K5	H—	sputum	H	+	—	0.003	fimbria—
17633	O18ac	K5	H—	U	S	+	—	0.004	fimbria—
31041	O18ac	K5	H—	B	S	+	+	0.003	
31042	O18ac	K5	H—	B	S	+	+	0.006	
41149	O18ac	K5	H—	F	E	+	+	0.003	
43398	O18ac	K5	H—	F	H	+	+	0.009	
52772	O18ac	..	H—	lung	S(D)	—	+	0.004	fimbria+
67020	O18ac	K5	H—	B	S	+	+	0.005	
67021	O18ac	..	H—	B	S	—	+	0.004	fimbria+
95367	O18ac	K5	H—	F	E	+	—	0.008	fimbria—
97158	O18ac	K5	H—	F	E	+	+	0.004	
97159	O18ac	K5	H—	F	E	+	+	0.004	
97166/3	O18ac	K5	H—	F	H	+	+	0.003	
98479	O18ac	..	H7	U	ABU	—	—	0.006	fimbria+
496	O75	K5	H—	U	Pn	+	+	0.006	
499	O75	..	H7	U	C	—	+	0.002	fimbria+
845	O75	K5	H—	throat	H	—	+	0.003	
153	O78	..	H—	CSF	M	—	+	0.001	
87216	O78	..	HNT	U	C	—	+	0.001	
92389	O78	..	HNT	F	H	—	+	0.001	
97978	O78	..	H—	F	H	—	—	0.001	fimbria+
101999	O78	..	H—	F	H	—	—	0.001	fimbria+

For explanation see Table I

(MSHA), or existence of fimbrial structures. It should be noted that the presence of fimbria-like appendages was examined by electron microscopy in case when either MRHA and/or MSHA could not be shown.

Properties of *E. coli* strains falling into the other group (AC_{50}, 0.001–0.009) is demonstrated in Table II. All strains had O antigens O2, O4, O6, O18ac and O75, and 20 out of the 46 strains harboured antigen K5. These strains varied on the basis of origin, haemagglutinating capacity and possession of fimbrial structures.

All strains exerted haemagglutinating activity or had fimbrial structure.

Discussion

On the basis of special characteristics we have previously distinguished [4] in addition to urinary tract infections (UTI) two groups of extraintestinal diseases caused by *E. coli* (1) "Meningitis of the newborn" was usually associated with *E. coli* having antigen K1, belonging to serogroups O7, O18 and in many cases lacking MRHA activity; (2) in causative agents of "sepsis and organotropic diseases" the $MRHA^+$ marker and in case of serogroup O18, the presence of antigen K5 were common. Strains, causing meningitis in elder children or adults and UTI belonged to the latter category.

The present examination revealed a close association between O and K antigens and AC_{50} pattern. Namely, all those strains, that belonged to serogroups O1:K1, O2:K1, O7:K1, O18ac:K1, O83:K1 and spontaneously agglutinable:K1, showed AC_{50} in a narrow range of phosphate molarity: 0.01–0.04 (AC_{50} group 1). Meanwhile, strains with other O and K antigens (O2:K5, O4, O6, O18ac:K5, O75:K5, O78) belonged to AC_{50} group 2 ranging between 0.001–0.009.

These two different groups of O and K antigens and, accordingly the corresponding AC_{50} groups in some extent, are associated with two groups of extraintestinal infections, too [4]. This sharp separation of the two AC_{50} groups may reflect differences existing between adhesive properties of these two main pathogenicity groups of *E. coli*. Besides, although only few strains were examined, the correlation between different properties supports the concept that *E. coli* strains with identical markers have a clonal connection. Finally it also proves the previous concept [5] that there is a correlation between antigens and adsorption character.

There was one exception: a culture belonged to AC_{50} group 1 although it ought to be classified rather into pathogenicity group 2 on the basis of its O antigen. As the K antigens of serogroup O4 strains were not examined, it remains to be determined whether or not in surface structure strain No. 892 differs from the other O4 strains.

In their experiments Wadström et al. [11] could demonstrate differences between CFA^+ and CFA^- strains on the basis of hydrophobic character measured with different concentrations of ammonium sulphate. However, the data presented here do not allow to explain the nature of surface structure taking part in the inhibition of bacterial adsorption to $Al(OH)_3$ gel.

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AGE DEPENDENT EFFECT OF TOXIC AND DETOXIFIED ENDOTOXINS ON THE NATURAL ANTI-DNA ANTIBODY LEVEL IN RATS

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Toxic endotoxin (LPS) and its irradiated detoxified form (RD-LPS) induced only small changes in the DNA level in the sera of 4, 12 and 18 months old rats. Changes in the anti-DNA titres were age-dependent. In 4 months old rats one or two injections of the LPS preparations failed to increase the circulating anti-DNA level. In 12 and 18 months old rats both toxic and detoxified LPS preparations raised the anti-DNA titres. The second LPS injection evoked a smaller response in 18 months old rats than the first one.

The aging process is generally characterized by the decline of immune reactivity. Winchurch et al. [1] have found that endotoxin (LPS) treatment plays a contributory role in the immune response. Nariuchi and Adler [2] assumed rather a qualitative than a quantitative decrease in B cell function. Bános et al. [3] have reported that the reduced cellular immune reaction of old mice to virus infection was restored by treatment with radio-detoxified endotoxin (RD-LPS).

Fujiwara et al. [4] have observed an enhanced autoantibody production to bromeline-treated mouse erythrocytes in old mice, which could be due to expansion of B cell clones rather than to polyclonal B cell activation. Fish and Ziff [5] have concluded that the basic factor in the development of autoimmune diseases is the generation of B cell activation. Primi et al. [6] and Furnie et al. [7] have shown that LPS stimulates autoreactive clones. Furnie et al. [7] and Izui et al. [8] have demonstrated that LPS treatment causes a significant release of DNA into the circulation and leads to the formation of anti-DNA antibodies in mice. Fisbach et al. [9] after LPS treatment have found a temporary production of antibodies to polyadenylic acid (poly- A). Antibodies to native DNA were also induced by LPS, but the magnitude of the response was much lower when compared to the poly-A antibodies. When

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LPS was inactivated by complexing with polymyxin B and injected in vivo, no response was observed. Reeves et al. [10] have demonstrated in young mice prone to autoimmune diseases an anti-DNA production after repeated treatment with polyinosinic polycytidilic acid. After repeated treatments with LPS the anti-DNA antibody production was less pronounced. The effect of RD-LPS — which retains some beneficial effects of LPS [11] — has not yet been investigated. The anti-DNA antibody production was followed not only in rodents but in healthy adults and patients. Silvestries et al. [12] showed some autoantibodies in young and old persons. The level of anti-DNA antibodies was similar in both groups whereas the IgM type rheumatoid factor (IGM-RF) was threefold higher in the elderly subjects.

The aim of present work was to study the dynamics and quantity of DNA released into the circulation after treatment with toxic LPS or RD-LPS, and to follow the anti-DNA production in young and also in old rats.

Materials and methods

Animals. CFY rats (LATI Gödöllő, Hungary), 4, 12 or 18 months old of both sexes were used. Each group contained 3 to 8 animals. All experiments were performed at least twice.

Endotoxins. Endotoxin was prepared by warm phenol-water method [13] from *Escherichia coli* 089. For detoxification, the endotoxin was dissolved in distilled water and exposed to ^{60}-Co-gamma irradiation (Sovatom) as described by Previte et al. [14] and modified by us [11]. The dose for detoxification was 150 kGy.

Treatment of animals. The animals were treated intraperitoneally with 100 or 500 μg of toxic LPS or RD-LPS. Six weeks later each group was treated again with the same type of LPS. The dose of endotoxin was either the same as at the first time, or after a lower primary dose (100 μg) the rats were given a higher dose (500 μg). Serum samples were taken before the LPS injections and on different days during the response.

Measurement of serum DNA level. Blood was taken from the rats at different intervals after the treatment. Before performing the test serum samples of 3–8 rats of the same group and time point were pooled. The determination was carried out by a competitive radioimmunoassay, using the 125-I DNA tracer of Amersham anti-DNA kit. As anti-DNA antibody a human serum from an SLE patient with known anti-DNA activity was used. The inhibition of the binding of labelled DNA to the anti-DNA autoantibody was inhibited by the rat sera assumed to be proportional to their DNA content. Results are given as per cent of the value before LPS treatment.

Determination of anti-DNA level in serum samples was carried out by ELISA. For the investigations double stranded DNA (DS-DNA) was purified from chicken erythrocytes and dissolved in 0.1 M ammonium acetate. Single stranded DNA (SS-DNA) was prepared by heating the DNA solution to 100 °C for 10 min followed by immediate cooling down to 4 °C. Microtiter plates with 96 wells were coated overnight at 4 °C with 100 μl/well of 1 mg/ml SS-DNA or DS-DNA solution. The plates were washed 3 times with washing buffer (phosphate-buffered saline containing 0.01% merthiolate and 0.05% Tween 80). The plates were stored at 4 °C filled up with washing buffer until used. For the determination of anti-DNA levels the plates were incubated with 100 μl of 1:100 or 1:200 dilution (in PBS–Tween) of serum samples or with 100 μl of standard serum dilution for 1 h at 37 °C. The plates were washed again with PBS–Tween and after adding 100 μl of 10^{-3} dilution of peroxidase-conjugated rabbit IgG specific to rat IgG, were incubated for 1 h at 37 °C. After repeated washings 100 μl of diluted orthophenilene-diamine in citric-phosphat buffer were added to the plates. After incubation in the dark, the colour developed and 50 μl of H_2SO_4 were added to each well. The absorbance was measured at 492 nm. As reference, a pooled standard serum was tested in each series and the individual results were calculated on the basis of the standard serum samples. Serum samples were pooled. Each titration was performed in two parallels.

Results

Changes of serum DNA level after treatment with LPS or RD-LPS. In the 4 and 12 months old rats (Table I), 3–7 days after the administration of 100 μg LPS or RS-LPS, a moderate decrease of serum DNA content was observed which returned to the original level. After treatment with 500 μg LPS, the magnitude of alteration in the early period was more expressed. In 18 months old rats the DNA level remained around the original level, but on day 6 and 8 an elevation appeared. Toxic and detoxified preparations in the two doses evoked nearly the same alteration.

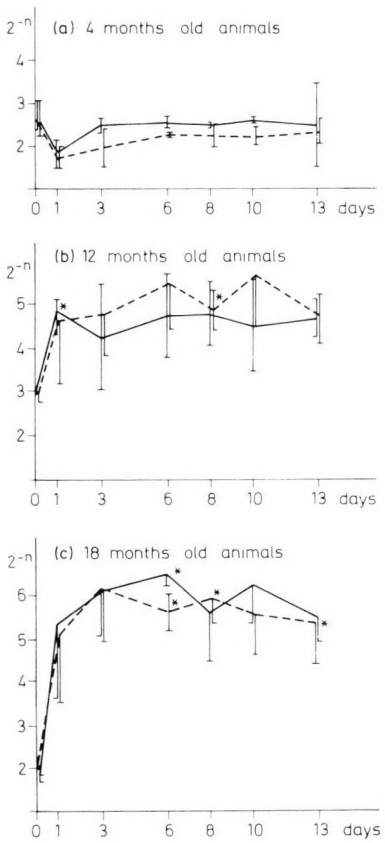

Fig. 1. Effect of 500 μg LPS or RD-LPS treatment on the anti-DS-DNA titre of 4, 12 and 18 months old rats (mean $\pm$ SE). Experiments were repeated in 4 and 12 months old animals 2 times, in 18 months old rats 3 times. Groups represent the results of pooled serum samples of 5–10 animals in the 4 months old group and of 4–5 rats in 12 and 18 months old groups. The asterisks mean that titres are significantly higher ($p < 0.05$) than that of the 4 months old animals on the same day. The titre increase is significant ($p < 0.05$) in 12 months old rats from the first day, in 18 months old rats from the third day. ——— LPS; – – – – – RD-LPS

The effect of LPS or RD-LPS treatment on the anti-DS-DNA level of 4, 12 and 18 months old rats. Before treatment with endotoxin preparation the anti--DS-DNA titre was low in all investigated groups of animals (Fig. 1). After treatment with LPS or RD-LPS the anti-DNA production seemed to be age dependent. In young animals no anti-DNA elevation appeared after the treatment. In 12 and 18 months old animals the treatment with both doses of both preparations evoked increase of anti-DS-DNA titres (Fig. 1b). In 18 months old rats the anti-DS-DNA titre became higher than it was seen in 12 months old animals (Fig. 1c). After 6 weeks the titres returned to the initial low levels (Fig. 2). The anti-SS-DNA titre showed in all age-groups similar changes to that of the anti-DS-DNA.

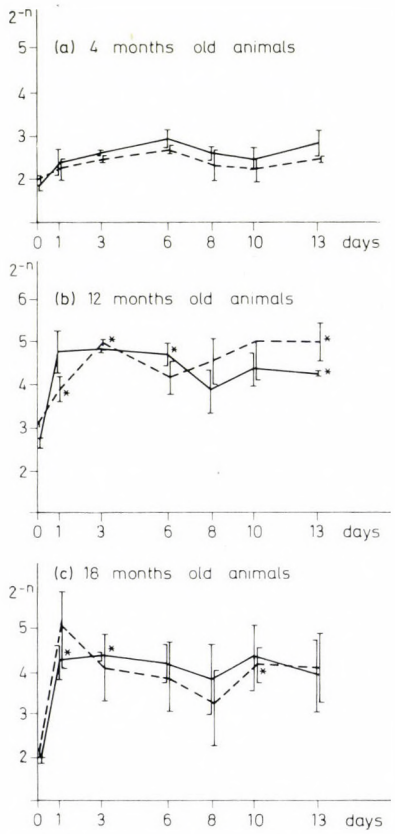

Fig. 2. Effect of repeated treatment with 500 μg LPS or RD-LPS on the anti-DS-DNA titre in 4, 12 and 18 months old rats (mean $\pm$ SE). Serum samples of 5–8 animals were pooled in the 4 months old group, of 3–5 animals in the 12 and 18 months old groups. The asterisks mean that titres are significantly higher than the titres of 4 months old animals on the same day. Titres were significantly higher after the second treatment than before the second treatment of 18 months old rats in all cases and time points while in the 12 months old animals after secondary LPS treatment and on day 3 and 13 after the second RD-LPS injection. For other details see Fig. 1

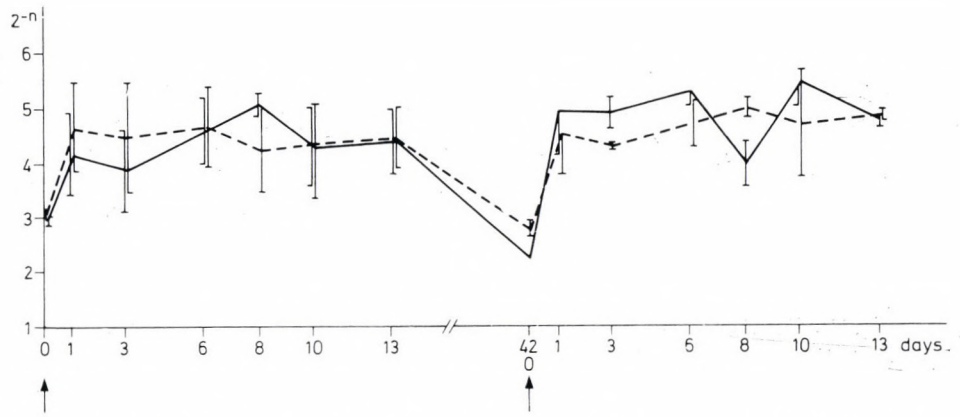

Fig. 3. Anti-DS-DNA titre in 12 months old animals after treatment with 100 μg LPS or RD-LPS, and after repeated treatment by the same dose of the same preparation (mean $\pm$ SE). After the first treatment, determination was repeated 4 times and each group contained 4–5 animals, while during the secondary response sera were tested 2–3 times. Arrows: LPS or RD-LPS treatment. For other details see Fig. 1

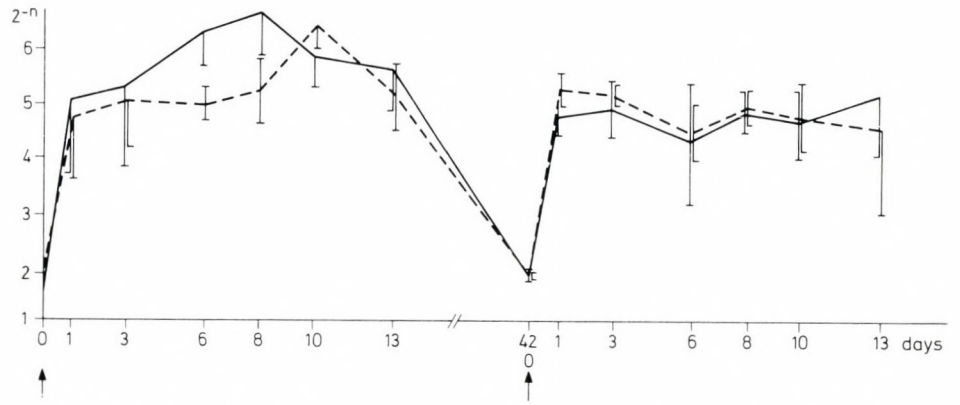

Fig. 4. Changes in anti-DS-DNA levels in 18 months old rats after two injections of 100 μg LPS or RD-LPS. The testing of each sample was carried out 4 times after the first treatment and 3 times after the second. Each group contained 4–5 animals. Arrows: LPS or RD-LPS treatment. For other details see Fig. 1

The effect of repeated LPS or RD-LPS treatment on the anti-DS-DNA titre. In 4 months old animals even the second injection of LPS or RD-LPS could not elevate the anti-DNA titre (Fig. 2a). In the 12 and 18 months old animals repeating the injection of both preparations and both doses again a significant increase of the anti-DNA level (Fig. 2b) was observed. In nearly all cases the titre was significantly higher than before the second treatment (Fig. 4). If the lower first dose was followed by a higher second one, the response was similar to the effect of a second small dose.

Discussion

Lipopolysaccharides are frequently used in the study of autoimmunity [6, 8, 15], since after injection into mice, they produce anti-erythrocyte and anti-DNA autoantibodies. In our experiments the effect of LPS and of RD-LPS was studied to reveal similarities or differences between these two preparations. It has been postulated that the effect may differ in young and old animals.

In all three age-groups we assayed DNA release in the blood. During the first day after LPS or RD-LPS treatment we did not measure the DNA liberation and thus could not demonstrate the very early changes in the DNA level after LPS treatment as Furnie et al. [7] did. From the first day in the 4 and 12 months old rats we observed only a decrease of the DNA level, but with a

Table I

Relative changes in DNA content of sera after LPS or RD-LPS treatments

Age of animals, months	Treatment, μg		Days after treatment 1	3	6
4	LPS	100	87.89 ± 11.37	80.93 ± 10.34	80.36 ± 8.36
		500	73.75 ± 14.08	68.73 ± 13.85	88.84 ± 0.26
	RD-LPS	100	92.32 ± 10.80	73.78 ± 7.81	91.59 ± 4.37
		500	89.36 ± 2.43	9.49 ± 8.13	82.87 ± 6.57
12	LPS	100	92.82 ± 14.51	78.98 ± 1.89	81.98 ± 12.83
		500	61.10 ± 2.78	68.01 ± 11.43	101.43 ± 27.59
	RD-LPS	100	74.55 ± 5.87	54.48 ± 11.34	72.13 ± 12.21
		500	62.50 ± 1.06	88.99 ± 12.47	76.24 ± 9.97
18	LPS	100	96.95 ± 5.66	94.89 ± 3.14	109.82 ± 6.59
		500	112.71 ± 29.78	90.63 ± 3.35	117.60 ± 8.75
	RD-LPS	100	81.00 ± 4.35	88.06 ± 4.35	93.98 ± 3.51

Age of animals, months	Treatment, μg		Days after treatment 8	10	13
4	LPS	100	78.49 ± 7.07	61.87 ± 10.03	94.65 ± 8.55
		500	79.03 ± 14.49	82.05 ± 8.51	90.08 ± 12.26
	RD-LPS	100	93.55 ± 5.86	80.54 ± 5.17	100.34 ± 9.63
		500	75.20 ± 11.68	92.05 ± 7.64	90.97 ± 16.05
12	LPS	100	81.96 ± 11.83	84.58 ± 15.97	87.43 ± 12.22
		500	76.66 ± 2.48	102.64 ± 10.25	89.46 ± 20.99
	RD-LPS	100	92.06 ± 17.91	87.53 ± 17.95	102.46 ± 8.39
		500	79.09 ± 17.25	65.37 ± 15.13	77.39 ± 14.71
18	LPS	100	102.76 ± 2.49	96.74 ± 4.75	99.26 ± 5.38
		500	116.04 ± 11.76	106.04 ± 3.87	123.60 ± 0.35
	RD-LPS	100	102.05 ± 7.08	78.74 ± 0.20	103.97 ± 11.47

All data represent mean ±SE of 2–4 repeated measurements in per cent of DNA content before treatment. Each determination was carried out with the pooled serum of at least 4 animals

tendency to normalization (Table I). The tendency of changes caused by LPS and RD-LPS did not differ from each other, only the magnitude of alteration was different. In 18 months old rats not only the decrease but some elevation of the DNA level was characteristic particularly after a higher dose. It is likely that the decreased level of DNA is due to antigen-antibody complexes, but in the present work DNA-anti-DNA complexes were not determined.

Before the first treatment and also 6 weeks after the first LPS dose, the anti-DNA titre was low in all investigated age groups. This result is in good agreement with the results of Niva et al. [16], who observed a transient antibody production after a common viral infection. The autoantibodies disappeared after 6 to 8 weeks. In 4 months old rats there was no increase of the anti-DNA titre. In the old animals (12 and 18 months) after treatment with both endotoxin preparations and both doses, a rapid increase of circulating autoantibodies have been observed. The rapid increase of the titres raises the question, as to whether the response is a real primary or a secondary one, or in the earliest time after the treatment the already synthesized anti-DNA only gets into the circulation after the LPS or RD-LPS stimuli. It is possible that after this mobilization a new autoantibody production is taking place. The other possibility is that after the first treatment the quickly appearing high antibody titre was a consequence of a secondary type reaction. In the 18 months old rats after the second treatment, particularly at the higher dose, the response was lower than after the first treatment. This decrease was statistically significant on the 6th day after the second LPS dose. The cause of this phenomenon is still obscure. One can speculate that it is still a late effect of endotoxin tolerance, but it is possible that the capacity of the immune system determines this lower secondary response. From these results, it is clear that what we observed was not real primary antibody response, and that the secondary response differed also from the typical secondary type response. It may also be concluded that the effect of LPS and RD-LPS did not differ markedly. Autoantibody production after the first treatment seems only transient. Further experiments at cellular level will presumably provide better understanding of the mechanism of stimulatory action of LPS and RD-LPS.

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THE EFFECTS OF CANNABINOIDS AND CANNABISPIRO COMPOUNDS ON *ESCHERICHIA COLI* ADHESION TO TISSUE CULTURE CELLS AND ON LEUKOCYTE FUNCTIONS IN VITRO

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Δ^9-Tetrahydrocannabinol, cannabidiol, cannabidiolic acid, tetrahydrocannabidiolic acid, cannabispirol, acetylcannabispirol, cannabispirone, and cannabispirenone in a low concentration did not affect the adhesion of *Escherichia coli* on cultured HEp-2 cells. Cannabinoids at 10^{-6} M increased the chemiluminescence of human polymorphonuclear leukocytes, while the cannabispiro compounds failed to enhance the oxidative burst of leukocytes. In lymphocyte and granulocyte function tests (E- and EA-rosette formation, blast transformation of T-lymphocytes in the presence of phytohaemagglutinin and concanavalin-A, ADCC and phagocytosis) all compounds displayed immunosuppressive effect at 1.5×10^{-5} M. Tetrahydrocannabidiolic acid exerted the weakest immunosuppression on human leukocyte functions.

Cannabinoids have stereospecific effects in man and animals [1, 2]. Investigations into the antibacterial activities of several cannabinoids have shown that cannabidiolic acid exerts antibacterial activity against Gram-positive bacteria [3, 4]. Tetrahydrocannabidiolic acid has a special kind of antibacterial effect on *Escherichia coli*, inhibiting the plasmid replication Various compounds such as Δ^9-tetrahydrocannabinol and cannabinol exert an inhibitory effect on intercellular plasmid transfer, but Δ^8-tetrahydrocannabinol and cannabinol have only very moderate effects [5].

The chemical structure-dependent antibacterial and plasmid curing effects of some newly discovered cannabispiro compounds have been established. These compounds inhibit mating pair formation, which is essential for

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bacterial conjugation, and some of them inhibit the penetration and synthesis of plasmid DNA during bacterial growth [6].

These compounds also influence the bacterial pili, and in this way inhibit conjugation. It was therefore considered worthwhile to study the effects on other plasmid-mediated and pili-dependent processes, e.g. the adsorption of bacteria on tissue culture cells. We were interested in the chemical structure-dependent antibacterial effects of the compounds and in their possible effects on the host defence mechanism. Their action on some immunological reactions were tested in vitro. We report here the effects of some cannabinoids and cannabispiro compounds on bacterial adhesion to HEp-2 tissue culture cells. Further, the membrane effects of the derivatives on the chemiluminescence of human granulocytes and the reactions of human leukocytes were investigated in vitro.

Materials and methods

Compounds. Δ^9-Tetrahydrocannabinol, cannabinol and cannabidiolic acid were kindly provided by Keizo Watanabe, United Nations Laboratories, Narcotics Laboratory Section, Vienna, Austria. Cannabispirol, acetyl-cannabispirol, cannabispirone and cannabispirenone were isolated by Y. Shoyama and I. Nishioka in 1978. Tetrahydrocannabidiolic acid was produced by Alkaloida, Tiszavasvári, Hungary. The chemical structures of the compounds are shown in Fig. 1.

Bacterial strain. Escherichia coli K12/R144 drd-3 was kindly provided by I. B. Holland, Department of Genetics, University of Leicester, UK. For the cultivation of bacteria, tryptone-yeast extract (MTY) broth and agar plates were prepared according to Alföldi et al. [7].

Determination of bacterial adhesion to HEp-2 monolayers. HEp-2 monolayers were prepared in 2 cm^2 multiwell dishes (Costar tissue culture cluster, USA) using 5.0×10^5 dissociated cells as inoculum from a confluent culture. Confluent cell layers resulting after 24 h of growth were washed three times with HEPES–Hanks solution, and 0.5 ml HEPES–Hanks solution containing cannabinoids or cannabispiro compounds at twice the desired final concentration was added to each well. Next, 0.5 ml *E. coli* K12 R144 or other *E. coli* suspension, grown in Brain Heart Infusion broth and washed twice with HEPES–Hanks solution, was added to each well. The concentration of the cultures was adjusted to OD 0.2 at 620 nm, which corresponded to 2.2 to 2.8×10^8 bacteria/ml. After 20 min incubation at 37 °C, the unbound bacteria were removed by washing three times with 1.0 ml HEPES–Hanks solution. To determine the bound bacteria, the monolayer was dissociated into single cells by the addition of 1.0 ml trypsin solution; the latter contains 0.25% trypsin (Difco) and 0.05 mM EDTA in HEPES–Hanks solution (pH 8.2). After 20 min incubation at 37 °C, serial dilution were prepared and 0.1 ml aliquots of the dilutions were plated on MTY agar plates for counting the number of colony formers [8].

Preparation of polymorphonuclear granulocytes. Twenty ml freshly drawn heparinized blood (10 U/ml) from healthy human donors were diluted with 6.0 ml of 6% dextran solution. The blood was allowed to sediment in a test tube at 37 °C for 60 min in a carbon dioxide incubator. After 60 min, the upper layer was collected and put into a centrifuge tube, to which the same volume of RPMI was added. After mixing, the PNG sample was centrifuged at 1500 rpm for 10 min in an MSE minor centrifuge. The sediment was resuspended in 2.0 ml RPMI medium containing 10% fetal calf serum. The cells were diluted ten-fold, then tested for viability with trypan blue solution (5.0 mg/ml) and diluted to give a final cell concentration of 2.5×10^6/ml by the addition of RPMI. The cell suspension was stored at 4 °C in a refrigerator until use.

Chemiluminescence assay. Two hundred μl of the above-mentioned cell suspension (2.5×10^5 PNG) were incubated with 100 μl of a definite concentration of psychopharmacon for 2 h at 4 °C. Preliminary investigations showed that preincubation resulted in less variations and better reproducibility. Thereafter, the incubation mixture was warmed to 37 °C and 10 μl

TPA (12-*O*-tetradecanoyl-phorbol-13-acetate, 100 μg/ml TPA in RPMI) were added to each sample to elicit the respiratory burts of PNG. One hundred μl of the drug, and then the pretreated and TPA-induced cells, were pipetted into glass vials which contained a final volume of 4.0 ml PBS, including buffer and luminol (10^{-6} M). The vials were mixed and placed in the scintillation counter, which was then closed. Repetitive counts were recorded every 5 min. The chemiluminescence was registered for 120 min in a computerized Packard Tricarb. The initial zero time was spurious as a result of the luminescence of the vials, but later on the measurements were entirely reproducible since the resting values without stimulus extrapolate to the zero time value [9].

Data presentation. The integral was calculated for the registered impulse rates over the whole period and compared for drug-incubated versus nontreated cells. This quotient, the CL index, was multiplied by 100 and was given in relation to the concentration of the psychopharmacon [9].

Erythrocyte rosette formation assay. Mononuclear cells were separated on a Ficoll–Uromiro gradient and treated with the drugs, then mixed with 30 parts of sheep red blood cells and incubated at 37 °C for 5 min. The samples were centrifuged and incubated at 4 °C for 14 h, when the number of rosette-forming cells was determined by the method of Aiuti et al. [10]. The mononuclear cells were treated with the tested compound at a concentration of 1.5×10^{-5} M. The results are expressed as percentages of rosette-forming T-cells.

EA rosette estimation of the number of Fc-bearing lymphocytes. In this assay the number of red blood cells covered with antibody was determined. Mononuclear cells separated on Ficoll–Uromiro gradient and treated with the tested drug at 1.5×10^{5} M were mixed with 0 Rh^{+} human anti-D treated red blood cells and incubated at 37 °C for 5 min. Samples were centrifuged and incubated for 1 h at 4 °C. The number of rosette-forming cells was expressed in percentage [11].

Blast transformation of T-lymphocytes in relation to the capacity of cellular immune response. Mononuclear cells were separated on Ficoll–Uromiro gradient. Then 2×10^{5} cells were treated with the cannabinoids at 1.5×10^{-5} M and incubated in the presence of 10 μg/ml PHA and 5 μg/ml Con A for 72 h in RPMI medium containing 10% fetal calf serum. Cells were cultivated in Greinier microtiter plates. The transformation rate was estimated after the addition of 0.5 μCi ^{3}H-thymidine to the samples and incubation for 5 h. The controls did not contain the mitogens. Results were expressed in cpm as the incorporated radioactivity by transformed cells minus radioactivity of control cells.

ADCC (antibody-dependent cytotoxicity) assay. 0 Rh^{+} human red blood cells were used as target, and a moncyte-free mononuclear cell suspension was applied which was treated with 1.5×10^{-5} M of drug as effector in 1:10 ratio. The reaction was mediated by red blood cell specific anti-D antibody. The assay was carried out in Greiner microtiter plates, where the controls did not contain antibody. The results were expressed in terms of the amount of ^{51}Cr released during the lysis of the target according to Urbaniak [12]; the test culture result minus control/total activity $\times 100$ expressed the cytotoxicity as a percentage.

Assay for phagocytotic activity of neutrophilic granulocytes. The method of Klebanoff and Clark [13] was applied. The blood sample was allowed to sediment in dextran, then suspended in Ca^{++} and Mg^{++}-free Hanks medium and layered onto Ficoll–Uromiro Gradient. After centrifugation, the red blood cells were removed by lysis. The granulocytes obtained were activated with ^{125}I-labelled zymosan in the presence of the tested drug at 1.5×10^{-5} M concentration. Labelling of zymosan particles with ^{125}I was performed at 37 °C for 30 min together with the opsonisation with fresh mixed human serum. After 60 min incubation with granulocytes the samples were precipitated with TCA then the radioactivity of samples was measured. Basic activity was determined in the presence of ^{125}I without adding zymosan to granulocytes. The phagocytosis index was calculated as the quotient of zymosan-activated sample cpm and basic activity cpm. All immunological assays were performed in three independent experiments with cells from three different healthy persons.

Results

Cannabinoids and cannabispiro compounds in high concentration only were able to inhibit the adhesion of *E. coli* to HEp-2 monolayer cells. The effect was slight, but it was dependent on the drug concentration. As Table I

shows, tetrahydrocannabidiolic acid, which has a plasmid-curing activity, did not differ considerably in effect from compounds which do not influence bacterial plasmid replication.

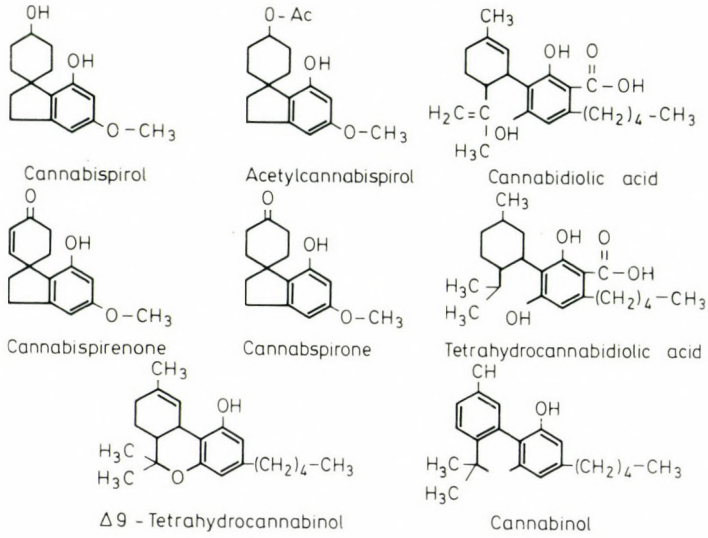

Fig. 1. Cannabinoids and cannabispiro compounds

Clearly, both cannabinoids and cannabispiro compounds exert a certain effect on the surface properties of bacteria (e.g. bacterial fimbriae) and/or tissue culture cells, which leads to the partial inhibition of bacterial adhesion.

The membrane alteration of tissue culture cells in model experiments was followed by measuring the phorbol ester-induced chemiluminescence of human neutrophilic granulocytes in the presence of the cannabis derivatives.

The cannabis derivatives increased the chemiluminescence in pretreated cells at as low as 1.5×10^{-6} M, while in a higher concentration all of the derivatives exerted an inhibitory effect on the chemiluminescence, perhaps some of them acting as a free radical scavanger. It was interesting that cannabispiro compounds had inhibitory effects on the chemiluminescence of leukocytes, which were more expressed than those of cannabis compounds.

As Table II shows, cannabispiro compounds exhibit an immunosupressive effect, while the majority of the cannabis compounds tested have a biphasic effect: at low concentration they enhance, whereas at high concentration inhibit chemiluminescence.

The immunomodulatory effects of the cannabis and cannabispiro compounds were studied in experiments on the activity of T and B-lymphocytes

Table I

Adhesion of E. coli to HEp-2 monolayers in the presence of cannabinoids and cannabispirol compounds

Compounds	Concentration, M	Viable bacteria, bound %
$^{2}\Delta^{9}$-Tetrahydrocannabinol	1.5×10^{-6}	1.40
	1.5×10^{-5}	0.90
Cannabinol	1.5×10^{-6}	1.20
	1.5×10^{-5}	0.80
Cannabidiol	1.5×10^{-6}	1.62
	1.5×10^{-5}	1.10
Cannabidiolic acid	1.5×10^{-6}	1.45
	1.5×10^{-5}	1.04
Tetrahydrocannabidiolic acid	1.5×10^{-6}	1.30
	1.5×10^{-5}	1.12
Cannabispirol	1.5×10^{-6}	1.26
	1.5×10^{-5}	0.80
Acetylcannabispirol	1.5×10^{-6}	1.20
	1.5×10^{-5}	0.80
Cannabispirone	1.5×10^{-6}	1.32
	1.5×10^{-5}	0.67
Cannabispirenone	1.5×10^{-6}	1.28
	1.5×10^{-5}	0.69
Control	—	1.28

Table II

Modulation of chemiluminescence reaction of TPA-induced polymorphonuclear granulocytes in the presence of cannabinoids and cannabispiro compounds

Compounds	Chemiluminescence as per centage of the control			
	1.5×10^{-7}	1.5×10^{-6}	1.5×10^{-5}	7.5×10^{-5} M
Δ^{9}-Tetrahydrocannabinol	100	112	96	91
Cannabinol	100	140	82	60
Cannabidiol	100	190	126	25
Cannabidiolic acid	100	341	165	56
Tetrahydrocannabidiolic acid	100	168	118	78
Cannabispirol	100	110	152	150
Acetylcannabispirol	100	86	45	90
Cannabispirone	100	96	103	17
Cannabispirenone	120	34	18	4

Table III

Effects of cannabinoids and cannabispiro compounds on the cellular immune response of human leukocytes in vitro

Compounds	Rosette formation, %		Mitogen-induced transformation of lymphocytes (cpm)		ADCC activity, %	Phagocytic index
	E	EA	PHA	ConA		
Δ^9-THC	46	18	17.853	10.611	25	110.4
Cannabinol	42	16	18.956	15.638	35	143.4
Cannabidiolic acid	28	12	2.724	18.707	35	31.7
Tetrahydrocannabidiolic acid	20	36	25.327	22.020	29	70.1
Cannabispirol	36	32	11.790	12.040	26	58.0
Acetylcannabispirol	20	18	14.193	13.820	32	65.9
Cannabispirenone	22	30	24.955	28.702	32	45.5
Cannabispirone	20	32	24.146	32.003	35	75.4
Control	55	44	22.790	35.256	32	48.4

The compounds were applied in a concentration of 1.5×10^{-5} M; they were toxic at 1.5×10^{-4} M in trypan blue assays

and phagocytic functions. As the results in Table III show, all of the tested derivatives inhibited several granulocyte and lymphocyte functions to various extents e.g. E and EA rosette formation, phytohaemagglutinin and concanavalin A-induced transformation of T-lymphocytes, ADCC and phagocyte activities. On the basis of the index it seems that the phagocytosis activity is increasing in the cases of Δ^9-THC and cannabinol, but the cannabidiolic acid inhibits this function.

To summarize the results relating to the cannabinoids, tetrahydrocannabidiolic acid inhibited *E. coli* adhesion to HEp-2 cells, enhanced the chemiluminescence of leukocytes and had the weakest inhibitory effect on leukocyte functions. In the group of cannabispiro compounds cannabispirone inhibited the adhesion of *E. coli* to tissue culture cells more effectively than acetylcannabispirol and cannabispirenone did. In addition, cannabispirone had the weakest inhibitory effect on the phorbol ester-induced chemiluminescence and cellular immune response of human leukocytes.

Discussion

Generally a correlation exists between inhibition of chemiluminescence and phagocytic activity of leukocytes by the compounds applied in a relatively high concentration.

The findings presented here suggest that the introduction of different substituents might modify the dose-dependent antibacterial and the immuno-

suppressive effects of the cannabinoids and cannabispiro compounds. The question arises as to whether the compounds effective against bacteria do or do not influence some factors of the natural host resistance to various kinds of infection. Previous experiments showed that one of the cannabinoids, tetrahydrocannabidiolic acid, and one of the cannabispiro compounds had plasmid curing effects [5, 6]. Further, all of the compounds tested inhibited the adhesion of *E. coli* to the tissue culture cells possibly through an interaction with bacterial fimbriae [6]. The two groups of drugs, with the exception of tetrahydrocannabidiolic acid and cannabispirenone exerted a marked inhibitory effect on the function of human leukocytes. The inhibition of natural host resistance to infections by cannabinoids is interesting and important.

It can explain, for instance, the higher incidence of an immunodeficient state among drug abusers than in other members of the population. Though cannabinoids have a rather low plasma concentration in man [14], theoretically they can result in the immunosuppressive effect of cannabinoids and cannabispiro compounds.

Various effects of cannabinoids are both in vivo and in vitro metabolic processes [15–17].

The different pharmacological potencies of cannabinoids may possibly be due to their different activities on the central nervous system, in consequence of their different affinities to the hypothetical receptor [18]. It has been reported that Δ^9-THC is distributed almost equally between triglyceride and phospholipid phases, but the amide exhibits a strong preference for phospholipids, which can be interpreted as high relative membrane affinity. It has been shown that some derivatives can affect the immunocompetent cells directly [19]. Besides the cannabinoids, cannabispiro compounds have recently been isolated from *Cannabis sativa* by Shoyama and Nishioka [20]. These might have either the same or different action from cannabinoids on bacteria and immunocompetent cells. Moreover, different cannabinoids may have similar metabolites, which can act similarly on the biochemical processes on the cells [21]. From a practical point of view it is important that in the USA, herpes infections reached a nearly epidemic incidence [22] when marijuana was in extensive use. The major psychoactive component of this drug, Δ^9-THC, has been shown to be immunosuppressive [23]. It decreases the host resistance to a number of infectious agents, diminishing both humoral and cellular responses in a variety of animals and man as well [24–26].

Studies conducted in this laboratory have also demonstrated that, through their action on the various functions of T and B lymphocytes, cannabinoids and cannabispiro compounds possibly can decrease host resistance in vitro. This decreased resistance can lead to an increased sensitivity to infections and possibly to the spread of malignant diseases.

On the basis of these experiments the immunosuppressive effects of the drugs tested cannot be predicted for individuals exposed to marijuana, since dosages and route of administration are not representative of human exposure. In addition the drugs may interact with other systems in vivo. Further experiments need to give definitive answers whether or not the cannabinoids and spiro compounds increase the susceptibility of human drug abusers to various infectious diseases or which isomers of tetrahydrocannabidiolic acid have immunomodulating effect.

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ELIMINATION OF NON-SPECIFIC NUCLEASES FROM RESTRICTION ENDONUCLEASE PREPARATIONS BY DIFFERENT BINDING ON FREE DNA LIGAND

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In the purification of a novel restriction endonuclease (an AvaIII isoschizomer, isolated in this laboratory) standard methods were insufficient to eliminate non-specific nuclease contaminations. Taking advantage of the specific site recognition and binding of the restriction endonuclease on DNAs, a method is described for the simple extraction of non-specific nucleases. DNA substrates without recognizable sites do not bind the restriction endonuclease, while non-specific nucleases are absorbed to, and eliminated with, the DNA via gel filtration chromatography under special conditions.

A novel restriction endonuclease has recently been isolated in this laboratory (patent pending). In order to analyse the basic properties of the enzyme a high quality purification protocol had first to be established. Standard chromatographic methods failed to eliminate the trace amounts of non-specific contaminating nucleases. Experiments with carboxymethyl-cellulose, phosphocellulose, DEAE-cellulose, heparin-agarose, Blue-Sepharose, phenyl-Sepharose, hydroxyl-apatite and gel filtration media in different combinations were equally frustrating.

Finally, a method based on differential affinity binding of nucleases on DNAs was successfully applied. Chromatographies on single-stranded or double-stranded DNAs bound to different solid supports are widely exploited as final steps in restriction endonuclease purification protocols [1–5], but these chromatography media must be prepared prior to use in the laboratory, or they are expensive if commercially available.

In our approach the ligand DNA is not insolubilized to any solid supports thus avoiding expenses and complications of the laboratory-made preparations. The DNA is free, and since there is no recognizable nucleotide sequence on this DNA for the site-specific restriction endonuclease (the point of the theory), the enzyme remains unbound in the solution. Non-specific nucleases, however, bind to the ligand DNA in the presence of EDTA in a low ionic strength buffer. In this way the otherwise unremovable non-specific

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nucleases share the diffusion and mobility characteristics of the ligand DNA, and they can be eliminated without any further complications in the exclusion volume of a usual gel filtration chromatography, together with the carrier DNA.

Materials and methods

Chemicals. Most of the chemicals used were of analytical grade (Renal, Hungary). Sepharose 6B (Pharmacia) and Whatman P11 phosphocellulose were used. Ethidium bromide and agarose (Type I, low EEO) were purchased from Sigma.

Bacterial strain and cultivation. The bacterial cells were cultivated in two litres of Bacto Brain Heart Infusion (Difco) medium, and were harvested in the late logarithmic growth phase determined spectrophotometrically at 550 nm. Cultivation at 37 °C lasted for about 10 h without shaking. The cells were washed in 10 mM TrisHCl pH 8, 1 mM EDTA, and stored at −20 °C.

Preparation of the crude extract. The cells were suspended in two volumes of extraction buffer: 20 mM TrisHCl pH 8, 7 mM 2-mercaptoethanol, 5 mM EDTA, 200 μg/ml RNase (bovine pancreas, Reanal, Hungary). An MSE ultrasonic desintegrator was used (maximal output, 5 min at 0 °C) and then 0.2% Triton X100 was added. The cell debris was sedimented by ultracentrifugation (Janetzky VAC601, 30 000 rpm, 1 h, +4 °C). The supernatant was further purified by the addition of activated phosphocellulose powder in a batch procedure for 20 min at +4 °C. The supernatant, obtained after pelletting the phosphocellulose by centrifugation (Janetzky K 23, 4000 rpm, 5 min, +4 °C) was immediately applied to gel filtration chromatography.

Gel filtration chromatography. Sepharose 6B chromatography was carried out in each case in a column (1.5 $cm^2 \times 80$ cm) prewashed with the elution buffer containing 20 mM TrisHCl pH 8, 7 mM 2-mercaptoethanol, 5 mM EDTA, with or without 1 M NaCl (see later). One or two ml fractions were collected and directly tested for DNA and nuclease content.

Substrate DNAs. In tests of nuclease activity the substrate was lambda phage DNA purchased from Reanal. PM2 DNA was a generous gift from Dr. P. Medveczky.

Restriction endonuclease digestion. Digestions were carried out in the medium ionic strength buffer suggested by Maniatis et al. [6] in 20 μl at 37 °C for 1 h. The reaction was stopped by the addition of 2 μl of the electrophoresis marker solution (100 mM EDTA, 20% succrose, 0.1% bromphenol blue) and the digest was analysed by agarose gel electrophoresis.

Agarose gel electrophoresis. A horizontal electrophoresis system was used with submerged gel, the DNA fragments were run in 1% agarose. The buffer and voltage were the same as in [7]. The DNA fragments were visualized by ethidium bromide (0.5 μg/ml) staining and photographed under UV illumination.

Results and discussion

Extrinsic, heterologous DNA. In the first experiments, applying the principle described, the nuclease-contaminated end-product of the usual purification procedures was the object of our attempts to get rid of a non-specific nucleases. PM2 DNA was resistant to the cleavage of the novel restriction endonuclease in previous tests demonstrating the absence of the recognition sequence, and accordingly, no binding of the site-specific enzyme could be assumed. PM2 DNA (500 μg) was added to 2 ml of the partially purified preparation in the presence of 5 mM EDTA, and prior to gel filtration the reaction was allowed to stand at 0 °C for half an hour. The result of the Sepharose 6B chromatography is shown in Fig. 1, panel A: PM2 DNA eluted in the exclusion

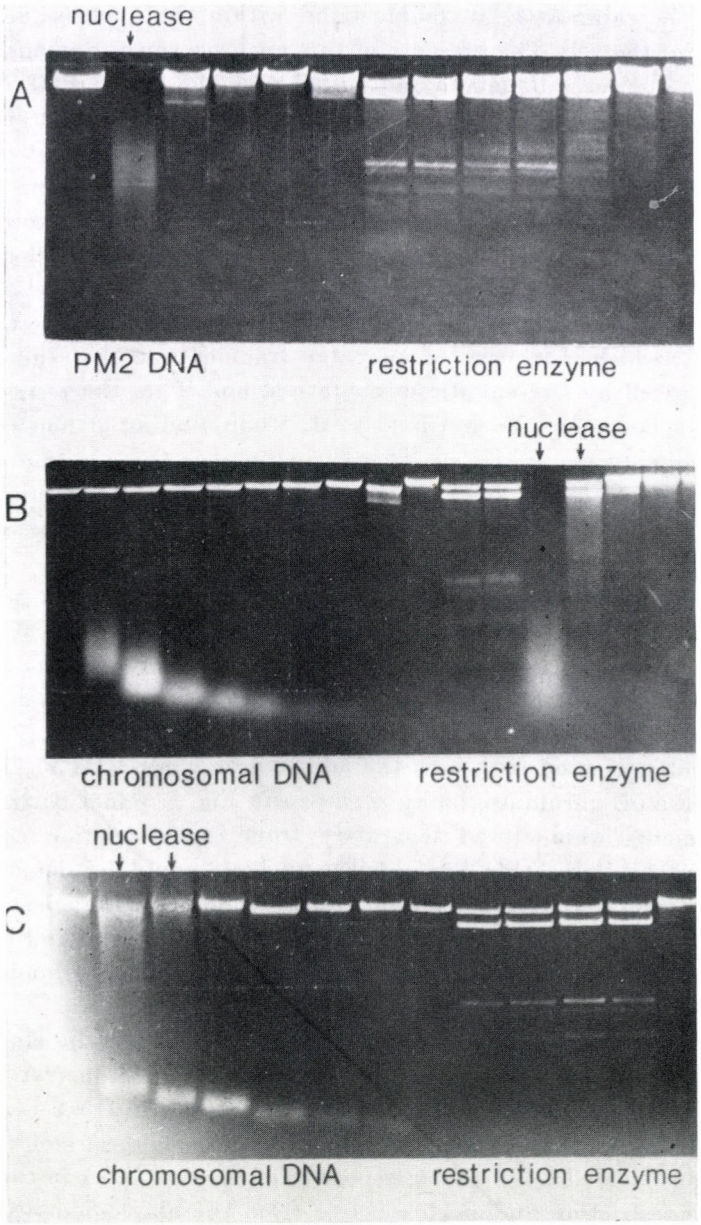

Fig. 1. Panel A. The activity test of fractions from Sepharose 6B chromatography of the nuclease contaminated, and PM2 treated restriction enzyme preparation is demonstrated by agarose gel electrophoresis. Each fraction (2 μl) was tested in 20 μl test buffer with 1 μg lambda DNA for 1 h at 37 °C and applied onto the agarose gel from left to right according to elution. *Panel B.* The result of activity test of fractions from Sepharose 6B chromatography of the crude extract containing 1 M NaCl and 5 mM EDTA. Conditions and arrangement of test aliquots on the gel are as indicated for panel A. *Panel C.* Activity test of fractions from Sepharose 6B chromatography of the crude extract containing 5 mM EDTA without sodium chloride. Assay conditions and arrangement of test aliquots on the gel are as indicated for panel A

volume of the column (faint double band within the nuclease smear in the second slot of the gel). The presence of this nuclease smear demonstrates, that non-specific nucleases bound to and eluted with the ligand PM2 DNA, separately from the restriction endonuclease activity shown in test slots of later fractions.

Intrinsic, chromosomal DNA. Subsequent experiments were based on a somewhat different rationale. In most cases bacterial chromosomal DNA is protected against the cleavage of its own restriction endonucleases by the modification of the recognition sequences via site-specific methylase mediated methylation. It was reasonable to suppose that also in this case the chromosomal DNA, which is in form of sonicated fragments in the crude extract, is modified as well by site-specific methylation, and if so, the restriction endonuclease can not recognize and bind to it, while binding of non-specific nucleases is probably not inhibited. This theory makes the addition of extrinsic ligand DNA superfluous, since some modifications in the conditions of crude extract and gel filtration chromatography could render the chromosomal DNA an optimal affinity chromatography ligand.

According to generally accepted methodology [2, 8–10], gel filtration chromatographies are carried out mostly in high ionic strength buffers to diminish non-specific interactions and aggregations of proteins and nucleic acids. In testing optimal conditions for nuclease — chromosomal DNA binding the conventional buffer (1 M NaCl, 20 mM TrisHCl pH 7.5, 7 mM 2-mercaptoethanol) was used first with the addition of 5 mM EDTA. The result of the Sepharose 6B chromatography is shown in Fig. 1, panel B: the sonicated DNA fragments were eluted separately from the restriction endonuclease activity, but no detectable non-specific nuclease could be demonstrated in association with the DNA. Instead, the smear of non-specific nucleases appeared in the elution range of the restriction endonuclease, in slots 13 and 14. Thus the usual high ionic strength buffer proved to be inhibitory for binding of non-specific nucleases to the chromosomal DNA.

In the next step sodium chloride was omitted from the elution buffer, whereas 5 mM EDTA was invariably present. As it is demonstrated in Fig. 1, panel C, the low ionic strength buffer did not interfere with DNA — non-specific nuclease binding, since these nucleases eluted with the highest molecular weight fractions of sonicated DNA (nuclease smear of the test DNA in the slots indicated). The restriction endonuclease, free from the above-demonstrated contaminating nucleases eluted in subsequent fractions. With two additional purification steps (hydroxylapatite and DEAE-cellulose chromatographies) a nuclease-free restriction endonuclease preparation could be produced from these fractions (published in detail elsewhere).

The method described is of upmost simplicity, because instead of sophisticated chromatography media, devices or chemical compounds, just some slight

and reasonable modifications in well established methods are necessary. In the system described the method works satisfactorily, but the principle possesses certain inherent limitations. Predictably not all of the contaminating nucleases in different restriction endonuclease preparations belong to the sort that binds to the substrate DNA upon inhibition of its catalytic activity by EDTA. Secondly, the overall ionic charge of the restriction endonuclease molecule must also be taken into consideration, because the principle described would work only in case of enzyme molecules with overwhelming negative charges, since a positively charged restriction endonuclease would also bind to the DNA in low ionic strength buffers via non-specific electrostatic forces. Thus the applicability of the method needs preliminary investigations in individual cases.

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DISTRIBUTION PATTERN OF ADENOVIRUS HEXON EPITOPES IN INFECTED CELLS DETERMINED WITH MONOCLONAL ANTIBODIES BY IMMUNOFLUORESCENCE ANALYSIS

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Two monoclonal antibodies (MAbs) specific for two distinct epitopes on the human adenovirus type 1 (AV1) hexon were used to determine the subcellular localization of hexon epitopes in the infected HEp-2 cells by indirect immunofluorescence. On the basis of cross-reactivity pattern of MAbs, presumably one of the epitopes is genus specific and the other should be intertype specific. The epitopes, i.e. the adenovirus hexons could be detected throughout the cell and could display different accumulation forms. Fluorescence appeared either in the cytoplasm only or both in the nucleus and the cytoplasm. In the cytoplasm the hexons could be found in diffuse or perinuclear distribution or accumulated into discrete spots. In the nucleus they formed granules or clusters or were diffusely distributed causing a bright fluorescence of the whole nucleus. The different accumulation forms appeared at the same time in different cells of a culture, but in one given cell the fluorescence always appeared first in the cytoplasm.

Monoclonal antibodies are widely applied in research laboratories and in clinical diagnostic medicine. There are research and diagnostic procedures in which immunofluorescence is still the most sensitive and reliable method [1, 2], providing a wide variety of quantitative immunological, histochemical and routine clinical rapid assays for bacterial, viral, and parasitic infections [3]. The involvement of MAbs in the immunofluorescence technique provides further benefits [4–7]. For instance, studies on the subcellular localization of a viral protein by immunofluorescence requires the availability of antibodies recognizing this unique protein [8–12].

The main structural adenovirus protein is the hexon capsomer, which consists of three identical polypeptide subunits. The hexon proteins are syn-

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thesized in the cytoplasm of the infected cell and are rapidly transported into the nucleus probably in trimetic form [13]. In our previous experiments a panel of MAbs directed against AV1 hexon was tested with purified hexon proteins of different adenovirus types. It was demonstrated that besides the genus specific epitope(s) there are a number of different intertype specific epitopes on the surface of the hexon [14]. In this paper we describe the localization, and the different accumulation forms of the hexon in the infected cells as detected by immunofluorescence with MAbs directed against two different epitopes.

Materials and methods

Production of hybridomas. Balb/c mice were immunized with crystallized AV1 hexon. The immunizing antigen was prepared as described previously [15]. The animals were injected twice with Al-precipitated antigen three weeks apart, and three days prior to the fusion, the spleen cell donors received an intravenous injection of antigen in PBS. Description of the cell lines, the fusion protocoll, and cultivation of fused cells could be found in details elsewhere [16]. The supernates of the individual cultures were tested by indirect ELISA (Enzyme Linked Immunosorbent Assay) for specific antibodies. The positive clones were injected intraperitoneally into mice and the developed ascitic fluid was sucked off from the abdomen, centrifuged, and stored at −20 °C.

Indirect ELISA. Wells of 96-well flat-bottomed polystirene plates (Falcon Micro Test T. C., or Novogen) were coated with purified hexon preparation of the homologous AV1 hexon as well as of ten different human heterologous hexon types and with two hexon types of animal origin for determining the production of AV1 hexon specific MAbs and for characterizing them by their cross-reactivity patterns. The bound MAbs were detected by horse radish peroxidase (HRPO) conjugated rabbit anti-mouse IgG (Human Institute for Serobacterial Production and Research, Hungary) using freshly prepared o-phenylene diamine (OPD) as substrate. The absorbance was determined by a Titertek Multiskan spectrophotometer equipped with a 492 nm filter.

Passive haemagglutination (HA) was carried out as described earlier [17].

Indirect immunofluorescence assay (IFA). HEp-2 cells were grown in Roux flasks and were infected with AV1 at a multiplicity of 10^4 CPU. After incubation at 37 °C for 18 h the infected cells were washed with Hanks' BSS. Cells were scratched off the glass wall, and suspended in 2 ml of Hanks' BSS. For IFA 0.1 ml aliquots of cell suspensions were airdryed on cover slips and fixed with acetone-methanol mixture (1:1) for 10 min. For the detection of bound MAbs anti-mouse IgG conjugated with fluorescein isothiocyanate (Human Institute for Serobacterial Production and Research, Hungary) was used. The results were evaluated by a Zeiss-Fluoval microscope at a magnification of 630.

Results

Characterization of MAbs. For the present studies two MAbs (1A3 and H12) were selected on the basis of their similar broad cross-reacting ability with the purified hexons of ten different human adenovirus types belonging to four subgenera, as well as on the basis of different reactivity with adenovirus hexons of simian and bovine origin. The cross-reactivity patterns were tested both in indirect ELISA and passive HA. Results are shown in Table I. In addition, these MAbs were tested against different hexon types for precipitin line formation in agar gel containing 5% PEG 4000 [18]. Both MAbs formed a

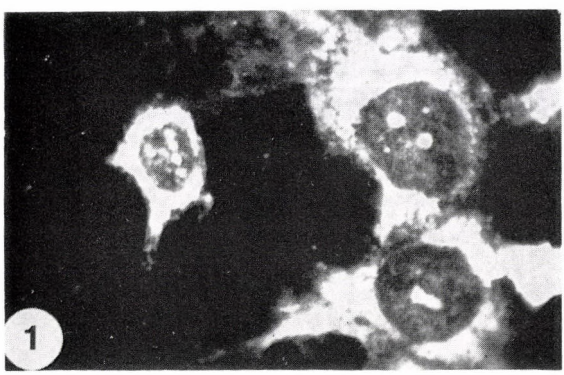
single precipitin line with AV1 hexon, as well as with SA7 (SAV 16) hexon. MAb 1A3 formed a line with bovine AV type 3 (BAV3), too, while MAb H12 did not. Furthermore, MAb 1A3 formed a line of double partial identity (double spur) with MAb H12 when they were placed in two juxtaposed wells. This phenomenon indicates that these MAbs identified two sterically distinct epito-

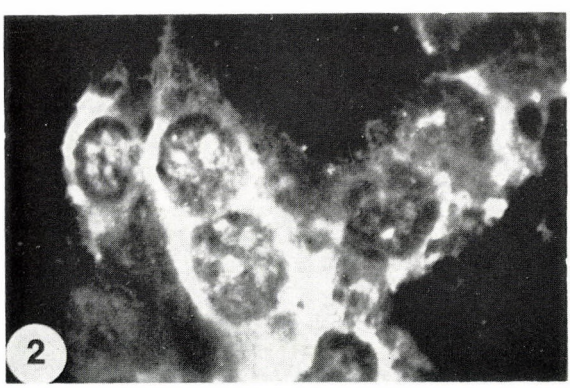
Table I

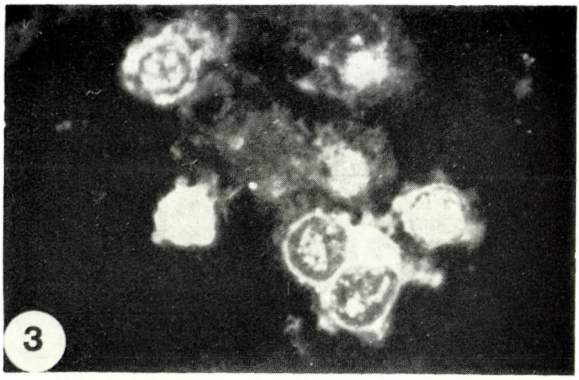
Cross-reactivity pattern of MAbs 1A3 and H12

MAbs		Human adenovirus types according to subgenera											Simian adenoviruses	Bovine adenoviruses
		A	B		C				D					
		12	7	35	1	2	5	6	8	9	10	13	SAV16	BAV3
1A3	E	+	+	+	+	+	+	+	+	+	+	+	+	+
	H	+	+	+	+	+	+	+	+	+	+	+	+	n.e.
H12	E	+	+	+	+	+	+	+	+	+	+	+	+	−
	H	+	+	+	+	+	+	+	+	+	+	+	+	n.e.

E = ELISA, H = passive hemagglutination, n.e. = not evaluable



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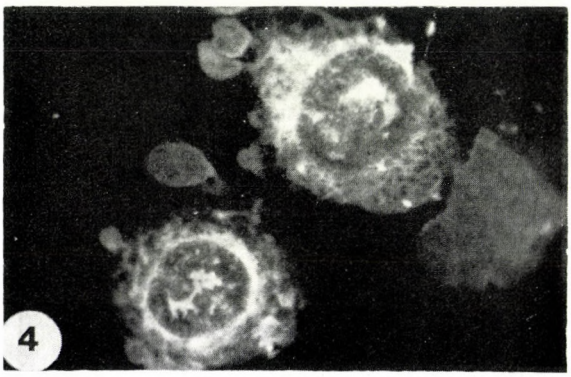
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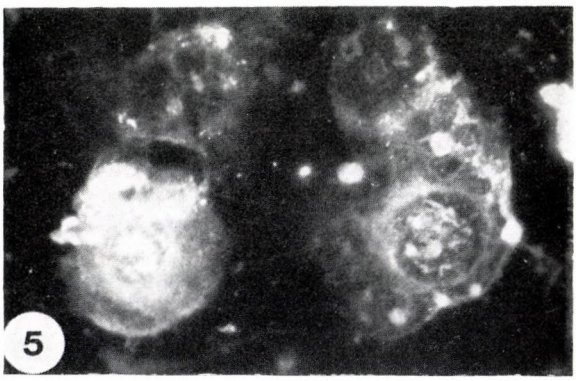
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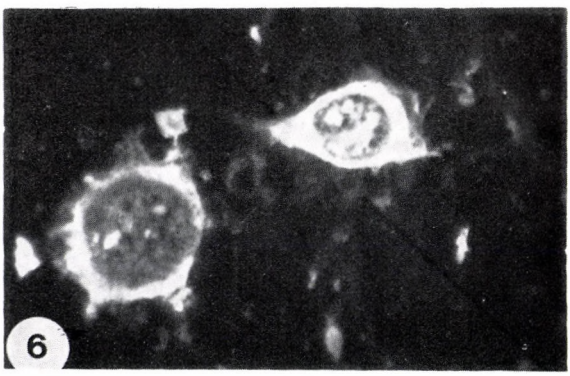
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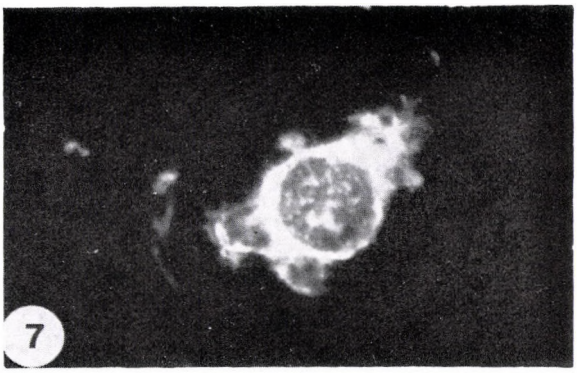
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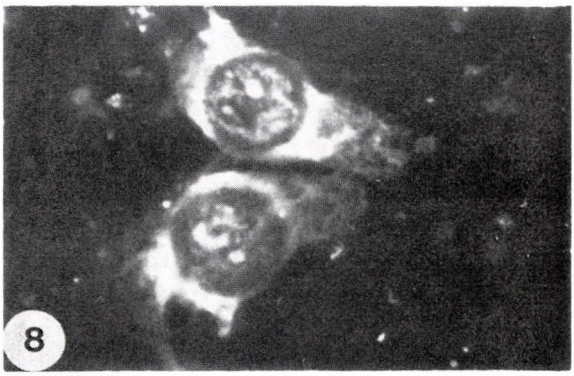
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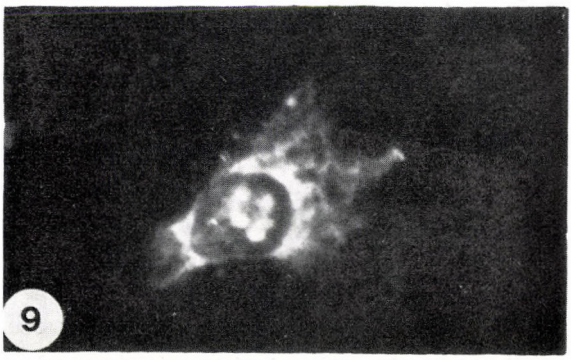
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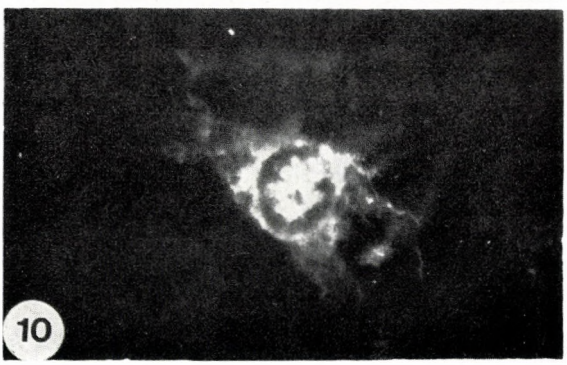
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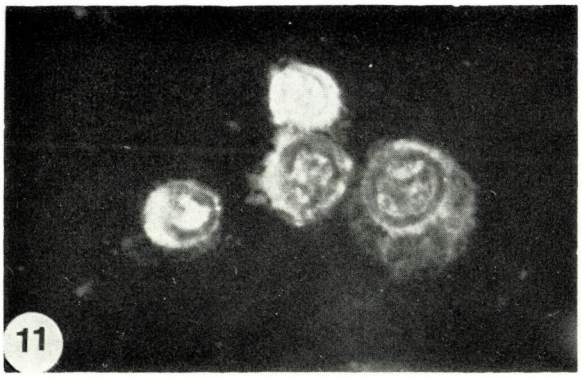

Figs 1–11. Different accumulation forms of hexon in the AV1 infected HEp-2 cells 18 h postinoculation detected by immunofluorescence with MAb 1A3 (Figs 1, 3, 4, 5, 6, 7, 10, 11) and with MAb H12 (Figs 2, 8, 9). See text for details

pes, in spite of their similar cross-reactivity patterns with human heterologous hexon types studied. The dissimilarity of the two MAbs were also demonstrated by the correlation coefficient calculated from their reactivity values and in competitive binding ELISA experiments. The details of these experiments were published elsewhere [19, 20].

Localization of adenovirus hexon epitopes in the infected cells. The fluorescence pattern shown by the MAbs in indirect IFA on HEp-2 cells at the same stage of infection i.e. 18 h postinoculation, displayed a wide diversity, indicating that the hexon antigens appeared throughout the cell. They could be found both in the cytoplasm and in the nucleus showing different forms of accumulation. The most characteristic forms of hexon accumulation revealed by the distribution of fluorescence were the following. One of the most frequent finding was the localization of the hexon antigens in the cytoplasm in large patches near the nucleus, the fluorescence filling up the larger part of the cytoplasm, while the nucleus seemed to be intact (Figs 1 and 2). Another form of cytoplasmic appearence was the thin confluent perinuclear accumulation (Fig. 3). The hexon epitopes could also be found in the cytoplasm diffusely distributed with a netlike structure (Fig. 4). There were also brightly fluorescent spots and patches of varying size in the cytoplasm (Fig. 5). The whole cytoplasm could sometimes be strongly fluorescent (Fig. 6). Fine granulate (Fig. 7) or cluster-like fluorescence (Fig. 8) or separated larger bright spots in the nucleus (Fig. 9) occurred besides the different forms of cytoplasmic fluorescence. Incidentally the whole central part of the nucleus was brightly fluorescent (Fig. 10). In other cases the cells became rounded and only the remnants of the cytoplasm could be seen, while the whole nucleus was brightly fluorescent (Fig. 11).

The different distribution forms of hexon epitopes could be seen at the same time in the different cells of the culture (Figs 1, 3, 5, 6, 11) and the intensity of the fluorescence varied with the infected cells.

The hexon epitopes could be detected in the cytoplasm without appearing in the nucleus, but if present in the nucleus, they were always revealed in the cytoplasm as well.

No difference was observed between the fluorescence patterns of the two MAbs recognizing different hexon epitopes.

Discussion

With the help of two MAbs recognizing two different hexon epitopes, it was proved by indirect IFA that the recognized epitopes could be detected all over the infected cells and could display different accumulation forms. The epitope recognized by MAb 1A3 may be assumed to be the genus specific epitope because of the wide cross-reactivity spectrum with human and animal adenovirus hexon preparations. On the basis of the cross-reactivity pattern and competition results of MAb H12 the other epitope could be intertype specific [19, 20]. Considering that multiple copies of these epitopes occur on one complete hexon [21] and the MAbs can only react with complete hexons [22], the localization of a given epitope might mean the localization of the complete hexon in the infected cell. Accordingly, the adenovirus hexons can be found in the cytoplasm of the infected cell in diffuse or perinuclear distribution or they can be accumulated into spots. In the nucleus they formed granules or clusters or they can be diffusely distributed (the whole nucleus is brightly fluorescent). This phenomenon may be of some use from a diagnostic viewpoint. The finding that the hexons are localized throughout the infected cell is consistent with the replication cycle of adenoviruses. As the hexon proteins are synthesized in the cytoplasm of the cell and then quickly transported to the nucleus [13, 24] and this procedure continues for several hours, it may be expected that the hexon should be demonstrable both in the cytoplasm and in the nucleus. The different accumulation forms may represent the different transportation stages of the hexon proteins into the nucleus.

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EFFECT OF ORGANIC VOLATILES FROM *SACCHAROMYCES* ON THE SPORE GERMINATION OF FUNGI

(A NOTE)

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Common volatile organic compounds (acetaldehyde, ethylacetate, ethanol, n-propanol, isobutanol, 2-methyl-butanol, 3-methyl butanol) tested singly and in combination inhibited the spore (conidium) germination of *Helminthosporium oryzae*, *Cercospora personata*, *Cunnighamella blakesleeana*, *Colletotrichum capsici*, and *Alternaria solani*.

Glen and Hutchinson [1], Robinson and Park [2] and Robinson et al. [3] identified some organic volatiles from *Saccharomyces cerevisiae* and showed their inhibitory action on the germination of *Aspergillus niger* and *Pestalotia rhododendri*. Deshmuk [4] has reported the effects of some organic volatiles on dermatophytes. The present study deals with the effect of these organic volatiles on some common plant pathogens.

Materials and methods

The paired Petri dish technique of Dennis and Webster [5] was used to study the effects of organic volatiles (acetaldehyde, ethylacetate, ethanol, n-propanol, isobutanol, 2-methyl-butanol, and 3-methyl-butanol) against 5 test pathogens (*Helminthosporium oryzae*, *Cercospora personata*, *Cunninghamella blakesleeana*, *Colletotrichum capsici* and *Alternaria solani*).

The test pathogens were grown on Sabouraud's Dextrose Agar (dextrose, 40 g; peptone, 10; agar, 15 g; water 1 litre) for 7 days at 28 ± 1 °C. Then sterilized peptone discs (3% agar and 1% peptone, 0.6 mm thick and 4 mm in diameter) were made and were stuck on the sealed slide in the upper lid of the Petri dish. The spore (conidium) suspension of the test pathogens was inoculated over each disc and 2 ml of the pure organic volatiles were kept in the lower lid singly and in various combinations. The Petri dishes were placed in mouth to mouth contact and were sealed in the rim by cello-tape to avoid any outward diffusion, then incubated at 28 ± 1 °C for 24 h. After this the percentage of spore germination was recorded.

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Table I

Effect of organic volatiles applied singly

Organic volatiles	Spore germination, %									
	H. oryzae		*C. personata*		*C. blakeslelana*		*C. capsici*		*A. solani*	
	test	control	test	control	test	control	test	control	test	control
Acetaldehyde	48.2	100	92.1	100	72.1	69	12.0	92	32.1	89
Ethylacetate	39.1		42.6		32.0		12.2		14.0	
Ethanol	23.4		17.4		28.0		14.0		17.1	
n-Propanol	90.9		89.5		84.0		23.4		28.2	
Isobutanol	92.1		84.3		72.0		16.2		32.0	
2-Methylbutanol	33.2		32.2		82.0		72.2		42.7	
3-Methylbutanol	42.3		16.1		91.3		33.0		14.2	

Each reading is an average of 3 determinations. Test: % spore germination in the presence of volatiles. Control: agar discs with test organisms in the absence of volatiles

Table II

No.	Organic volatiles	Spore germination, %									
		H. oryzae		*C. personata*		*C. blakes-lelana*		*C. capsici*		*A. solani*	
		test	control	test	control	test	control	test	control	test	control
1	Acetaldehyde+ethyl-acetate	42.1	100	14.0	100	34.0	69	42.0	92	13.0	89
2	Ethylacetate+ethanol	23.3		23.2		34.0		23.0		21.7	
3	Acetaldehyde+ethyl-acetate+ethanol	14.5		42.9		23.0		14.1		19.0	
4	Ethanol+n-propanol	23.7		33.4		42.5		22.0		16.1	
5	n-Propanol+n-butanol	14.9		34.3		82.5		14.0		22.7	
6	Isobutanol+n-propanol+ +n-butanol	16.3		14.2		89.4		23.1		14.6	
7	Ethanol+n-propanol+ +n-butanol	23.0		17.0		82.3		42.0		21.7	
8	Isobutanol+acetaldehyde	24.0		19.9		82.7		13.7		3.3	
9	Ethylacetate+ +n-butanol+n-propanol	23.4		22.7		83.4		12.7		4.2	
10	2-Methylbutanol+ +3n-butanol	17.5		32.7		89.7		21.3		10.0	
11	Isobutanol+2-methyl-butanol+3-m-butanol	16.7		14.2		84.0		23.7		10.1	
12	Mixture of all 7 volatiles	12.9		16.7		82.0		10.1		14.0	

Each reading is an average of 3 determinations. Test: % spore germination in the presence of volatiles. Control: agar discs with test organisms in the absence of volatiles. Combinations of volatiles are in equal proportions (1 ml each).

Results and discussion

Table I shows that, when applied singly, all but one organic volatiles suppressed the spore germination of all test pathogens. *C. blakesleeana* was suppressed only by ethanol and ethylacetate, but stimulated by the rest of the volatiles. This organism is known to utilize the hydrocarbons for its growth [6]. Comparatively high germination was observed in *H. oryzae* and *C. personata* against n-propanol and isobutanol.

Table II, which shows the complex interaction of organic volatiles in various combinations, clearly depicts that certain combinations are effective suppressants against all the test pathogens. The spore germination of *C. blakesleeana* was suppressed considerably by combinations 1, 2, 3 and 4, but on the contrary it was stimulated by the rest of the combinations. In some cases, especially with combinations 7 and 9 the effect of ethanol and ethylacetate was masked by n-propanol and n-butanol, respectively. Thus the growth of *C. blakesleeana* was found to be stimulated. In many cases the suppression of spore germination was more definite with combined as compared to single volatiles (Table II).

It was interesting to note that the mixture of all volatiles was responsible for remarkable inhibition of spore germination in all cases barring the 7 cases of *C. blakesleeana* where stimulations were seen. In all combinations *A. solani* germination was inhibited to a greater extent.

Keeping these aspects in view this study can be used as a useful adjunct in controlling the prevalance of these plant pathogens in the soil by *Saccharomyces* spores which are responsible for the production of these volatiles.

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