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Investigation of the immune status of mice during and following selective decontamination of the digestive tract

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SUMMARY

Selective decontamination of the digestive tract (SDD) employs oral antibiotics to eliminate aerobic Gram-negative bacilli while retaining the anaerobic flora. A combination of SDD and parenteral cefotaxime has recently been reported to strikingly reduce the incidence of infection in patients treated in an intensive therapy unit. The present study describes the effects of SDD and of cefotaxime on the immune response of mice to protein antigens. The *in vivo* cellular response to ovalbumin and sheep red blood cells was unchanged. However, SDD appeared to decrease the *in vitro* mitogenic response of spleen cells to phytohaemagglutinin, and cefotaxime similarly affected the response to Concanavalin A. The antibody response to sheep red blood cells was increased in the period after discontinuation of SDD. The antibody response was otherwise not affected. These results indicate that SDD is unlikely to have adverse effects on the immune response to protein antigens.

INTRODUCTION

Selective decontamination of the digestive tract (SDD) eliminates or markedly reduces the numbers of potentially pathogenic aerobic Gram-negative bacilli (AGNB) and of yeasts from the gastrointestinal tract, while retaining the normally predominant anaerobic flora. Maintenance of the anaerobic flora during SDD prevents overgrowth with drug-resistant strains, a phenomenon termed colonization resistance (CR) (1).

The technique has been used with some success to reduce infections of endogenous origin in leucopenic patients (2–4). More recently, it has been shown to induce a striking reduction in the incidence of unit-acquired infections in intensive care patients (5, 6). In these latter studies, SDD was achieved with the oral administration of a mixture of polymyxin E (P), tobramycin (T) and amphotericin B (A). In addition to SDD, parenteral cefotaxime (CTX) was used to provide broad-spectrum systemic antibiotic cover during the first 4 days of intensive therapy unit admission.

The success of SDD regimens in reducing endogenous infection due to AGNB is ascribed to the marked reduction in the numbers of these organisms detected in the oropharynx and gastrointestinal tract of treated patients (5, 6). This

assumption is entirely reasonable. However, the patients' immune status must also be important, both during the period of SDD and following the abrupt withdrawal of antibiotic support on cessation of the regimen.

It is generally thought that the intestinal flora is a modulator of the immune response both in the gut and systemically (7–9). Gut decontamination induces gross changes in the flora of the intestinal tract and is also associated with the release of lipopolysaccharide and other degradation products from killed bacteria (10–12). These regimens may thus have significant effects on immune function, and there is some experimental evidence to support this view. Removal of both aerobic and anaerobic flora from the gut (total decontamination of the digestive tract) has been shown to affect the systemic immune response in mice (13) and in dogs (14). Prolonged selective decontamination in piglets (15) has been associated with granulocytopenia, the disappearance of IgG^+ cells from the spleen, a marked increase in IgA^+ cells in the spleen and an altered antibody response to protein antigens.

In these circumstances there is legitimate cause for concern that SDD may alter the immune response in patients. It is therefore pertinent to question the effects on immune function of the SDD regimen itself.

The present study describes the effects on immune responses to systemic antigens in mice of an SDD regimen identical to that described for clinical use in ITU patients. The possibility of additional effects due to the systemically administered CTX is also studied. A mouse model was employed because of the intractable limitations imposed by human studies and because the bacteriological consequences of SDD in mice are extensively described (1, 16, 17). The results do not support the concept that SDD is associated with significant alterations to the immune response to protein antigens.

METHODS

Animals

BALB/c or C3H/He female mice, aged 8–16 weeks were used. Mice were randomly assigned to batches and co-housed wherever experimental conditions permitted. All mice were housed in the same room in the animal unit. Cages were changed daily to minimize coprophagy. Water and standard laboratory chow were given *ad libitum*.

Bacteriology

Faeces collected from mice were homogenized immediately in brain heart infusion broth (Oxoid). For quantitative estimates, serial tenfold dilutions were plated on MacConkey agar (Oxoid) and 5% blood agar and incubated at 37 °C for 24 h. Aerobic Gram-negative bacilli and enterococci (EC) were identified by Gram stain and colonial morphology. For qualitative estimates, faeces from four mice were pooled, the faecal suspension was streaked on to the same media, and the presence and approximate prevalence of organisms was noted.

Antibiotics

The SDD regimen employed has been employed previously (17). Briefly, 0·5 mg of polymyxin E (Colomycin powder, Pharmax, Bexley, Kent, UK), 0·4 mg of

tobramycin (Nebcin injection, Eli Lilly, Basingstoke, Hants, UK) and 0.8 mg of amphotericin B (Fungilin suspension, Squibb, Houndslow, Middx, UK) per mouse per day were administered by gavage in aqueous suspension. If used, 3 mg of cefotaxime (Claforan injection, Roussel, Wembley Park, Middx, UK) was given by a single intraperitoneal (i.p.) injection each day. Total gut decontamination was induced with a regimen of 8 mg bacitracin + 24 mg streptomycin (Sigma, Poole, Dorset, UK) (18) in aqueous solution per mouse per day given by gavage. In all cases, sham treatments were added to equalize the amount of insult in each group. The different regimens employed are abbreviated as follows: selective decontamination of the digestive tract (SDD), selective decontamination of the digestive tract with parenteral CTX (SDD/C), total decontamination of the digestive tract (TDD).

DTH studies

Mice were injected subcutaneously into a footpad with 50 μl of grade III (Sigma) ovalbumin (OVA) in complete Freund's adjuvant. Three to 4 weeks later, they were challenged in the contralateral footpad with 100 μl of heat aggregated OVA (19). Alternatively, mice were immunized with 10^7 fresh sheep red blood cells (SRBC) given i.p. After 6 days they were challenged with 10^8 SRBC injected into the right footpad.

Measurements of footpads were made with a callipers. For OVA, footpad estimations were reported as the mean difference in footpad thickness before and after challenge. For SRBC, footpad estimations were reported as the mean difference between the right and left footpad on the same mouse. Results are the mean of three measurements made 24 or 48 h after challenge.

Antibody studies

Mice employed in DTH studies with OVA were bled 14 days after challenge. Alternatively, 10^8 SRBC were administered i.p. and the mice were bled after 5 days.

All sera were separated and inactivated at 56 °C for 30 min. An ELISA assay (20) was used for estimation of anti-OVA. Sera were tested for α-SRBC activity in a haemagglutination assay employing 0·25% SRBC suspended in phosphate-buffered saline. Plates were incubated at room temperature for 24 h.

Estimation of in vitro *mitogen response*

Mice were killed by cervical dislocation and the spleens were aseptically removed and forced through a sterile stainless steel mesh. Spleen cells were pooled and suspended in RPMI 1640 w/o HEPES + 5% newborn calf serum. Wells of microtitre plates were inoculated with 2×10^5 cells per well. Concanavalin A (ConA), or phytohaemagglutinin (PHA) (5 mg l^{-1}) or saline were added to the cells from each group of mice in sextuplicate. Plates were incubated for 3 days at 37 °C in 5% CO_2 and were pulsed for 4 h with 3·7 MBq methyl-[^{3}H]thymidine ([^{3}H]TdR, Amersham, UK) per well. Cells were harvested onto glass fibre disks with a TiterTek cell harvester and assayed in a liquid scintillation spectrometer.

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Statistics

Multiple simultaneous comparisons were made using Scheffé's test. Where data were not normally distributed, the Kruskal and Wallis test or Wilcoxon's Sum of Ranks test with a correction for multiple testing was employed. Comparisons of two sets of data were made using Student's *t*-test, or Wilcoxon's Sum of Ranks test in the case where data were not normally distributed.

RESULTS

Bacteriology

Unless otherwise stated, the results shown in Table 1 are representative of the bacteriological findings obtained from the different groups of mice studied. The total viable count of AGNB was not affected by 3 days treatment with SDD or SDD/C, but these organisms were reduced to undetectable levels after treatment for 4 or 12 weeks. By contrast, even prolonged administration of CTX alone had no effect on total numbers of AGNB. Levels of enterococci were usually unaffected by SDD. However, an apparent overgrowth of these organisms was consistently seen after 3 days treatment with SDD/C or CTX alone. Despite the continued presence of CTX, this increase did not persist, and after 12 weeks treatment levels were lower than those in the controls. Throughout these studies, the total viable count (not shown) was little affected by the use of SDD, SDD/C or CTX. The total decontamination regimen (TDD) proved to be very effective and aerobic bacteria were not detected in samples taken after 6 weeks treatment.

Effect of treatment of gross anatomy

Mice of the same age were sham-treated or given SDD, SDD/C or CTX for 12 weeks. The various groups showed no significant difference (Scheffé's test) in body weights after treatment (data not shown).

Groups of mice were treated for 6 weeks either with TDD, SDD, SDD/C or with CTX alone. Immediately after cessation of treatment, the mice were killed, weighed, and their caeca were removed and weighed. The results are shown in Table 2. It can be seen that a significant change in caecal size occurred only in the mice receiving TDD.

Estimation of in vitro *mitogen response*

Four groups of eight mice were treated with SDD, SDD/C, CTX only, or saline. After 12 weeks, mice were sacrificed and spleen cells were incubated with mitogens and pulsed with [^{3}H]TdR. The results are shown in Table 3. It can be seen that there was a significant decrease in uptake in the ConA treated cells from CTX treated mice, and a significant decrease in uptake in PHA treated cells from SDD- and SDD/C-treated mice. Significance was estimated by Wilcoxon's sum of ranks test ($P < 0{\cdot}05$ in all cases).

Delayed-type hypersensitivity to OVA

Mice were treated with SDD, SDD/C, CTX only, or saline for 12 weeks. After 8 weeks of treatment, they were immunized, and challenged 4 weeks later, with

Table 1. *The effect of antibiotics on aerobic Gram-negative bacilli and enterococci in the faeces of mice*

		AGNB			EC		
		log			log		
Duration	Treatment	c.f.u./g	S.D.	P/N	c.f.u./g	S.D.	P/N
3 days	SDD/C	5·4	0·1	2/3	10·6	0·1	3/3
	SDD	6·2	0·8	3/3	6·4	1·0	3/3
	CTX	4·1	1·6	2/3	9·5	0·8	3/3
	None	5·3	1·5	3/3	7·2	0·5	3/3
4 weeks	SDD/C	NS		0/3	5·7	0·7	3/3
	SDD	NS		0/3	6·6	0·6	3/3
	CTX	6·0	1·3	3/3	6·0	1·3	3/3
	None	5·0	1·4	3/3	5·9	1·8	3/3
12 weeks	SSD/C	NS		0/4	4·2	0·6	4/4
	SDD	NS		0/4	NS		0/4
	CTX	6·9	0·9	4/4	3·3	0.7	4/4
	None	5·1	1·0	4/4	6·5	0·8	4/4
6 weeks	TDD	NS		0/12	NS		0/12

Results shown as $\log_{10}$ colony forming units (c.f.u.)/g wet faeces. Mean of results from positive mice.

S.D., standard deviation; AGNB, aerobic Gram-negative bacilli; SDD/C, selective decontamination of the digestive tract and parenteral cefotaxime; SDD, selective decontamination of the digestive tract; CTX, parenteral cefotaxime only; TDD, total decontamination of the digestive tract; NS, not seen ($< 1 \times 10^3$ c.f.u./g wet faeces); P/N, number of positive mice/number of mice tested.

Table 2. *Effect of 6 weeks of antibiotic treatment on the ratio of mouse body weight : caecal weight*

Treatment	No. of mice	Body Wt / Caecal Wt	S.D.
SDD/C	7	48·46	9·15
SDD	6	55·33	14·75
CTX	8	60·63	11·14
TDD	12	20·28*	2·18
None	4	42·63	3·58

SDD/C, selective decontamination of the digestive tract and parenteral cefotaxime; SDD, selective decontamination of the digestive tract; CTX, parenteral cefotaxime only; TDD, total decontamination of the digestive tract; S.D., standard deviation;

* $P < 0{\cdot}01$ against all other groups by Wilcoxon's sum of ranks test.

OVA. There was no significant difference (Scheffé's test) between any of the groups (Table 4).

Delayed-type hypersensitivity to SRBC

Mice undergoing treatment with SDD were immunized after 2 weeks with SRBC and the footpad swelling was measured after further challenge. Alternatively, mice were immunized and challenged after completing a course of SDD. No

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Table 3. *Uptake of [^3H]TdR by spleen cells from treated and control mice treated for 12 weeks*

	Mitogen Treatment							
	SDD/C		SDD		CTX		Sham*	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
None	2·10	0·36	2·12	0·24	2·45	0·12	2·05	0·26
ConA	4·14	0·23	4·24	0·10	3·60	0·15†	4·04	0·26
PHA	2·27	0·38†	3·43	0·45†	4·08	0·29	4·23	0·36

SDD/C, selective decontamination of the digestive tract and parenteral cefotaxime; SDD, selective decontamination of the digestive tract; CTX, parenteral cefotaxime only.

Results shown as log (d.p.m./10^6) cells.

* Gavage and i.p. injection with saline only.

† $P < 0·05$ compared to cells from sham-treated mice by Wilcoxon's sum of ranks test.

Table 4. *Delayed hypersensitivity in mice treated for 12 weeks: mean difference (in mm) in footpad thickness 24 h and 48 h after challenge with OVA*

Treatment*	No of mice	24 h		48 h	
		Mean	S.D.	Mean	S.D.
SDD/C	7	0·31	0·087	0·18	0·050
SDD	8	0·34	0·076	0·20	0·031
CTX	5	0·22	0·085	0·15	0·083
Sham	8	0·28	0·116	0·16	0·071

SDD/C, selective decontamination of the digestive tract and parenteral cefotaxime; SDD, selective decontamination of the digestive tract; CTX, parenteral cefotaxime only; S.D., standard deviation.

* Immunized after 8 weeks, challenged 4 weeks later: total treatment 12 weeks.

Table 5. *Delayed hypersensitivity: mean difference (in mm) in footpad thickness 24 h after challenge with SRBC*

Treatment before immunization	No. of mice	Swelling	
		Mean	S.D.
Untreated	6	0·47	0·07
2 weeks SDD	6	0·62	0·37
1 week SDD + 1 week*	5	0·55	0·17
1 week SDD + 2 weeks*	8	0·28	0·09

SDD, selective decontamination of the digestive tracts; S.D., standard deviation.

* Period between cessation of SDD and immunization.

significant changes (Scheffé's test) attributable to SDD were seen in any of the mice (Table 5).

No AGNB were detected in the faeces of the mice treated with SDD at the time of immunization or challenge. Both AGNB and EC were present 1 and 2 weeks after cessation of SDD, but not in abnormally high numbers.

Table 6. *Serum antibody response to OVA in mice after 12 weeks of SDD*

Expt no.	Treatment	No. of mice	Ab	S.D.
1	SDD/C	10	21	8·1
	None	8	11	5·4
2	SDD	6	17	6·9
	None	8	20	11·7
3	CTX	6	23	9·0
	None	8	24	12·6

SDD/C, selective decontamination of the digestive tract and parenteral cefotaxime; SDD, selective decontamination of the digestive tract; CTX, parenteral cefotaxime only; Ab, antibody levels; S.D., standard error of mean.

Table 7. *Serum antibodies against SRBC in mice: haemagglutination titres*

Treatment before immunization	No. of mice	Median titre	Inter-quartile range	*P* value*
None	4	384	160–512	NS
3 days SDD/C	8	192	128–256	NS
3 days SDD	8	256	128–256	NS
3 days CTX	7	256	256–256	NS
3 days water	8	128	128–256	NS
2 weeks SDD	4	384	64–512	NS
8 weeks SDD	7	512	0–512	NS
8 weeks water	7	256	256–1024	NS
1 week SDD+5 days†	5	512	384–512	0·023
1 week SDD-2 weeks†	6	512	256–512	0·031

SDD/C, selective decontamination of the digestive tract and parenteral cefotaxime; SDD, selective decontamination of the digestive tract; CTX, parenteral cefotaxime only; NS, not significant.

* *P* from standard deviate estimate from Kruskal and Wallis test.

† Period between cessation of SDD and immunization.

Serum antibody response to OVA

Mice were treated with SDD, SDD/C, CTX only, or saline for 12 weeks. After 4 weeks of treatment they were immunized and 7 weeks later they were challenged with OVA. Treatment was maintained until sampling for antibody studies. In a simultaneous comparison, no significant differences (Scheffé's test) were seen between any of the groups (Table 6). Although there is an apparent difference between the test and control group in experiment 1 (Table 6), this is due to an anomalously low result in the control group.

Serum antibody response to SRBC

Mice undergoing treatment with SDD/C, SDD, or CTX were immunized with SRBC on the last day of treatment. No significant differences from control mice were seen. Alternatively, mice were immunized 5 days or 2 weeks after completing a course of SDD. In these latter groups a significant increase in antibody titre was seen (Table 7). During administration of SDD or SDD/C, the bacteriological

findings conformed to the findings in Table 1. In addition, no AGNB were detected after 2 weeks of SDD. At both 5 days and 2 weeks after cessation of SDD, AGNB were present, but not in abnormally high numbers.

DISCUSSION

The results of this study suggest that this particular SDD regimen exerts only minimal effects on the immune response of adult mice to protein antigens. A similar conclusion applies to treatment with systemic CTX used alone or in combination with SDD.

No effects were seen in DTH studies with OVA or SRBC. In contrast spleen cells obtained from mice treated with SDD, SDD/C, or CTX showed a significantly reduced *in vitro* response to mitogens. However, the differing responses of spleen cells to PHA and ConA in these studies are difficult to explain as these two mitogens are thought to affect the same T-cell populations (21). The results related to cell-mediated immunity were thus either negative or anomalous. The serum antibody response to OVA and SRBC was unaffected by the three treatment regimens when these both preceded immunization and were continued until sampling for antibody studies. However, the antibody response to SRBC was significantly enhanced when immunization occurred after completion of SDD. This latter effect may be related to recolonization of the intestine with abnormally large numbers of AGNB, a phenomenon that has previously been demonstrated in this system following cessation of SDD (17). Such overgrowth may be associated with increased translocation of intestinal bacteria (22) and possibly increased levels of circulating lipopolysaccharide (LPS). LPS is a known B cell mitogen in mice (13) and is not thought to affect DTH reactions (13). However, it is doubtful if LPS migrates across the intestinal wall in normal mice (23), and the polymyxin used in the present study is known to complex with LPS (24).

We have reported previously (17) that the per-oral administration to mice of P, T and A results in the elimination of detectable aerobic Gram-negative bacilli from the faecal flora within 1 week, without affecting the total viable aerobic count, and that these effects were only slightly altered by the addition of parenteral CTX to the regimen. In the present study, these findings were confirmed. No AGNB were detected in the faeces of mice treated with SDD for more than 5 days. The removal of antibiotics resulted in a rapid recovery of the AGNB population, but the abnormally high levels of AGNB, seen during this period in an earlier study (17). were not seen. This may be due to co-housing of post-SDD with normal mice in the earlier study, whereas this was not done in the present study. The transient outgrowth of EC associated with CTX seen previously (17) was observed after 3 days treatment with CTX. On prolonged treatment, the population of EC returned to normal despite the continued presence of CTX in the regimen.

The lack of change in body weight in mice undergoing SDD is a good indication that the overall well-being of the animals was unaffected by even prolonged (12 weeks) SDD regimens. Subjectively, the animals also appeared to be healthy. The lack of increase in caecal size in mice treated with SDD suggests that the component of the intestinal flora associated with colonization resistance was intact in these animals (25). By contrast, and as expected (25), the mice treated

to induce total decontamination showed an increase in caecal size relative to body weight.

We conclude therefore that this SDD regimen probably has no significant effects on either the gross anatomy or on the immune response to systemic protein antigens in mice. This conclusion is not necessarily incompatible with the appreciable immunological effects previously reported following total gut decontamination of mice (13) or the use of nalidixic acid, kanamycin and nystatin to achieve SDD in piglets (15). Indeed, these differences further demonstrate the complexity of biological events associated with regimens that are increasingly employed to control infection in severely ill patients.

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