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# The relationship between faecal endotoxin and faecal microflora of the C57BL mouse

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SUMMARY

We studied the effect of oral selective antibiotic decontamination (SD) on the faecal endotoxin content and microflora in individual C57BL mice. Suppression of the coliform count was associated with an initial rise in faecal endotoxin concentration from 0·1 to 3·1 mg/g wet faeces during the first week of SD, which fell to 0·04 mg/g during the second week of treatment. Cessation of SD resulted in an immediate sharp increase in coliform count followed by its decline and gradual recovery to pre-treatment counts. Faecal endotoxin levels followed a parallel course. SD did not effect significantly the counts of lactobacilli, bacteroides and enterococci.

It appears that the coliform population is responsible for the overall level of faecal endotoxin, and that during the initial period of SD endotoxin levels are elevated, an effect which may be mediated by antibiotic-enhanced release of endotoxin.

## INTRODUCTION

Endotoxin (lipopolysaccharide) is associated with the outer part of the cell membrane of Gram-negative bacteria, and as such, the intestinal microflora represents a considerable potential source of bacterial endotoxin.

Intestinal endotoxin (IE) has been implicated in the pathogenesis of a variety of disease states (Morrison & Ulevitch, 1978; Rietschel *et al.* 1982). In particular, there has accumulated a body of evidence which suggests that IE may be involved in triggering or exacerbating graft-versus-host disease (GVHD) following allogeneic bone marrow transplantation (BMT) in murine models (Keast, 1973; Van Bekkum & Knaan, 1977; Skopinska, 1972) and in man (Vossen *et al.* 1981). Central to this hypothesis is the observation that total (Heit *et al.* 1979) or selective antibiotic decontamination (Van Bekkum *et al.* 1974) will prevent GVHD following BMT in mice.

Surprisingly, there have been no studies which compare in detail qualitative and quantitative relationships between the intestinal microflora and faecal endotoxin and the effect of antibiotics. Therefore we studied the microbial flora and the faecal endotoxin concentrations of five individual C57BL mice followed prospectively for 38 days, both before and after a period of antibiotic decontamination.

*Table* 1. Media and additives used for selection of faecal microflora

| Genera selected | Medium | Additives |
|---|---|---|
| Coliforms | MacConkey Agar | — |
| Enterococci | MacConkey Agar | — |
| Bacteroides | Wilkins–Chalgren Agar | Vancomycin 7·5 μg/ml<br>Kanamycin 75 μg/ml |
| Lactobacilli | Rogosa Agar | — |

All media obtained from Oxoid, Basingstoke, U.K.

## METHODS AND MATERIALS

*Animals and housing.* Five female C57BL mice, 16–18 weeks old, (Charles River, U.K.) identified by ear-piercing, were housed together in a sterile polycarbonate cage with autoclaved bedding and sterile food pellets (Lab Sure, P.R.D.). Acidified water (pH3), or antibiotic-water (see below), was given *ad lib.* Bedding and water were changed twice weekly.

*Antibiotics.* During the first week of treatment, neomycin sulphate 5 g/l, streptomycin sulphate 5 g/l and amphotericin B 100 mg/l were added to the drinking water. The amount of neomycin and streptomycin was halved during the second week of treatment (Van Bekkum *et al.* 1974).

*Faeces collection.* Twice weekly, individual mice were removed from the cage, 'scruffed' and encouraged to defaecate directly into two pre-weighed sterile bijoux.

The first bijou for bacteriological analysis, contained four 4 mm glass beads and 2 ml of 10 % glycerol broth (Crowther, 1971) which had previously been heated to 100 °C for 10 min and allowed to cool. The second bijou was plastic, pyrogen-free and was used for endotoxin determination.

Following collection, all containers were re-weighed and processed within 1 h or frozen to −20 °C.

*Bacteriology.* Inside an anaerobe chamber (Model 1024, Forma Scientific, Ohio) the specimen in glycerol broth was homogenized by vortexing for 30 sec. An aliquot of 100 μl was removed and tenfold serially diluted to $10^{-8}$ in 1/4 strength Ringers 1/4 peptone solution (previously heated to 100 °C for 10 min). Of each suitable dilution 100 μl was inoculated onto each of three selective media and spread (Table 1). All plates were incubated at 37 °C for 24–48 h, aerobically or anaerobically, as appropriate. To confirm the selective function of the plates, representative colonies were examined microscopically for Gram-staining characteristics and morphology.

*Faecal endotoxin estimation.* Faeces were homogenized in 5 ml of pyrogen-free water (PFW) and the suspension centrifuged at 1200 ***g*** for 10 min. The supernatant was filtered through a 0·2 μm Millipore filter (Millipore, Molsheim, France) into a sterile, pyrogen-free bijou and stored at −20 °C.

Endotoxin was assayed in batches, using a quantitative microassay as previously described (Cohen & McConnell, 1984). In brief, 50 μl of the test sample or standard was incubated in triplicate with 100 μl of Limulus amoebocyte lysate (LAL) (M.A.Bioproducts, Maryland, U.S.A.) at 37 °C for 1 h in a sterile microtitre plate (Sterilin, Teddington, U.K.). Fifty microlitres of a chromogenic substate (S 2423

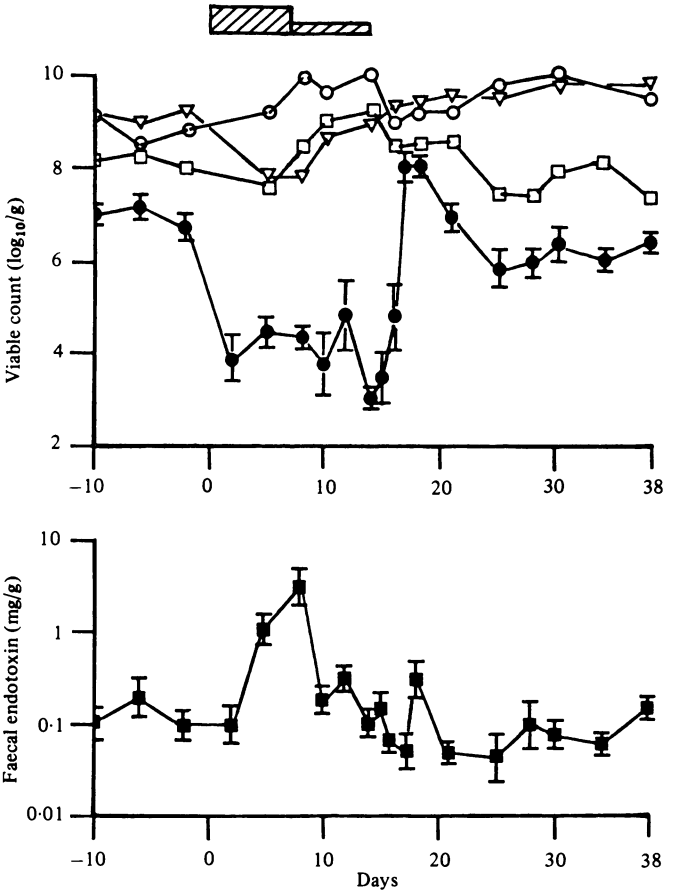


Fig. 1. The relationship between components of the faecal microflora (upper panel) and faecal endotoxin (lower panel) in five C57BL mice. Bars represent S.E.M. Hatched box indicates period of oral selective antibiotic decontamination (see text for details). ▽—▽, lactobacilli; ○—○, enterococci; □—□, bacteroides; ●—●, coliforms; ■—■, faecal endotoxin.

Kabi Diagnostics, Stockholm), reconstituted in THM buffer (0·1 M Tris-HCl, 0·1 M-$MgCl_2$, final pH 8·8) were then added rapidly and the optical density at 405 nm ($OD_{405}$) of each well measured automatically on a Multiskan Elisa plate reader (Flow Laboratories, Uxbridge, U.K.) at 1, 2 and 3 min. Each plate included appropriate controls and a set of standards.

The rate of change of the reaction ($\Delta OD/t$) for the standards was determined and plotted against log endotoxin concentration yielding a straight line, from which the endotoxin values of the unknown samples were obtained.

Faecal supernatants were diluted by a factor $10^3$ to $10^4$ in order to bring them into the standard range of the assay. Antibiotic concentrations at this dilution have no effect on the Limulus reaction (authors, unpublished observations).

## RESULTS

*Faecal endotoxin (FE).* Baseline pre-antibiotic levels ranged from 0·02 to 0·8 mg/g wet faeces (mean 0·13 mg/g, S.D. 0·1) (Fig. 1). During the first week of antibiotic treatment the FE concentration increased sharply to a peak of 3 mg/g before returning to baseline levels, which were maintained until 3 days following antibiotic withdrawal. Coincident with bacterial recolonization on the fourth day post-treatment there was a further sixfold rise in FE concentration to 0·3 mg/g, which by 1 week post-treatment had again fallen to 0·04 mg/g. Thereafter FE levels returned gradually to pre-treatment levels over the following 2 weeks.

*Coliforms.* Pre-treatment counts were $\log_{10}$ 7/g wet faeces (Fig. 1). Within 24 h of antibiotic treatment, the count had fallen by 3 logs and fell a further log during the second week of treatment. By the third day post-treatment, the coliform count had risen to $\log_{10}$ 8/g, thereafter gradually returning to pre-treatment levels.

*Enterococci, lactobacilli and bacteroides.* The counts of each genera remained stable throughout the experiment, uninfluenced by the antibiotic treatment (Fig. 1).

## DISCUSSION

Previous workers have shown that suppressing the intestinal microflora with non-absorbable antibiotics reduces the concentration of circulating endotoxin (Walker, Ledney & Galley, 1975; Ravin *et al.* 1960) and intestinal endotoxin (Ditter *et al.* 1984; Dankert, DeVries & Veninga, 1979). Dankert & DeVries showed that S.D. in humans reduced faecal endotoxin levels by at least 1000 times, although in some subjects FE levels remained high despite S.D. They suggested that the anaerobic Gram-negative flora might contribute to the pool of intestinal endotoxin, since oral metronidazole reduced FE levels further in those with persistently high levels (Dankert, DeVries & Veninga, 1979). Our results in mice demonstrate a close relationship between the coliform count and FE levels and argue against a significant contribution from the anaerobic flora.

The stability of the lactobacilli, bacteroides and enterococci counts confirms the selectivity of the antibiotic regimen. Apart from higher enterococci counts than observed generally, the numbers agree well with previous work on the quantitation of the faecal flora of mice (Russell, Schaedler & Dubos, 1962; Adrian *et al.* 1971).

The failure to eliminate the coliforms is surprising. The unpleasant taste of antibiotic-water usually results in decreased water intake for a few days. Despite this we observed an almost 50% fall in the coliform count with the first 24 h, and others have shown caecal antibiotic concentrations of four times the bactericidal level within 48 h of antibiotic-water administration and concomitant with a decreased water intake (van der Waaij & Sturm, 1968). Incomplete elimination of faecal coliforms has also been ascribed to airborne contamination leading to colonization with antibiotic-resistant organisms (van der Waaij, 1968; van der Waaij & Sturm, 1968).

Although the initial sharp rise in faecal endotoxin concentration might also be explained in part by a reduced fluid intake an additional factor may have been antibiotic-mediated release of endotoxin (Shenep & Kathryn, 1984). We and others have shown that rapidly-dividing coliform bacteria will release endotoxin into the

culture supernatant, and that this endotoxin release can be greatly enhanced by exposure to bactericidal antibiotics (Cohen & McConnell, submitted; Andersen & Solberg, 1980). The subsequent decline in FE levels to below normal after the initial rise is probably a reflection of decreased production from a much reduced coliform population. The relationship between coliform count and FE level is further supported by the parallel rise, fall and normalization after cessation of S.D.

There is mounting evidence of the involvement of endotoxin (especially intestinal endotoxin) in triggering or exacerbating GVHD in experimental mouse models of BMT (Keast, 1973; Van Bekkum & Knaan, 1977; Skopinska, 1972) and in man (Vossen *et al.* 1981). Our results are relevant to experimental mouse models of GVHD, since raised FE levels, through antibiotic-mediated release or secondary transient re-colonization with coliforms following cessation of S.D., may be important during critical periods of engraftment.

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