Lactulose inhibits endotoxin induced tumour necrosis factor production by monocytes. An in vitro study

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Abstract

Preoperative oral treatment with lactulose is used to prevent complications after surgery in patients with obstructive jaundice. The effect is perhaps the result of an inactivation of gut derived endotoxins but the exact mechanism of action is, however, unknown. Tumour necrosis factor is an important mediator of endotoxin toxicity. The cytokine tumour necrosis factor is mainly produced by mononuclear phagocytes. In this study, the effect of lactulose on the endotoxin induced tumour necrosis factor release by monocytes was investigated. The direct effect of lactulose on endotoxin was tested in a chromogenic limulus amoebocyte lysate assay. Polymyxin B a known inactivator of endotoxin was used as control in both experiments. Lactulose has a limited capacity to inactivate endotoxin as measured in the endotoxin assay. In contrast lactulose significantly reduced endotoxin induced tumour necrosis factor production by monocytes. In conclusion lactulose inhibits tumour necrosis factor production by a direct inhibitory effect on monocytes, rather than by inactivation of endotoxin. Because tumour necrosis factor is an important mediator of endotoxin toxicity, this inhibitory effect could explain the beneficial effect of lactulose in obstructive jaundice.

Gut derived endotoxins are thought to be responsible for postoperative complications such as renal insufficiency, gastrointestinal bleeding and infections, in patients with obstructive jaundice.[1 2] This theory is supported by the high incidence of endotoxaemia reported in these patients and the correlation that was found between the presence of endotoxaemia and occurrence of complications.[3–5]

Polymyxin B is a known antiendotoxin agent[6–8] and reduces mortality in rats, with experimental biliary obstruction, challenged with intragastric endotoxin.[9] Unfortunately, because of its renal toxicity, effective doses cannot be given to jaundiced patients.[10] Orally administrated bile acids have been used successfully to prevent endotoxin related complications after surgery in jaundiced patients[11 12] bile acids reduced absorption of endotoxins from the gastrointestinal tract resulting in a decrease of portal and systemic endotoxaemia and of postoperative renal complications.

Preoperative oral treatment with lactulose was found to be equally effective in preventing postoperative renal complications in patients with obstructive jaundice.[13] The effect of lactulose was explained by an inactivation of endotoxins from the gastrointestinal tract. Suggestive evidence for this hypothesis was the reduced limulus amoebocyte lysate activation by endotoxin in the presence of lactulose.[13 14] The mechanism through which lactulose reduces endotoxin toxicity, however, remains to be further clarified.

Tumour necrosis factor is reported to be an important mediator of endotoxin toxicity.[15–17] The cytokine tumour necrosis factor is mainly produced by mononuclear phagocytes in response to endotoxin.[18 19] These findings enabled us to develop an *in vitro* model to study the effect of different agents on the production of tumour necrosis factor as the mediator of endotoxin toxicity. Human peripheral blood monocytes were stimulated with endotoxin in the presence of the compound to be tested and tumour necrosis factor production was measured with an enzyme linked immunosorbent assay (ELISA).

To analyse if and how lactulose prevents the toxic effects of endotoxin, this *in vitro* model was used. The direct effect of lactulose on endotoxin was evaluated with a modified chromogenic limulus amoebocyte lysate assay. As lactulose preparations used in clinical practice can be divided in two groups, a crystalline pure lactulose and lactulose syrup containing galactose and lactose besides lactulose, both preparations were tested.

Methods

CHEMICALS AND ANTIBODIES

Lactulose syrup (Duphalac®) was kindly provided by Duphar (Weesp, The Netherlands). Crystalline lactulose (Legendal®) was a gift from Inpharzam (Almere, The Netherlands). Lactitol was kindly provided by CGA biochemicals (Gorichem, The Netherlands). Lipopolysaccharide *E coli* 0111:B4 chromatographically purified, phorbol myristate acid, Polymyxin B sulphate (8000 U/mg), latex beads (1·091 μm diameter), bovine serum albumin, and o-phenylenediamine were obtained from Sigma Chemical Co (St Louis, MO). Human recombinant tumour necrosis factor was a gift of BASF/Knoll AG (Ludwigshaven, FRG). Monoclonal antibodies against recombinant tumour necrosis factor were derived from hybridomas obtained by standard cell fusion procedures. Rabbit polyclonal antibodies against tumour necrosis factor were raised by immunising rabbits with recombinant tumour necrosis factor. Peroxidase conjugated goat antirabbit IgG was purchased from

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MONONUCLEAR CELLS FROM HUMAN PERIPHERAL BLOOD

Peripheral blood mononuclear cells from buffy coats of healthy blood donors (kindly provided by the local blood bank) were purified by buoyant density centrifugation on Lymphoprep (Nycomed AS, Oslo, Norway). The interface layer was collected and washed twice in Hanks balanced salt solution. The peripheral blood mononuclear cells were resuspended in culture medium, consisting of RPMI 1640 (Gibco, Paisley, Scotland) supplemented with 10% bovine calf serum (Hyclone, Logan, UT) and streptomycin 100 μg/ml penicillin 100 IU/ml (Flow, Irvine, UK), at a concentration of 5×10^7/ml. Monocytes were further isolated as described elsewhere.[20] In short, the suspension was spun in polypropylene tubes at 20 rpm, 4°C to induce monocyte clumping. The clumped monocytes were separated from lymphocytes by sedimentation on ice cold bovine calf serum for 20 min. Monocytes were resuspended in culture medium at a concentration of 10^5/ml and transferred to a 96 well tissue culture plate (Costar, Cambridge, MA) 100 μl/well. The remaining cells consisted of more than 90% monocytes as evaluated by phagocytosis and indirect immunofluorescence (data not shown).

TUMOUR NECROSIS FACTOR ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Tumour necrosis factor concentrations in the cell free supernatants of the cell cultures were determined with a tumour necrosis factor specific ELISA as described previous.[21] In short: 96 well immuno plates (Nunc, Roskilde, Denmark) were coated with a monoclonal antitumour necrosis factor antibody. Test samples were added to the plate and incubated for three hours at room temperature. A standard titration curve was obtained by making serial dilutions of a known sample of recombinant tumour necrosis factor. Next the plates were washed and sequentially incubated with rabbit antitumour necrosis factor immune serum and peroxidase conjugated goat antirabbit IgG. After adding substrate (o-phenylenediamine 0·43 mg/ml) the colour reaction was stopped and light absorption (at 492 nm) was measured with a microelisa autoreader (Flow, Irvine, UK). The ELISA has a lower detection limit of 10 pg/ml. The tumour necrosis factor-assay was neither affected by both lactulose preparations nor by Polymyxin B. Extinctions as measured with the ELISA of different concentrations of recombinant tumour necrosis factor as well as natural, monocyte derived, human tumour necrosis factor in presence of these compounds differed less than 5% from control values.

ENDOTOXIN ASSAY

Endotoxin was measured with a modified chromogenic limulus amoebocyte lysate test (Coatest® endotoxin, Kabivitrum, Stockholm, Sweden).[22 23] To evaluate the direct inactivating effect of lactulose on endotoxin crystalline lactulose or lactulose syrup dissolved in pyrogen free water (NPBI, Emmer-Compascuum, The Netherlands), at concentrations similar to those used in the cell culture experiments, were incubated for two hours at 37°C with different concentrations of lipopolysaccharide. As control Polymyxin B, a known inactivator of endotoxin, was incubated at a concentration of 25 μg/ml with lipopolysaccharide. After incubation the remaining endotoxin was measured with the limulus amoebocyte lysate assay. All glassware used in the experiments was rendered endotoxin free by heating for three hours at 180°C. Addition of lactulose or Polymyxin B just before the limulus lysate or the substrate step of the limulus amoebocyte lysate assay did not affect recovery of standard samples of lipopolysaccharide. This indicates that both the lysate reaction and the substrate activation are not influenced by both substances.

CELL CULTURE EXPERIMENTS

The isolated monocytes, as described above, were allowed to settle for two hours after which culture medium was replaced with culture medium to which combinations of lipopolysaccharide, phorbol myristate acid, crystalline lactulose, lactulose syrup, lactitol, and Polymyxin B were added. In the first series of experiments a serial dilution of lipopolysaccharide was preincubated for two hours with crystalline lactulose, lactulose syrup, lactitol, or Polymyxin B. In the second series of experiments different concentrations of crystalline lactulose, lactulose syrup, and Polymyxin B were preincubated for two hours with a serial dilution of lipopolysaccharide. In the third series of experiments phorbol myristate acid was used to stimulate the tumour necrosis factor production of monocytes, again after preincubation for two hours with crystalling lactulose, lactulose syrup, lactitol, and Polymyxin B. In both experiments monocytes stimulated with lipopolysaccharide or phorbol myristate acid in culture medium served as positive controls whereas monocytes cultured in culture medium were used as negative controls. All cultures were performed at 37°C in a moist 8% carbon dioxide air atmosphere. Lactulose syrup, crystalline lactulose, and lactitol used in the cell culture experiments were diluted in pyrogen free water in order to reach an osmolarity of 300 mosmol. Thereafter lactulose syrup was diluted in culture medium to obtain a concentration of ±7 mg/ml of lactulose. Crystalline lactulose and lactitol were diluted in culture medium to a concentration of ±10 mg/ml. The difference in lactulose content between the two lactulose solutions is caused by the presence of the monosaccharides lactose and galactose which account for 30% of the osmolarity of undiluted lactulose syrup. In all experiments osmolarity and pH of culture medium after addition of the different compounds were determined. Osmolarity ranged from 300 to 320 mosmol, pH from 7·14 to 7.24. The values of culture medium were 307 mosmol and pH 7·15.

TABLE Effect of lactulose and Polymyxin B on endotoxin measured with the limulus amoebocyte lysate assay

	A	B	C
Control	7·6 (8·1)	43·3 (14·0)	165 (18·8)
Crystalline lactulose	20·2 (13·4)	72·3 (8·6)	149·8 (20·9)
Lactulose syrup	2·3 (4·8)	14·5 (5·8)	85 (6·5)
Polymyxin B	1·8 (2·1)	6·7 (5·9)	11·2 (7·9)

Endotoxins (in three concentrations A, B, C), dissolved in endotoxin free water, were incubated with lactulose syrup (7 mg lactulose/ml), crystalline lactulose (10 mg lactulose/ml), and Polymyxin B (25 µg/ml) for two hours at 37°C. Untreated lipopolysaccharide dissolved in endotoxin free water served as control. Remaining endotoxin was measured with a modified chromogenic limulus amoebocyte lysate assay. Results, expressed in pg lipopolysaccharide/ml, are mean (SD) of 6 values.

Experiments lasted for 16–18 hours after which cell free culture supernatants were harvested for analysis. The supernatants were assayed for presence of tumour necrosis factor directly or were stored at −20°C until analysis. Experiments were performed in duplicate or quadruplicate as given below. All experiments were repeated at least three times using monocytes from different blood donors.

VIABILITY OF MONOCYTES

Viability of the monocytes was examined after the experiments by trypan blue exclusion and by phagocytosis of latex beads. Latex beads were washed four times in phosphate buffered saline and a 0·01% (v/v) suspension was made in culture medium. The latex beads were added to the monocytes. After incubation for two hours at 37°C remaining latex beads were removed and percentage phagocytosis was assessed by counting the monocytes containing two or more latex beads. The percentage trypan blue excluding, viable, cells was assessed by counting 200 cells per cell culture.

Results

EFFECT OF LACTULOSE AND POLYMYXIN B ON LIMULUS AMOEBOCYTE LYSATE ACTIVATION BY ENDOTOXIN

The concentrations of both lactulose prepara-

Figure 1: Inhibitory effect of lactulose syrup, crystalline lactulose, and Polymyxin B on tumour necrosis factor (TNF) production by monocytes stimulated with lipopolysaccharide (LPS). Human peripheral blood monocytes were incubated with a serial dilution of LPS and the same amounts of LPS after preincubation with lactulose syrup (7 mg lactulose/ml), crystalline lactulose (10 mg lactulose/ ml), and Polymyxin B (25 µg/ml). Tumour necrosis factor production was measured in cell free culture supernatant with an ELISA. Data points represent mean (SD) of 4 values.

tions used to inactivate endotoxin were similar to the concentrations used in the cell culture experiments. Lactulose syrup (Duphalac) inactivated low amounts of lipopolysaccharide significantly, however at increasing amounts of lipopolysaccharide this inactivating effect of lactulose decreased, being reduced to approximately 50% at an endotoxin concentration of 150 pg/ml (Table). The crystalline, 99% pure, lactulose (Legendal) did not inactivate lipopolysaccharide as measured with the limulus amoebocyte lysate assay.

As a positive control Polymyxin B, a known inactivator of lipopolysaccharide, was used. In contrast with both lactulose preparations Polymyxin B inactivated the amount of lipopolysaccharide used in this experiment nearly completely.

EFFECT OF LACTULOSE, LACTITOL, AND POLYMYXIN B ON THE TUMOUR NECROSIS FACTOR PRODUCTION BY MONOCYTES STIMULATED WITH ENDOTOXIN
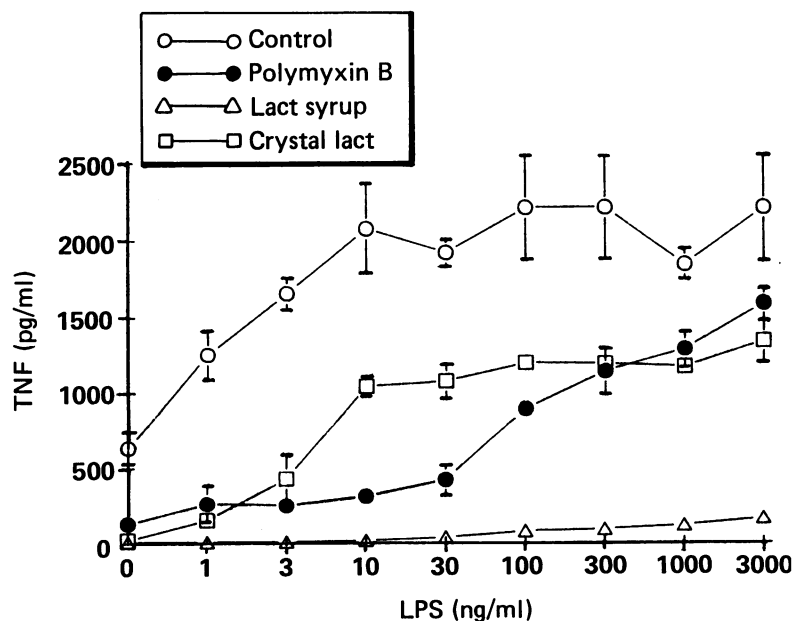
Stimulation of monocytes with endotoxin resulted in a high tumour necrosis factor release. Endotoxin dosages of 1 ng lipopolysaccharide/ml caused tumour necrosis factor productions which were on average above 1000 pg/ml (Figs 1–3). The tumour necrosis factor production rapidly increased at higher lipopolysaccharide dosages reaching a plateau at 30–100 ng lipopolysaccharide/ml, after which a much slower increase of tumour necrosis factor production in relation to the lipopolysaccharide dose was found. Measuring tumour necrosis factor production by monocytes was a highly sensitive and reproducible way to detect the presence of biological active lipopolysaccharide.

Both lactulose preparations inhibited tumour necrosis factor production by monocytes after stimulation with endotoxin significantly (Fig 1). Lactulose syrup appeared to be a more effective inhibitor of tumour necrosis factor production, than crystalline lactulose. At 7 mg lactulose/ml lactulose syrup reduced lipopolysaccharide induced tumour necrosis factor production by monocytes with 95% at all concentrations of lipopolysaccharide used, whereas crystalline lactulose in similar concentrations reduced tumour necrosis factor production with 15–20%. Lactitol at 10 mg/ml did not inhibit endotoxin induced tumour necrosis factor production by monocytes (data not shown in figure).

As expected Polymyxin B, a proven inactivator of endotoxins, inhibited tumour necrosis factor production by monocytes after stimulation with endotoxins. With 25 µg Polymyxin B/ml on average 30 ng lipopolysaccharide/ml was inactivated.

The effect of both lactulose preparations and Polymyxin B on the endotoxin induced tumour necrosis factor production by monocytes was further studied by testing different concentrations of these agents. Crystalline lactulose and lactulose syrup inhibited concentration dependent lipopolysaccharide induced tumour necrosis factor production by monocytes (Figs 2, 3). Lactulose syrup reduced lipopolysaccharide induced tumour necrosis factor production at 7,
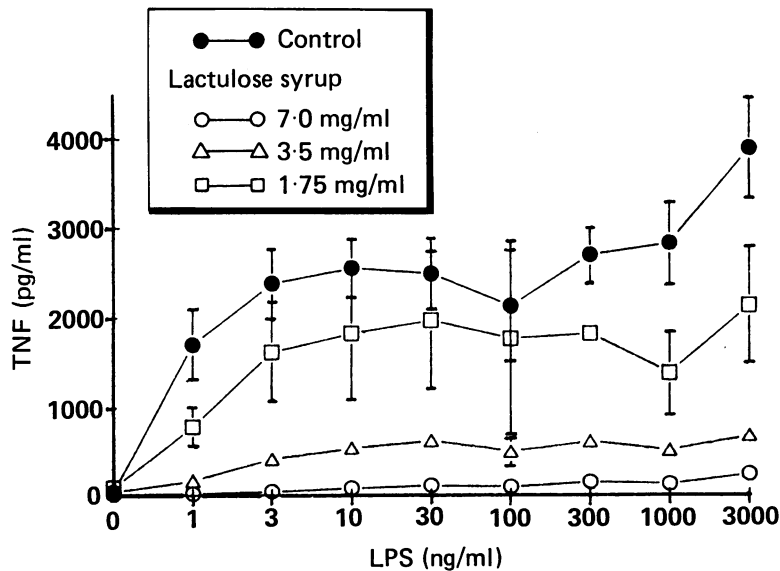
Figure 2: Effect of different concentrations of lactulose syrup on lipopolysaccharide (LPS) induced tumour necrosis factor (TNF) production by monocytes. Monocytes were incubated with a serial dilution of lipopolysaccharide preincubated with lactulose syrup in concentrations of 7, 3·5, and 1·75 mg lactulose/ml. Untreated lipopolysaccharide served as control. Tumour necrosis factor production was measured in cell free culture supernatants with an ELISA. Data Points represent mean (SD) of 4 values.

3·5, and 1·75 mg lactulose/ml with 95, 75, and 30% respectively. Crystalline lactulose reduced lipopolysaccharide induced tumour necrosis factor production with approximately 25, 15, and 0% at concentrations of 10, 5, and 2·5 mg lactulose/ml. This inhibitory effect of both lactulose preparations was independent from the concentration of endotoxin used. Polymyxin B also reduced concentration dependent the tumour necrosis factor production of monocytes stimulated with endotoxin. With concentrations of 25, 5, and 1 μg Polymyxin B/ml the effect of 30, 10, and 1 ng lipopolysaccharide/ml was inhibited. In contrast with lactulose, however, this inhibitory effect was limited to a maximum
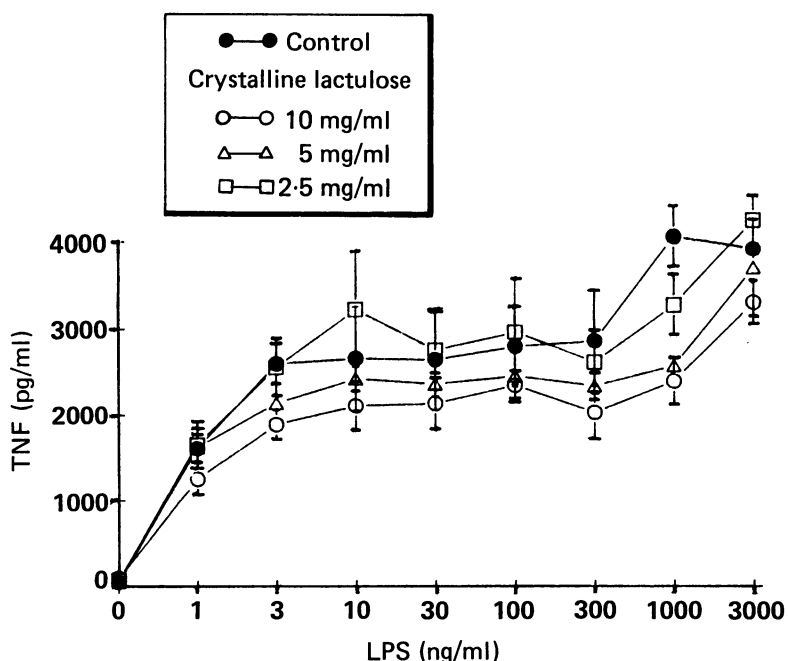

Figure 3: Effect of different concentrations of crystalline lactulose on lipopolysaccharide (LPS) induced tumour necrosis factor (TNF) production by monocytes. Monocytes were incubated with a serial dilution of LPS preincubated with crystalline lactulose in concentrations of 10, 5, and 2·5 mg lactulose/ml. Tumour necrosis factor production was measured in cell free culture supernatants with an ELISA. Data points represent mean (SD) of 4 values.

dose of endotoxin, after which tumour necrosis factor production of Polymyxin B treated cells paralleled that of control monocytes (Fig 4).

TUMOUR NECROSIS FACTOR PRODUCTION BY MONOCYTES AFTER STIMULATION WITH PHORBOL MYRISTATE ACID, EFFECT OF LACTULOSE, LACTITOL AND POLYMYXIN B

To further analyse the effect of both lactulose preparations, lactitol, and Polymyxin B, on tumour necrosis factor production by monocytes, the phorbol myristate acid induced tumour necrosis factor production by monocytes was studied. Phorbol myristate acid induces tumour necrosis factor production by an activation of the intracellular messenger protein kinase C.

After stimulation with phorbol myristate acid monocytes produced high amounts of tumour necrosis factor. Crystalline lactulose and lactulose syrup in concentrations shown, significantly reduced phorbol myristate acid induced tumour necrosis factor production by monocytes (Fig 5). This reduction was equivalent to the inhibitory effect of both crystalline lactulose and lactulose syrup on endotoxin induced tumour necrosis factor production by monocytes. Lactitol did not inhibit phorbol myristate acid induced tumour necrosis factor production.

Polymyxin B did not affect phorbol myristate acid induced tumour necrosis factor production by monocytes, this in contrast to the endotoxin induced tumour necrosis factor production.

VIABILITY OF THE MONOCYTES AFTER THE EXPERIMENTS

After the cell culture experiments the viability of the monocytes was tested by trypan blue exclusion and phagocytosis of latex beads. Directly after removing supernatants for tumour necrosis factor measurements trypan blue solution or latex beads in culture medium were added to the cell cultures. Trypan blue exclusion was greater than 85% in all experiments. Phagocytosis of latex beads was clearly delayed, but not significantly reduced, after incubation with lactulose syrup whereas preincubation with crystalline lactulose or Polymyxin B did not affect phagocytosis as compared with control cells.

Discussion

The results of our experiments show that crystalline lactulose (Legendal) did not inactivate endotoxin as determined with the limulus amoebocyte lysate assay. Lactulose syrup (Duphalac) did inactivate low amounts of lipopolysaccharide, this effect decreased with increasing lipopolysaccharide concentrations.

Both lactulose syrup and crystalline lactulose inhibited tumour necrosis factor production of monocytes stimulated with lipopolysaccharide significantly. Polymyxin B also inhibited tumour necrosis factor production by monocytes after stimulation with endotoxin but the kinetics were
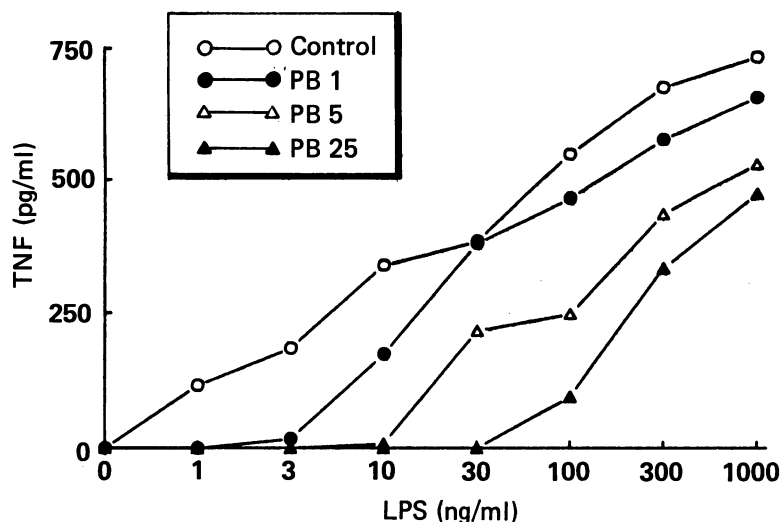
Figure 4: Effect of different concentrations of Polymyxin B on lipopolysaccharide (LPS) induced tumour necrosis factor (TNF) production by monocytes. Monocytes were incubated with a serial dilution of lipopolysaccharide preincubated with Polymyxin B in concentrations of 25, 5, and 1 µg/ml. Tumour necrosis factor production was measured in cell free culture supernatants with an ELISA. Results from a representative experiment are shown.

monocytes was not affected. Lactitol a disaccharide clinically used for similar indications as lactulose did not reduce tumour necrosis factor production by monocytes, indicating that the observed inhibitory effect is specific for lactulose.

Experiments with phorbol myristate acid, a direct stimulator of tumour necrosis factor production by monocytes which acts through activation of the intracellular messenger protein kinase C, supplied further evidence for a direct inhibitor effect of lactulose on monocytes. Both lactulose preparations inhibited the phorbol myristate acid induced tumour necrosis factor production by monocytes in a fashion similar to the inhibition of lipopolysaccharide induced tumour necrosis factor production. In contrast Polymyxin B, as expected, did not reduce tumour necrosis factor production by monocytes stimulated with phorbol myristate acid.

The data show that both lactulose preparations inhibit lipopolysaccharide induced as well as phorbol myristate acid induced tumour necrosis factor production by monocytes. In all cell culture experiments, however, lactulose syrup was more effective than similar amounts of crystalline lactulose in reducing tumour necrosis factor production by monocytes.

The effect of Polymyxin B as described in our experiments is similar to results of a study by Duff et al.[24] In these experiments the effect of Polymyxin B on production of endogenous pyrogen by monocytes after stimulation with endotoxin was studied. They found that lipopolysaccharide induced production of endogenous pyrogen was inhibited by Polymyxin B, whereas production of endogenous pyrogen after stimulation of monocytes with killed staphylococci could not be reduced by polymyxin B. This indicated that Polymyxin B inactivated lipopolysaccharide but did not affect the monocytes. The inhibitory effect of Polymyxin B on lipopolysaccharide but not on cells was further supported by results of Spear et al, who used Polymyxin B to inactivate contaminating lipopolysaccharide in mixed lymphocyte reactions and found that Polymyxin B did not affect [3H]thymidine incorporation.[25] Polymyxin B was used successfully as an antiendotoxin drug in a number of in vivo experiments, but its high toxicity severely limits its clinical use.[6-10] In contrast with Polymyxin B little is known of the antiendotoxin effect of lactulose.

Orally administrated lactulose prevented endotoxin related complications after surgery in jaundiced patients.[13] Moreover galactosamine induced liver necrosis, caused by endotoxins from the gastrointestinal tract, was prevented by oral pretreatment with lactulose.[14] Both studies reported a strongly reduced activation of the limulus amoebocyte lysate assay by endotoxin in presence of lactulose but these data differ from our observations. We found that lactulose inactivates only small amounts of endotoxin. The results of our study indicate, however, that the efficacy of lactulose can be explained by a direct effect on mononuclear phagocytes resulting in a reduced production of the mediator of endotoxin toxicity. Lehman et al showed that tumour necrosis factor appeared to be a central

clearly different from the inhibition caused by lactulose. Tumour necrosis factor production of the monocytes stimulated with Polymyxin B treated lipopolysaccharide parallelled that of control cells when a maximum amount of lipopolysaccharide was passed. Apparently Polymyxin B could only inactivate a certain amount of endotoxin related to the amount of Polymyxin B used. In contrast both lactulose preparations inhibited tumour necrosis factor production by monocytes independent from the concentration of endotoxin used. These data indicate that where Polymyxin B was effective by actually inactivating endotoxin, both lactulose preparations probably prevented the production of the mediator of endotoxin toxicity by a direct effect on the cells. The inhibitory effect of both lactulose preparations or Polymyxin B in the experiments could not be explained by a cytolytic effect on the cells as the viability of
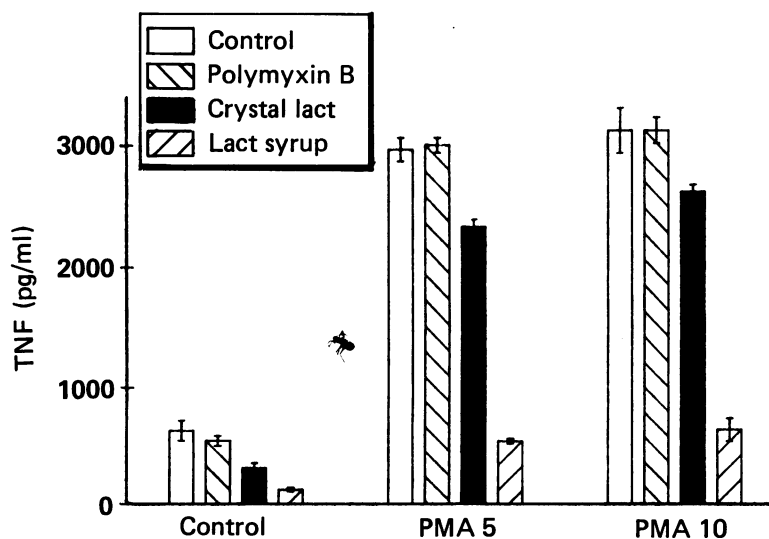

Figure 5: Effect of both lactulose preparations and Polymyxin B on the phorbol myristate acid (PMA) induced tumour necrosis factor production by monocytes. Monocytes were incubated with PMA in concentrations of 5 and 10 ng/ml in the presence of lactulose syrup (7 mg lactulose/ml), crystalline lactulose (10 mg lactulose/ml), and Polymyxin B 25 µg/ml. Unstimulated monocytes and monocytes stimulated with PMA served as control. Bars represent mean (SD) of 6 values.

mediator of endotoxin mediated mortality in mice treated with D-galactosamine and endotoxin.[19] These results indicate that inhibition of tumour necrosis factor production can prevent endotoxin induced complications.

That lactulose treatment not only causes an acidification and/or hyperosmolarity of the bowel lumen, but can also have an effect on metabolism of bacteria and different cells in the bowel wall was suggested by a number of studies on lactulose treatment of hepatic encephalopathy in patients with liver cirrhosis. Diminished ammonia production after lactulose treatment was explained by alteration of bacterial flora in the gut, by changes of bacterial metabolism, and recently by an effect of lactulose on metabolism of crypt and villus cells of the bowel wall.[26-30]

Lactulose under normal conditions is only absorbed in small amounts from the gastrointestinal tract (approximately 1·5% of the administrated oral dose is excreted in urine).[31] In situations of mucosal damage, stress, or infection, however, the absorption of lactulose from the bowel is greatly increased.[32-34] It is in these situations where substantial amounts of lactulose penetrate the bowel wall that it is probably most effective. Our observations show that lactulose can have a direct effect on cell function. Lactulose inhibited the release of tumour necrosis factor, the mediator of endotoxin toxicity, by an inhibitory effect on mononuclear phagocytes. The effect of lactulose could not be explained by an inactivation of endotoxin. Hypothetically this inhibitory effect of lactulose on the production of tumour necrosis factor may explain the beneficial effect of lactulose as supportive preoperative treatment in jaundiced patients.

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