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Endotoxaemia and cytokine production in experimental colitis

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Systemic endotoxaemia is a well recognized feature of inflammatory bowel disease but its pathogenic role remains uncertain. This study examined plasma endotoxin and cytokine concentrations and the acute-phase protein response in a hapten-induced model of experimental colitis. On days 2, 8 and 14 after induction of colitis with trinitrobenzenesulphonic acid in ethanol (TNBS–E), plasma endotoxin, immunoglobulin (Ig) G and IgM endotoxin-core antibody (EndoCAb), tumour necrosis factor (TNF), interleukin (IL) 6 and α_2-macroglobulin (α_2M) concentrations and colon macroscopic inflammation score were determined. At all time points there was significant colonic inflammation when compared with control values ($P < 0{\cdot}0001$). Animals treated with TNBS–E had raised concentrations of endotoxin at all time points ($P < 0{\cdot}04$). In TNBS–E-treated animals EndoCAb concentrations were reduced on day 2 ($P < 0{\cdot}0001$) and later increased. There were increases in IL-6 and α_2M concentrations in TNBS–E-treated animals but no significant change in TNF concentrations. Endotoxin concentrations correlated with macroscopic inflammation score, IL-6 and α_2M concentrations. There was a less consistent correlation between EndoCAb concentrations and these parameters. These results suggest that endotoxin is a mediator of the systemic response in this model of experimental colitis.

In inflammatory bowel disease (IBD) intraluminal antigens may initiate or exacerbate mucosal inflammation[1]. Possible antigens include whole bacteria[2–5] and their products such as endotoxin[6], formylated oligopeptides[7] or peptidoglycans[8]. Following disruption of the mucosal barrier, bacteria or their products may enter the portal circulation and overwhelm hepatic clearance capacity, resulting in systemic bacteraemia and endotoxaemia.

Endotoxaemia is a well recognized feature of both ulcerative colitis and Crohn's disease[6,9]. However, its precise role in the inflammatory process remains uncertain. In experimental animals administration of endotoxin increases mesenteric vascular and gastrointestinal permeability and promotes bacterial translocation[10–12]. Endotoxin is thought to be responsible for most, if not all, of the features of Gram-negative septicaemia[13]. There is evidence that these effects of endotoxin are mediated by cytokines such as tumour necrosis factor (TNF), interleukin (IL) 1 and IL-6[14,15]. Production of these proinflammatory cytokines is increased in IBD[16–18] and may contribute to the hepatic synthesis of acute-phase reactants such as α_1-glycoprotein and C-reactive protein. These acute-phase proteins have been shown to correlate with clinical indices of disease activity[19].

The aim of this study was to investigate the hypothesis that endotoxin penetrates the mucosal barrier in a model of experimental colitis[20] and contributes to both mucosal inflammation and systemic disease activity.

Materials and methods

Male Wistar rats ($n = 72$) weighing 275–325 g were studied. They were housed in groups of six in wire-mesh cages and in controlled conditions (temperature 22°C; 12 h light–dark cycle). Animals were given free access to standard laboratory pelleted formula and tap water.

Induction of colitis

Animals were fasted for 20 h before induction of colitis. They were anaesthetized by intramuscular injection of 1 ml/kg Vetalar (ketamine 100 mg/ml; Parke-Davis, Pontypool, UK) and 0·5 ml/kg Rompun (xylazine 2 per cent solution; Bayer UK, Bury St Edmunds, UK). A 5-Fr polypropylene cannula (Bardic feeding tube 1732; Bard, Sunderland, UK) was inserted into the rectum until the tip was 8–10 cm proximal to the anus at approximately the splenic flexure. A solution of 30 mg 2,4,6-trinitrobenzenesulphonic acid (Sigma Chemicals, Poole, UK) in 0·5 ml 50 per cent ethanol (TNBS–E) was instilled. Controls were given an isovolumetric bolus of normal (0·9 per cent) saline. The rats were then maintained in a supine Trendelenberg position until recovery from anaesthesia to prevent early leakage of the intracolonic instillate.

Assessment

The experimental end points were 2, 8 and 14 days (12 TNBS–E-treated animals and 12 saline controls at each time point). Under general anaesthesia, as before, a midline laparotomy incision was made and blood was aseptically withdrawn from the inferior vena cava with a heparinized syringe (20 units heparin per ml blood). The colon was then excised for inflammatory assessment[21] by an independent pathologist using a colon macroscopic inflammation score (CMS) of 0–10.

Plasma analysis. Blood was centrifuged (400*g*) for 10 min at 4°C and plasma was stored in sterile cryotubes (Nunc 363401; Intermed, Roskilde, Denmark) at −80°C until analysis.

Endotoxin was assayed with a chromogenic Limulus lysate assay (Coatest Endotoxin; Kabi Diagnostica, Molndal, Sweden) as previously described[22]. A microtitre plate system was used. The assay sensitivity was 8·3 pg/ml.

Endotoxin-core antibody (EndoCAb) immunoglobulin (Ig) G and IgM concentrations were determined with a microtitre plate enzyme-linked immunosorbent assay (ELISA)[23]. Results were expressed as a percentage of values derived from normal controls.

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TNF was analysed with a microtitre plate bioassay incorporating WEHI 164 subclone 13 cells[24]. The assay sensitivity was less than 1·0 pg/ml.

IL-6 was analysed with a microtitre plate bioassay incorporating B9 hybridoma cells[25]. The assay sensitivity was 33 pg/ml.

α_2-Macroglobulin (α_2M) was assayed with a single radial immunodiffusion assay[26]. The assay sensitivity was 0·05 g/l.

Tetanus toxoid antibody response

In a separate experiment to assess the specificity of the IgM EndoCAb response, 20 rats were immunized on two occasions (56 and 28 days before induction of colitis) with tetanus toxoid vaccine (Tetavax; Merieux, Maidenhead, UK) and blood samples were taken before induction of colitis ($n = 13$) or instillation of saline ($n = 7$) and again 8 days later. Plasma samples were analysed for tetanus toxoid and EndoCAb using the same microtitre plate ELISA as above. For tetanus toxoid antibody determination, plates were coated with *Clostridium tetani* instead of endotoxin.

Statistical analysis

The Mann–Whitney *U* test was used for comparison between groups and correlations were assessed with the Spearman rank correlation coefficient. $P < 0·05$ was considered significant.

Results

Assessment of illness

Administration of TNBS–E resulted in diarrhoea, rectal bleeding and increased mucus production with associated anorexia, weight loss and reduced activity. These changes were most marked during the first 2 days. Normal bowel function, weight gain and activity were observed in the saline controls throughout the experimental period.

Colonic inflammation

In comparison with saline controls, animals treated with TNBS–E had a significant increase in the CMS (*Table 1*).

Endotoxin-core antibody and tetanus toxoid titres

Systemic endotoxin concentrations were significantly increased in animals given TNBS–E compared with controls at all time points (*Table 2*). Plasma EndoCAb concentrations were decreased on day 2 and later increased in animals treated with TNBS–E compared with saline controls (*Table 2*). In contrast there was no significant difference in tetanus toxoid concentrations 8 days after induction of colitis (*Fig. 1*).

Cytokine and acute-phase protein response

There was no significant difference in plasma TNF concentrations in animals given TNBS–E at any time point compared with controls (*Table 3*). Plasma IL-6 concentrations were increased on days 2 and 8 (*Table 3*). Plasma α_2M concentrations in TNBS–E-treated animals were increased at each time point (*Table 3*).

Table 1 Colon macroscopic score after intracolonic instillation of saline or trinitrobenzenesulphonic acid in ethanol

Study	Saline control	TNBS–E (colitis)	*P**
Day 2	0·5 (0–1)	9 (8–10)	<0·0001
Day 8	1 (0–1)	5 (3–7)	<0·0001
Day 14	0 (0–1)	5 (2–7)	<0·0001
Tetanus toxoid (day 8)	0 (0–1)	7 (5–9)	0·0001

Values are median (95 per cent confidence interval). TNBS–E, trinitrobenzenesulphonic acid in ethanol. *Mann–Whitney *U* test

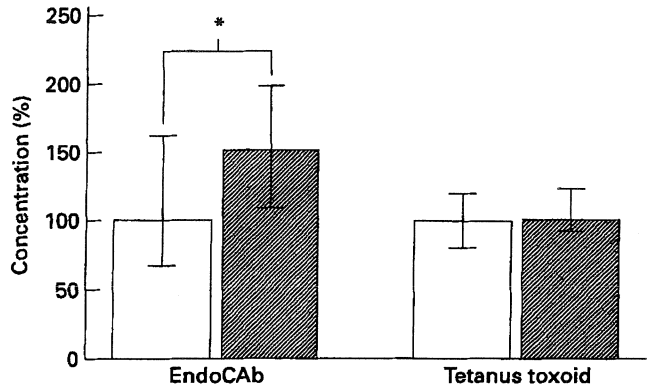

Fig. 1 Tetanus toxoid study. Plasma endotoxin-core antibody (EndoCAb) and tetanus toxoid concentrations before and 8 days after induction of colitis. Values are median (95 per cent confidence interval). □, Day 0; ▨, day 8. *$P = 0·01$ (Mann–Whitney *U* test)

Table 2 Plasma endotoxin and endotoxin-core antibody concentrations

Assay	Day	Saline control	TNBS–E (colitis)	*P**
Endotoxin (pg/ml)	2	0·0 (0·0–14·4)	357·6 (15·9–7297·0)	0·0005
	8	0·0 (0·0–0·0)	0·0 (0·0–73·7)	0·039
	14	0·0 (0·0–0·0)	12·7 (0·0–18·5)	0·033
Immunoglobulin G EndoCAb (%)	2	98 (85–118)	53 (50–68)	<0·0001
	8	135 (103–180)	161 (119–241)	n.s.
	14	110 (95–125)	136 (102–181)	0·005
Immunoglobulin M EndoCAb (%)	2	144 (113–187)	79 (72–96)	<0·0001
	8	144 (103–180)	219 (138–333)	0·02
	14	154 (127–190)	204 (95–265)	n.s.

Values are median (95 per cent confidence interval). TNBS–E, trinitrobenzenesulphonic acid in ethanol; EndoCAb, endotoxin-core antibody; n.s., not significant. *Mann–Whitney *U* test

Table 3 Plasma tumour necrosis factor, interleukin 6 and α_2-macroglobulin concentrations

Assay	Day	Saline control	TNBS–E (colitis)	*P**
TNF (pg/ml)	2	0·6 (0·0–4·1)	2·0 (0·0–20·5)	n.s.
	8	1·3 (0·0–2·2)	2·7 (0·0–4·7)	n.s.
	14	1·8 (1·0–3·2)	2·5 (1·3–4·5)	n.s.
IL-6 (pg/ml)	2	0·0 (0·0–96·0)	882·0 (334·0–3421·0)	<0·0001
	8	0·0 (0·0–0·0)	42·5 (0·0–289·0)	0·02
	14	0·0 (0·0–0·0)	0·0 (0·0–178·0)	n.s.
α_2M (g/l)	2	0·15 (0·07–0·32)	4·96 (4·32–7·20)	<0·0001
	8	0·08 (0·03–0·11)	0·51 (0·31–1·24)	<0·0001
	14	0·00 (0·00–0·07)	0·42 (0·10–1·44)	<0·0001

Values are median (95 per cent confidence interval). TNBS–E, trinitrobenzenesulphonic acid in ethanol; TNF, tumour necrosis factor; IL-6, interleukin 6; α_2M, α_2-macroglobulin; n.s., not significant. *Mann–Whitney *U* test

Table 4 Spearman rank correlation coefficient and *P* values for the relationship between endotoxin and endotoxin-core antibody concentrations and mucosal and systemic disease activity

		CMS		IL-6		α_2M	
Assay	Day	r_s	*P*	r_s	*P*	r_s	*P*
Endotoxin	2	0·64	0·03	0·74	0·008	0·58	0·05
	8	0·58	0·04	0·55	0·05	0·67	0·01
	14	0·64	0·04	0·75	0·02	n.c.	
IgG EndoCAb	2	n.c.		n.c.		n.c.	
	8	n.c.		n.c.		n.c.	
	14	0·68	0·02	n.c.		n.c.	
IgM EndoCAb	2	n.c.		n.c.		n.c.	
	8	n.c.		0·59	0·03	n.c.	
	14	0·81	0·004	n.c.		0·78	0·007

CMS, colon macroscopic inflammation score; IL-6, interleukin 6; α_2M, α_2-macroglobulin; Ig, immunoglobulin; EndoCAb, endotoxin-core antibody; n.c., no significant correlation

Relationship between systemic endotoxaemia, mucosal inflammation and systemic disease activity

In TNBS–E-treated animals, endotoxin concentrations correlated positively with CMS, IL-6 and α_2M (*Table 4*). The correlation of systemic EndoCAb concentrations with these parameters is less consistent (*Table 4*). In TNBS–E-treated animals IL-6 correlated with α_2M on day 2 ($r_s = 0{\cdot}83$ $P = 0{\cdot}001$,), day 8 ($r_s = 0{\cdot}86$ $P = 0{\cdot}0003$,) and day 14 ($r_s = 0{\cdot}71$, $P = 0{\cdot}02$). TNF concentrations did not correlate with systemic endotoxaemia.

Discussion

Systemic endotoxin concentrations were found to correlate with the severity of colitis as assessed by the CMS during both early and late disease. This is in keeping with the results of the clinical studies of Colin *et al.*[9] and Aoki[27]. In association with systemic endotoxaemia there was an initial reduction in both IgG and IgM EndoCAb concentrations in the experimental animals, with a subsequent increase. This may represent a consumption of antibody followed by a B-cell response and increased antibody production. A significant increase in IgG EndoCAb concentration has also been reported in patients with active Crohn's disease[28]. In the present study there was also an increase in the IgG EndoCAb concentration in controls on day 8 compared with day 2. This may be due to transient endotoxaemia secondary to minor mucosal trauma associated with the administration of saline. This would be in keeping with endotoxaemia that is a recognized feature following colonoscopy and radiological studies of the colon[29,30].

Circulating concentrations of TNF were not significantly raised in animals with experimental colitis in this study. This, however, does not exclude TNF as a major mediator in the inflammatory process. It is possible that the effects of TNF are short-lived or that TNF stimulates the inflammatory cascade within the mucosa without entering the systemic circulation in significant quantities. Recent studies showing increased mucosal and faecal TNF concentrations in experimental and clinical IBD[31–33] suggest that release of TNF may be important at the mucosal level.

In contrast to TNF, plasma concentrations of IL-6 were significantly increased in the experimental group. In addition, the IL-6 concentration correlated with systemic endotoxin and α_2M concentrations. This suggests that the effect of endotoxin on the acute-phase protein response may be mediated by an IL-6-dependent pathway. As the half-life of IL-6 is short (5 min) the raised circulating concentrations observed in this study suggest that there is continuous stimulation of IL-6-producing cells by endotoxin, of which there is an abundant supply within the intestinal lumen.

In conclusion, experimental induction of colonic inflammation is associated with significant systemic endotoxaemia, a cytokine response and acute-phase protein synthesis. These features are also seen in patients with IBD[6,27,29] and have been previously described in a variation of this animal model[34]. The positive correlation of systemic endotoxin concentration with the severity of colitis, the plasma cytokine concentration and the acute-phase protein response suggest that, in this model, gut-derived endotoxin may drive the systemic inflammatory response. The contribution that gut-derived endotoxin makes to this response could be further delineated by studying this model in a germ- and endotoxin-free environment. Alternatively the ability of an antiendotoxin antibody to abrogate the inflammatory response would provide confirmatory evidence for an aggravating role of endotoxin on mucosal inflammation.

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