

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_2 M) concentrations and colon macroscopic inflammation score were determined. At all time points there was significant colonic inflammation when

compared with control values ($P < 0.0001$). Animals treated with TNBS-E had raised concentrations of endotoxin at all time points ($P < 0.04$). In TNBS-E-treated animals EndoCab concentrations were reduced on day 2 ($P < 0.0001$) and later increased. There were increases in IL-6 and α_2 M concentrations in TNBS-E-treated animals but no significant change in TNF concentrations. Endotoxin concentrations correlated with macroscopic inflammation score, IL-6 and α_2 M 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 inflammation¹. Possible antigens include whole bacteria^{2–5} and their products such as endotoxin⁶, formylated oligopeptides⁷ or peptidoglycans⁸. 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 septicaemia¹³. 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 activity¹⁹.

The aim of this study was to investigate the hypothesis that endotoxin penetrates the mucosal barrier in a model of experimental colitis²⁰ 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 assessment²¹ by an independent pathologist using a colon macroscopic inflammation score (CMS) of 0–10.

Plasma analysis. Blood was centrifuged (400g) 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 described²². 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 cells²⁴. The assay sensitivity was less than 1.0 pg/ml.

IL-6 was analysed with a microtitre plate bioassay incorporating B9 hybridoma cells²⁵. The assay sensitivity was 33 pg/ml.

α_2 -Macroglobulin (α_2 M) was assayed with a single radial immunodiffusion assay²⁶. 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

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

Study	Saline control	TNBS-E (colitis)	<i>P</i> *
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

Table 2 Plasma endotoxin and endotoxin-core antibody concentrations

Assay	Day	Saline control	TNBS-E (colitis)	<i>P</i> *
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

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_2 M concentrations in TNBS-E-treated animals were increased at each time point (Table 3).

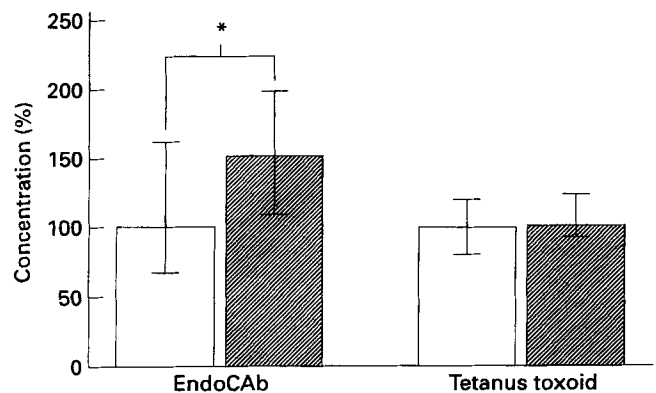


Fig. 1 Tetanus toxoid study. Plasma endotoxin-core antibody (EndoCAB) and tetanus toxoid concentrations before and 8 days after induction of colitis. Values are median (95 per cent confidence interval). □, Day 0; ▨, day 8. * $P = 0.01$ (Mann-Whitney *U* test)

Table 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_2 M (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_2 M, 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

Assay	Day	CMS		IL-6		α_2 M	
		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_2 M, 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_2 M (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_2 M on day 2 ($r_s = 0.83$ $P = 0.001$), day 8 ($r_s = 0.86$ $P = 0.0003$), and day 14 ($r_s = 0.71$, $P = 0.02$). 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 Aoki²⁷. 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 disease²⁸. 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 IBD³¹⁻³³ 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_2 M 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 model³⁴. 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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References

- Shorter RG, Huizenga KA, Spencer RJ. A working hypothesis for the etiology and pathogenesis of nonspecific inflammatory bowel disease. *Am J Dig Dis* 1972; 17: 1024-32.
- Seneca H, Henderson E. Normal intestinal bacteria in

- ulcerative colitis. *Gastroenterology* 1950; **15**: 34–9.
- 3 Ambrose NS, Youngs D, Burdon DW, Keighley MRB. Changes in intestinal microflora in Crohn's disease. *Br J Surg* 1982; **69**: 681 (Abstract).
 - 4 Heddle RJ, Shearman DJC. Serum antibodies to *Escherichia coli* in subjects with ulcerative colitis. *Clin Exp Immunol* 1979; **38**: 22–30.
 - 5 Auer IO, Roder A, Wensinck F, van de Merwe JP, Schmidt H. Selected bacterial antibodies in Crohn's disease and ulcerative colitis. *Scand J Gastroenterol* 1983; **18**: 217–23.
 - 6 Wellmann W, Fink PC, Benner F, Schmidt FW. Endotoxaemia in active Crohn's disease. Treatment with whole gut irrigation and 5-aminosalicylic acid. *Gut* 1986; **27**: 814–20.
 - 7 Nast CC, LeDuc LE. Chemotactic peptides. Mechanisms, functions, and possible role in inflammatory bowel disease. *Dig Dis Sci* 1988; **33**(Suppl): 50–7S.
 - 8 Sartor RB, Bond TM, Schwab JH. Systemic uptake and intestinal inflammatory effects of luminal bacterial cell wall polymers in rats with acute colonic injury. *Infect Immun* 1988; **56**: 2101–8.
 - 9 Colin R, Grancher T, Lemeland J-F *et al.* Recherche d'une endotoxémie dans les entéro-colites inflammatoires cryptogénétiques. *Gastroenterol Clin Biol* 1979; **3**: 15–19.
 - 10 Hutcheson IR, Whittle BJ, Boughton-Smith NK. Role of nitric oxide in maintaining vascular integrity in endotoxin-induced acute intestinal damage in the rat. *Br J Pharmacol* 1990; **101**: 815–20.
 - 11 Fink MP, Antonsson JB, Wang H, Rothschild HR. Increased intestinal permeability in endotoxic pigs. *Arch Surg* 1991; **126**: 211–18.
 - 12 Deitch EA, Berg R, Specian R. Endotoxin promotes the translocation of bacteria from the gut. *Arch Surg* 1987; **122**: 185–90.
 - 13 Shubin H, Weil M, Nishijima H. Clinical features associated with Gram-negative bacteremia. In: Urbaschek B, Urbaschek R, Neter E, eds. *Gram-negative Bacterial Infections and Mode of Endotoxin Actions: Pathophysiologic, Immunological, and Clinical Aspects*. Wien: Springer, 1975: 411–18.
 - 14 Beutler B, Cerami A. Cachectin and tumor necrosis factor as two sides of the same biological coin. *Nature* 1986; **320**: 584–8.
 - 15 Wong GG, Clark SC. Multiple actions of interleukin 6 within a cytokine network. *Immunol Today* 1988; **9**: 137–9.
 - 16 Gardiner KR, Halliday MI, Lloyd F, Stephens S, Rowlands BJ. Circulating tumour necrosis factor in active inflammatory bowel disease. In: Faist E, Meakins JL, Shildberg FW, eds. *Host Defense Dysfunction in Trauma, Shock and Sepsis: Mechanisms and Therapeutic Approaches*. Berlin: Springer, 1993: 699–703.
 - 17 Ligumsky M, Simon PL, Karmeli F, Rachmilewitz D. Role of interleukin 1 in inflammatory bowel disease: enhanced production during active disease. *Gut* 1990; **31**: 686–9.
 - 18 Mahida YR, Kurlac L, Gallagher A, Hawkey CJ. High circulating concentrations of interleukin-6 in active Crohn's disease but not ulcerative colitis. *Gut* 1991; **32**: 1531–4.
 - 19 Weeke B, Jarnum S. Serum concentrations of 19 serum proteins in Crohn's disease and ulcerative colitis. *Gut* 1971; **12**: 297–302.
 - 20 Morris GP, Beck PL, Herridge MS, Depew WT, Szwczuk MR, Wallace JL. Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology* 1989; **96**: 795–803.
 - 21 Wallace JL, MacNaughton WK, Morris GP, Beck PL. Inhibition of leukotriene synthesis markedly accelerates healing in a rat model of inflammatory bowel disease. *Gastroenterology* 1989; **96**: 29–36.
 - 22 Gardiner KR, Anderson NH, McCaigue MD, Erwin PJ, Halliday MI, Rowlands BJ. Adsorbents as antiendotoxin agents in experimental colitis. *Gut* 1993; **34**: 51–5.
 - 23 Jackson SK, Parton J, Shorthead G, Stark JM, Thompson EN. Serum immunoglobulins to endotoxin core glycolipid: establishment of normal concentrations. *Arch Dis Child* 1990; **65**: 768–70.
 - 24 Espevik T, Nissen-Meyer J. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J Immunol Methods* 1986; **95**: 99–105.
 - 25 Helle M, Boeije L, Aarden LA. Functional discrimination between interleukin 6 and interleukin 1. *Eur J Immunol* 1988; **18**: 1535–40.
 - 26 Jennings G, Elia M. The acute-phase response to turpentine-induced abscesses in malnourished rats at different environmental temperatures. *Metabolism* 1992; **41**: 141–7.
 - 27 Aoki K. A study of endotoxaemia in ulcerative colitis and Crohn's disease. I. Clinical study. *Acta Med Okayama* 1978; **32**: 207–16.
 - 28 Gardiner KR, Barclay GR, McCaigue MD *et al.* Endotoxin, tumour necrosis factor and anti-core glycolipid antibodies in inflammatory bowel disease. *Br J Surg* 1991; **78**: 1488 (Abstract).
 - 29 Kiss A, Ferenci P, Graninger W, Pamperl H, Potzi R, Meryn S. Endotoxaemia following colonoscopy. *Endoscopy* 1983; **15**: 24–6.
 - 30 Williams SM, Harned RK. Recognition and prevention of barium enema complications. *Curr Probl Diagn Radiol* 1991; **20**: 123–51.
 - 31 Murch SH, Braegger CP, Walker-Smith JA, MacDonald TT. Location of tumour necrosis factor α by immunohistochemistry in chronic inflammatory bowel disease. *Gut* 1993; **34**: 1705–9.
 - 32 Braegger CP, Nicholls S, Murch SH, Stephens S, MacDonald TT. Tumour necrosis factor alpha in stool as a marker of intestinal inflammation. *Lancet* 1992; **339**: 89–91.
 - 33 Neilly P, Campbell G, Gardiner K, Kirk S, Rowlands B. Faecal and systemic tumour necrosis factor in experimental inflammatory bowel disease. *Intensive Care Med* 1994; **20**(Suppl 1): S71 (Abstract).
 - 34 Brand HS, Maas GV, Bosma A, van Ketel RJ, Speelman P, Chamuleau RAFM. Experimental colitis in rats induces low-grade endotoxemia without hepatobiliary abnormalities. *Dig Dis Sci* 1994; **39**: 1210–15.