Non-lymphoid and lymphoid cells in acute, chronic and relapsing experimental colitis

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(Accepted for publication 19 October 1994)

SUMMARY

In rodents, intracolonic administration of ethanol 30% induces an acute colitis, while administration of 2,4,6-trinitrobenzene sulphonic acid (TNBS) in ethanol induces a longer lasting colitis. In the acute and chronic stages of experimental colitis, lymphoid and non-lymphoid cells were studied in the colon by immunohistochemistry. During the acute inflammation a high damage score of the colon was observed, which was related to an increase in the number of macrophages and granulocytes. Also a change in distributional patterns of macrophage subpopulations was found. The chronic stage of TNBS–ethanol-induced colitis was characterized by an increase in the number of lymphocytes, especially T cells. These data suggest that macrophages and granulocytes are important in the acute phase of experimental colitis, while lymphocytes play a pivotal role in the chronic stage. As most inflammatory bowel disease (IBD) patients have relapses during the chronic disease, we attempted to induce a relapse during experimental colitis by giving a second i.p. or s.c. dose of TNBS. This resulted in increased damage scores of the colon, new areas of ulceration and a further increase in macrophage numbers. No effect on the number of granulocytes was seen. These results indicate that it is possible to mimic relapses in experimental colitis by a second administration of TNBS, and suggest that the rats had been sensitized by the first dose of TNBS, given into the colon.

Keywords TNBS colitis immunohistochemistry rat

INTRODUCTION

The etiology of inflammatory bowel diseases (IBD), like Crohn's disease (CD) and ulcerative colitis (UC), remains unclear. Several studies indicate the involvement of macrophages and dendritic cells during active IBD. Large numbers of macrophages can be detected in the colon of patients with CD [1,2]. Furthermore, differences are found, not only in the number, but also in the heterogeneity of macrophage subpopulations and dendritic cells in colon from IBD patients compared with healthy controls and compared with intestinal inflammation due to other causes than CD and UC [1,3]. After treatment, the appearances returned to normal, indicating that the alterations seen in the macrophage populations in IBD are not primary to the disease process [4]. Although the recruitment of macrophages into the gut in IBD may be secondary to unknown primary events in the mucosa, these data indicate that various subpopulations of local non-lymphoid cells such as macrophages and dendritic cells could be important in the pathogenesis of IBD. Macrophages can play a role in the etiology of IBD by producing several cytokines, which are important mediators of inflammation and immunity. Tissue concentrations of these cytokines correlate with disease activity because they are dramatically increased with active inflammation and tend to revert to normal during remission [5].

Several animal models for intestinal inflammation are described, and they have yielded much information on the onset and course of acute stages of intestinal inflammation. Morris et al. [6] have developed an animal model in which colonic inflammation lasts at least 2 months, and which is induced by a single rectal administration of 2,4,6-trinitrobenzene sulphonic acid (TNBS) in ethanol. In this model it is hypothesized that ethanol functions as a mucosal barrier-breaker, enabling TNBS to bind covalently to proteins of colonic cells and modify cell surface proteins. These altered cells can then be taken up by macrophages, which become activated and may present antigen to T cells [7].

Although this model induces a long lasting colitis, the inflammation gradually heals and damage scores improve.
Furthermore, no relapses are seen in this model, whereas they are an important feature of the human disease. To be able to study chronic colitis with relapses, the effect of repeated TNBS
administration in intestinal inflammation and in mimicking relapses was evaluated. The aim of the present study was to investigate the contribution of macrophage subpopulations and of lymphocytes in the colon, during acute, chronic and relapsing experimental colitis.

MATERIALS AND METHODS

Animals and drugs
Male specific pathogen-free (SPF) Wistar rats, weighing about 200 g, were obtained from Harlan (Zeist, The Netherlands). They were kept under routine conditions with free access to commercial rat food and tap water.

TNBS was obtained from Sigma Chemical Co. (St Louis, MO).

Experimental design

Induction of colitis in groups A, B and C. Colitis was induced by intracolonic administration of ethanol 30% or by TNBS dissolved in ethanol, as described by Morris et al. [6] with minor modifications. Under Hypnorm anaesthesia, each rat received ethanol or 30 mg TNBS in 0.25 ml ethanol 30% using a rubber catheter (diameter 1.5 mm) inserted approximately 8 cm into the colon. After induction of colitis, the catheter was removed. The rats were checked daily with respect to their general condition, body weight and consistency of stools. In all groups, rats were killed under anaesthetics by CO₂ intoxication. Morphological changes of the colon were examined by at least three independent investigators. Any visible damage was scored on a 0–5 scale, as described by Morris et al. [6]. Parts of inflamed and non-inflamed colon were collected and frozen in liquid nitrogen for immunohistochemistry. Different cell types were studied using immunohistochemistry, and quantification of these cells was done by IBAS (Interaktives Bild Analysen System). Each of the following experiments was performed at least twice.

Group A. Colitis was induced at day 0 in a group of 12 rats with ethanol 30%. Four rats were killed at three different time points: day 7, day 14 and day 42.

Group B. Colitis was induced at day 0 in a group of 24 rats with 30 mg TNBS in ethanol 30%. Three animals were killed at eight different time points, i.e. day 1, day 7, day 14, day 28, day 42, day 56, day 70 and day 84.

Group C. Another group of 16 rats was used to study the effect of repeated TNBS administration on the course of the disease. For this purpose, 10 rats received an i.p. or s.c. injection of 30 mg TNBS (in 0.2 ml saline) 5 weeks after the induction of TNBS–ethanol-mediated colitis. Four control rats received saline either intraperitoneally or subcutaneously on the same day, and two others received no further treatment after induction of colitis. The animals were killed 7 days after the second administration of TNBS (or saline).

Immunohistochemistry

Cryostat sections of 8 μm were picked up on slides and air-dried. After fixing the slides for 10 min in pure acetone, a two-step immunoperoxidase staining was used. Slides were incubated horizontally for 60 min at room temperature with a solution of the first step MoAb in 0.01 M PBS pH 7.4, with 0.5% bovine serum albumin (BSA). The slides were washed three times in PBS and subsequently incubated with peroxidase-conjugated rabbit anti-mouse serum, dilution 1:200 (Miles, Elkhart, IN) in PBS with 0.5% BSA and 1% normal rat serum, for 30 min. After being rinsed in PBS (3 × 10 min), sections were stained for peroxidase activity with 3,3'-diaminobenzene-tetra-hydrochloride (Sigma, St Louis, MO) in 0.5 mg/ml Tris–HCl pH 7.6 containing freshly added 0.01% H₂O₂ [8]. After the slides had been washed in PBS, they were lightly counterstained with haematoxylin, dehydrated and mounted in Entellan (Merck, Darmstadt, Germany). Slides that were used for quantification with the Interactive Image Analysis System (IBAS) were counterstained using 0.01% nuclear fast red (Merck) in 5% Al₂(SO₄) to give good discrimination between cells and background. Control slides were incubated in PBS with 0.5% BSA in the first step, instead of the first specific antibody, and examined for non-specific staining. Staining with MoAbs ED1, ED2, ED3 (all raised in our laboratory), OX6, OX19, and OX33 (Serotec, Paris, France) was carried out on consecutive sections. ED1, ED2 and ED3 are all macrophage markers; ED1 recognizes CD68 [9,10], ED2 recognizes a differentiation membrane antigen [9,11], and ED3 recognizes sialoadhesin [9,12,13]. OX6 recognizes MHC class II antigen [14], OX19 is a T cell marker which recognizes CD5 [15] and OX33 is a B cell marker which recognizes CD45RA [16].

Quantification

Quantification was done by IBAS (Kontron Elektronik, Munich, Germany), an image analysis system. After appropriate noise reduction and contrast enhancement, eight microscopic fields of each colon were studied in three sections. Selection of measured fields was done by random assignment. Per microscopic field (obj. magnif. ×40) positive cells could be discriminated from the homogeneous, relatively colourless background. This was done in a semi-automatic way, i.e. the thresholds of the critical grey values were set interactively for each measurement. Thus the degree of colouration of a specimen did not affect the morphometric measurements. The percentage of positive cell staining was determined:

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\text{Percentage positive cell staining} = \frac{\text{field area of positive cells (in pixels)}}{\text{total field area (in pixels)}} \times 100% 
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To test the reproducibility of the quantification, the same fields were measured at different time points. Furthermore, to test the variation within the colon of individual rats, different fields of the same colon were measured. In both cases the observed differences were less than 0.5%. The use of IBAS to measure stained cells against a background in tissue sections was described before [17].

Statistical analysis

All data are expressed as mean ± s.d. The statistical significance of the differences was evaluated using the non-parametric Wilcoxon rank sum test. Statistical significance was defined as \( P < 0.05 \).
RESULTS

Group A: ethanol-induced colitis
All animals treated with ethanol 30% developed both clinical and histopathological symptoms, such as diarrhoea and transmural inflammation with or without ulceration. Mean damage scores are shown in Fig. 1.

Histologically an increase was seen in the number of macrophages and granulocytes, at day 7, especially in the submucosa (Fig. 2). After day 7 the number of granulocytes and macrophages started to decrease again. At day 14, there was no further significant difference in the number of macrophages between the ethanol-treated animals and healthy animals, but the number of granulocytes was still significantly increased in the submucosa. At day 42, no significant difference was observed between the ethanol-treated rats and healthy animals (Fig. 2). Local administration of ethanol had no effect on the number of T cells (results not shown).

Group B: TNBS-induced colitis
All animals treated with TNBS—ethanol developed both clinical and histopathological symptoms, such as diarrhoea and transmural inflammation with or without ulceration. Mean damage scores from day 1 to day 42 are shown in Fig. 1.

Histologically an influx was seen in the number of ED1+, ED2+ and ED3+ macrophages in the colon during the acute phase of inflammation. This increase in the amount of cells correlated with an increase in the damage score. In the submucosa, the number of ED1+ macrophages was significantly increased up to day 42 after induction of colitis. At day 7 the increase in ED1+ macrophages was as high as in the ethanol-treated group (Fig. 2b), but it lasted much longer. The number of ED2+ cells was significantly elevated up to day 84, whilst the amount of ED3+ cells was significantly increased from day 7 up to day 28. For all three subpopulations of macrophages, the highest influx in the submucosa was observed at day 7 after induction of colitis. In the mucosa, the influx was less than in the submucosa. ED3+ macrophages were not significantly increased in the mucosa, while the number of ED1+ cells was significantly elevated up to day 14. The amount of ED2+ macrophages was only significantly increased at day 14. Furthermore, we observed a redistribution of ED2+ macrophages from the basal to the upper part of the crypts. In the mucosa, all these subpopulations of macrophages showed the highest number of positive cells at day 14 after induction of colitis (Fig. 3a,b,c). Also, oedema of the submucosa with an enormous infiltration of cells was observed at day 7, while the mucosa at that time point was still almost normal. MHC class II expression, observed in the upper part of the lamina propria, was also significantly increased from day 7 up to day 28. In the submucosa, a significant increase in MHC class II expression was found from day 7 up to day 42 (Fig. 3d). In the mucosa as well as in the submucosa, we also observed an increased amount of granulocytes, in particular eosinophils and some neutrophils, from day 7 up to day 28 in the mucosa and up to day 14 in the submucosa (Fig. 3e). The amount of granulocytes in the submucosa at day 7 was as high as after administration of ethanol (Fig. 2c), but this increase lasted much longer. From day 7 an increase was seen in the number of T cells, and from day 14 also in the number of B cells (Fig. 4). These lymphocytes were not equally distributed over the tissue, but were concentrated in lymphoid accumulations in the submucosa.

Group C: repeated administration of TNBS
After a second administration of TNBS (either i.p. or s.c.) at day 35, after the initial exposure of the colon to TNBS—ethanol, damage scores were higher than in controls which received saline instead of TNBS (Fig. 1).

Histologically we found new areas of ulceration and an increase of macrophages, especially in the submucosa. For all three subpopulations of macrophages, this increase was significant in the submucosa, whereas in the mucosa only the increase in ED1+ macrophages was significant (Fig. 5a,b,c). Furthermore, we observed that the increase in the number of macrophages after the second administration of TNBS was comparable to the percentage of macrophages we found at day 7 after the first administration of TNBS. However, we did not observe a significant increase in the number of granulocytes compared with saline controls (Fig. 5d). Furthermore, there was no significant difference between the animals that received TNBS subcutaneously and the ones that received TNBS intraperitoneally for the second time.

DISCUSSION

Several studies report a general increase of mucosal macrophages and a change in subpopulations in IBD patients as recognized by immunohistochemistry [1–4]. The heterogeneity of these macrophages is more marked in inflamed than in uninflamed colonic mucosa. In the present study, we observed an increase in the number of macrophages in the colon, after administration of ethanol or TNBS—ethanol. After day 14, we no longer observed any significant differences with controls in...
Non-lymphoid and lymphoid cells in experimental colitis

Fig. 2. Difference in the percentage of macrophages and granulocytes between healthy controls (■) versus ethanol (■) and 2,4,6-trinitrobenzene sulphonic acid (TNBS)/ethanol-treated animals (■) on day 7, 14 and 42. (a) Difference in the percentage of ED1+ macrophages in the submucosa of the colon. (b) Difference in the percentage of granulocytes in the submucosa of the colon.

ethanol-induced colitis, which indicates that ethanol induces an acute inflammation. In TNBS–ethanol-induced colitis, a more chronic stage could be observed, which lasted about 8–12 weeks. Furthermore, after administration of ethanol as well as TNBS–ethanol, a change in the different subpopulations of macrophages was found. For example, ED3+ macrophages are usually only found in lymphoid organs. Outside the lymphoid organs, ED3 expression is only seen in several experimental autoimmune diseases, especially in the late and quiet effector stages [18,19]. This could indicate a suppressive role played by the ED3 determinant. In the present study, however, many ED3+ cells were found in the initial, acute phase of the inflammation. Recently it has been shown that ED3 is a marker for sialoadhesin, which functions as a macrophage restricted lymphocyte adhesion molecule [13]. The significance of the appearance of the ED3 determinant in acute and chronic inflammation remains to be established.

MHC class II expression was increased after induction of TNBS-mediated colitis. MHC class II is expressed by dendritic cells, macrophages, B cells, activated T cells, endothelial cells, and epithelial cells [20]. Although no relation was found between severity of inflammation in IBD patients and MHC class II expression on epithelial cells of these patients, unaffected areas showed no HLA-DR expression. In active IBD, colonic epithelial cells express HLA-DR, thus acting as antigen-presenting cells. Normal human epithelial cells present antigen to CD8+ T cells [21], while epithelial cells from IBD patients present antigen to CD4+ T cells [22]. This change in antigen presentation by epithelial cells may be a cause of IBD. In TNBS-induced colitis in the rat, however, no MHC class II expression was found on epithelial cells, which suggests that in rats expression of MHC class II on the epithelium does not play a role in the development of intestinal inflammation.

Lymphocytes are normally only present in the colon in small numbers, but after TNBS-induced colitis a slight increase was found in the number of B cells, and a striking increase was

Fig. 3. Macrophages, granulocytes and MHC class II expression in mucosa (——) and in submucosa (----) of 2,4,6-trinitrobenzene sulphonic acid (TNBS)/ethanol-induced colitis. (a) ED1+ macrophages. (b) ED2+ macrophages. (c) ED3+ macrophages. (d) IA (MHC class II)-positive cells. (e) Number of granulocytes.
Fig. 4. T cells (OX19 staining) and B cells (OX33 staining) in the colon of the rat. (a) T cells in the colon of a healthy rat. (b) T cells in the colon on day 14 after 2,4,6-trinitrobenzene sulphonic acid (TNBS)/ethanol-induced colitis. T cells are concentrated in lymphoid accumulations in the submucosa. (c) B cells in the colon of a healthy rat. (d) B cells in the colon on day 14 after TNBS/ethanol-induced colitis. B cells are concentrated in lymphoid accumulations in the submucosa.
observed in the number of T cells in the submucosa, especially at day 14 and day 28. Brandtzaeg et al. [23] also found a substantial rise in the number of B cells in IBD patients, although this increase was especially in the intestinal mucosa. The importance of T cells in experimental colitis was also reported by Sartor et al. [24], who observed that the chronic stage of peptidoglycan–polysaccharide complex (PG–PS)-induced enterocolitis was T cell-dependent. These data indicate that lymphocytes play a pivotal role in the chronic phase of both clinical and experimental intestinal inflammation. After the second administration of TNBS, a new influx of macrophages and lymphocytes was observed, mainly in the submucosa. The increase in these cell types was as high as the observed increase after the first administration of TNBS. On the other hand, the number of granulocytes was not increased, which indicates that these cells are not necessary to induce areas of ulceration. Taken together, the cell types that infiltrated into the submucosa are different after second administration of TNBS from the first administration, which implies that repeated administration of TNBS induces a prolonged chronic stage rather than a second acute stage. Furthermore, these data suggest that the rats had been sensitized by the first dose of TNBS, administered into the colon. That animals could be sensitized for TNBS was demonstrated before [25] by skin application or by subcutaneous injection of TNBS. Subsequent challenge in the gut then induced intestinal inflammation. However, in general, primary administration of antigens into the gastrointestinal tract often results in tolerance rather than stimulation of the immune system [26].

Usually in these studies the small bowel is examined, whereas in the present study the colon was investigated. Beagley [27] described differences in T cell populations and in expression of the T cell receptor (TCR) in small and large bowel. In mice, CD3+ intraepithelial lymphocytes (IEL) in small intestine were predominantly CD8+ (75%) and the αβ and γδ TCR were expressed almost equally. In contrast, 72% of the CD3+ IEL in the colon were CD4+, and over 85% expressed the αβ TCR. IEL in small intestine compared with colon may also have site-specific functions in both mucosal immune protection and tolerance induction [28], which could explain sensitization following colonic administration of antigen. Furthermore, differences in lamina propria lymphocytes between small intestine and colon should be taken into account.

In conclusion, these data suggest a pivotal role for macrophages and neutrophils in the acute stage of TNBS-induced colitis, and an important role for T cells in the chronic stage of the disease. Furthermore, mimicking a relapse of colitis in this model provides possibilities for studying new therapeutic approaches for the prevention of relapses in human IBD.

Fig. 5. Macrophages and granulocytes in mucosa and submucosa of the colon, on day 7 after second administration of 2,4,6-trinitrobenzene sulphonic acid (TNBS) (□) or NaCl (■). (a) ED1+ macrophages. (b) ED2+ macrophages. (c) ED3+ macrophages. (d) Number of granulocytes.

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