Clin Exp Immunol 1993; 92:303–307

Time-course of adhesion molecule expression in rectal mucosa of gluten-sensitive subjects after gluten challenge

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(Accepted for publication 1 February 1993)

SUMMARY

Adhesive interactions between endothelium and circulating cells, such as monocytes, neutrophils and lymphocytes, are crucial for localizing the inflammatory response. We investigated the inflammatory response of rectal mucosa to local gluten challenge as a dynamic model of antigen-induced tissue injury, during which the expression of adhesion molecules on leucocytes and endothelial cells could be sequentially observed. Expression of ELAM-1, ICAM-1 and VCAM-1 was monitored in 10 treated and eight untreated patients with gluten sensitivity (coeliac disease), and in five disease controls for up to 4 h (short challenge), while a further seven treated coeliacs were monitored for up to 24 h (long challenge) following rectal gluten challenge. In the former, the expression of VCAM-1 and ELAM-1 was significantly raised 4 h after gluten challenge compared with controls. VCAM-1 and ELAM-1 expression was also increased in mucosae of treated patients, but to a lesser extent. VCAM-1 expression continued to increase for up to 24 h after gluten, while ELAM-1 had begun to wane by 4 h, reaching basal levels by 24 h. In contrast, the expression of ICAM-1 did not change in any of the disease groups studied. These findings relate to significant increases in lymphocytes ($CD3^+$ cells) after 8 h, and neutrophils ($CD15^+$ cells) after 4 h in the lamina propria. This approach has permitted novel studies of the inflammatory response to a defined antigen in sensitized (gluten-sensitive) human patients.

Keywords coeliac rectal mucosa adhesion molecules lymphocyte neutrophil antigen challenge gluten

INTRODUCTION

The jejunal mucosal lesion of gluten sensitivity (GS) appears to be consistent with a T cell-mediated immune process in genetically predisposed individuals [1]. It seems certain that the spectrum of gluten sensitivity, with its accompanying series of mucosal changes, arises from the varied influences of sensitized mucosal T lymphocytes on intestinal mucosa. Although the major site of involvement is jejunal mucosa, which invariably results in the clinical syndrome of coeliac disease, similar responses are also mounted by rectal mucosa after exposure to gluten [1,2].

T cells play a critical role in both acute and chronic inflammatory processes. The localized adherence of T lymphocytes to vascular endothelium is a key event governing their emigration from the circulation into foci of chronic inflammation. This is regulated by the coordinated action of distinct families of adhesion molecules expressed on blood vessels and lymphocytes [3]. The capacity of lymphocytes to bind to vascular endothelium appears to be determined by their class, state of differentiation, as well as site of initial contact with antigen. This, in turn, determines the distribution of distinct lymphocyte subsets in particular organs or sites of inflammation, which is also influenced by local microenvironments in a dynamic manner [4].

The aim of this study was to investigate further the inflammatory response [2] of rectal mucosa to local gluten challenge, by studying the expression of the adhesion molecules ICAM-1, VCAM-1 and ELAM-1 relative to the observed time-course of the cellular infiltrate.

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PATIENTS AND METHODS

Patients

A total of 23 patients comprising five gastrointestinal disease controls (DC) and 18 proven gluten-sensitive patients, eight of whom had untreated coeliac disease (UCD) and 10 treated (TCD), were challenged rectally with a 6-g infusion of gluten (BDH). The disease controls comprised irritable bowel syn-

drome ($n=2$), iron deficiency anaemia ($n=2$), and giardiasis ($n=1$). A short challenge was performed by taking rectal biopsies before and 4 h after rectal gluten infusion. A further seven treated coeliac patients underwent a long challenge, in which rectal biopsies were taken before and at 4, 8, 12 and 24 h after challenge. These protocols were reviewed and approved by Salford District HA Ethics Committee.

Tissue processing

Rectal mucosal samples were orientated on dental wax, embedded in OCT, snap-frozen in liquid nitrogen-cooled isopentane and stored in liquid nitrogen. The specimens were sectioned at 6–7 μm with a Bright cryostat and kept at −20°C before immunohistochemical staining. The latter comprised a two-step indirect immunoperoxidase technique. Primary MoAbs were 1.3D5 (anti-ELAM-1), 1.4C11 (anti-ICAM-1), and 4.B2 (anti-VCAM-1) (British Biotechnology, Abingdon, UK) diluted 1:1000; CD3 (Dako, High Wycombe, UK) and CD15 (Dako) were used at 1:50 dilution. Primary MoAbs were incubated for 30 min and detected with peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako) at 1:25 dilution for 30 min. Peroxidase activity was visualized with DAB (Dako) substrate, the counterstain being haematoxylin. Sections were washed in Tris buffer-saline (pH 7·8, 0·05 M Tris and 0·15 M saline) for 5 min between each incubation and preserved in Apathy's Mounting Medium (BDH, Poole, UK).

Analysis

Expression of adhesion molecules was evaluated in terms of staining intensity and distribution pattern (microvasculature and/or cellular infiltrate) on sections containing a fair number of blood vessels (at least 10) and was graded on an arbitrary scale of nil staining (−)=0, weak staining (+)=1, moderate staining (++)=2, strong staining (+++)=3. To avoid intersubject variability, percentage change in the expression of adhesion molecules was calculated. Changes in the absolute number of cells expressing either CD3 or CD15 within surface and crypt epithelium and/or lamina propria were determined and finally standardized with reference to a constant test area of muscularis mucosae (10^4 μm^2) as described elsewhere [2]. Cells positive for either antibody were counted per section per time point along a measured length of muscularis mucosae (with a calibrated ocular graticule). Wilcoxon sum rank test and Mann–Whitney *U*-test were used in the analysis of paired, and unpaired, data respectively.

RESULTS

General observations

Endothelial expression of all three adhesion molecules, when found, was observed on every blood vessel present in the section. Before challenge, low levels of expression of ELAM-1 were comparable in each group, being confined to thin-walled venules (Fig. 1a) with only occasional weakly staining leucocytes. ICAM-1 expression was more pronounced, but similar, in specimens of all three study groups, being diffusely present on every vessel and on all cells present within lamina propria, particularly in the subepithelial region (Fig. 1b). Conversely, VCAM-1 expression was higher in untreated coeliac rectum ($P<0·01$) compared with either treated coeliac, or DC mucosae, but was confined largely to lamina propria mononuclear cells: its expression on the microvasculature (Fig. 1c) was either very weak or absent (see Table 1 for more detailed account).

Short (4 h) challenge

At the end of the short challenge, there was a significant and diffuse increase in expression of ELAM-1 on the microvasculature of untreated ($P\leqslant 0·01$) and treated ($P<0·05$) coeliac rectal mucosa, but no change in expression in disease controls. There was no detectable alteration either in density or distribution of ICAM-1 staining in either group of mucosae after challenge, compared with prechallenge appearances. Similarly, both groups of coeliac patients showed a response to gluten infusion with an increase in VCAM-1 expression on the cellular infiltration, and, to a far less detectable extent, on the blood vessels. When the percentage change in expression of adhesion molecules was calculated, both ELAM-1 and VCAM-1 showed a significant increase in expression in untreated coeliac rectum compared with disease controls ($P<0·05$, UCD *versus* DC). Though treated coeliac mucosae also showed increased expression of ELAM-1 and VCAM-1, it was not found to be significant.

Long (24 h) challenge

The findings during this challenge are summarized in Table 2. Vascular expression of ELAM-1 (treated coeliacs only) increased significantly by 4 h ($P\leqslant 0·05$) and returned to basal levels by 24 h. This increase paralleled the 4-h rise in the absolute number of lamina propria neutrophils ($CD15^+$) (Fig. 2a) measured at that time ($P<0·005$). Subsequently, ELAM-1 expression, and $CD15^+$ cells, declined and remained at low levels thereafter. ICAM-1 expression did not alter during challenge, remaining diffusely present on vasculature and lamina propria cells.

VCAM-1 expression increased significantly at 4 h ($P\leqslant 0·05$) and continued to rise for up to 24 h ($P\leqslant 0·01$), increasing by 99% at 4 h, 117% at 8 h, 120% at 12 h and 209% by 24 h. The continued, progressive rise in VCAM-1 expression reflected the change in absolute numbers of $CD3^+$ lymphocytes that entered the tissues, to result in progressive infiltration of lamina propria (Fig. 2b). The change in $CD3^+$ occurred at a slower tempo compared with neutrophils, and only became significant by 8 h post-challenge ($P<0·05$), remaining at that level for at least 24 h.

It should be noted that during the time course of both challenges, no discernible staining for any adhesion molecule was evident within surface or glandular epithelium, despite progressive infiltration by ($CD8^+$) intraepithelial lymphocytes (IEL).

DISCUSSION

The results of this dynamic study show that rectal mucosa of gluten-sensitized (coeliac) patients reveals increased expression of ELAM-1 and VCAM-1, but not ICAM-1, after local gluten challenge. In unchallenged rectal mucosa, VCAM-1 only was increased in untreated patients, while ELAM-1 and ICAM-1 expression showed no difference between either study group, although following challenge, increased expression of ELAM-1 and VCAM-1 did occur. This is the first *in vivo* experiment relating the temporal evolution of a lymphocytic and neutrophilic inflammatory infiltrate to the appearance of these adhesion

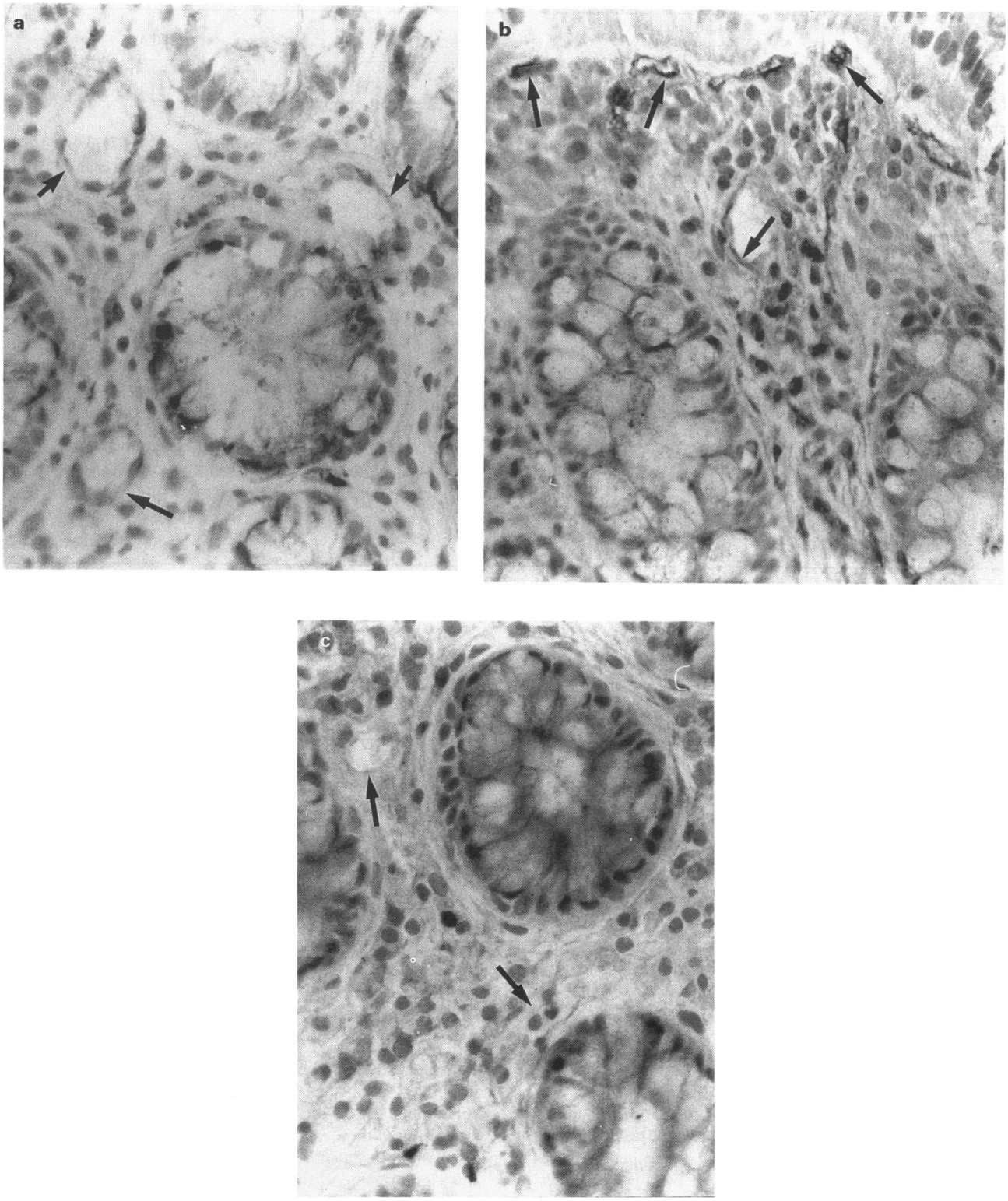

Fig. 1. Cryostat sections of the rectal mucosa in untreated coeliac disease. (a) A thin-walled venule with weak expression of ELAM-1 (arrows) is present. (b) ICAM-1 expression is evident on subepithelial and lamina propria vasculature (arrows) and diffuse staining of infiltrating mononuclears into lamina propria: there is no detectable staining of, or within, the surface epithelium. (c) VCAM-1 was not evident on mucosal microvasculature (arrowed), but was expressed on many cells within lamina propria. (× 40.)

Table 1. Expression of adhesion molecules before and at the end of a 4-h rectal challenge

	ELAM-1		VCAM-1		ICAM-1	
Category	Pre	Post	Pre	Post	Pre	Post
Untreated coeliac disease	1·0† (0·5–1·75)	2·25**† (1·75–2·75)	1·25† (0·5–1·75)	2·625**† (2·25–3·0)	2·625† (2·25–3·0)	3·0† (2·75–3·0)
	Moderately on all venules	Increased	Moderately on mononuclear cells	Increased	Strongly on all vessels	No change
	Weakly on few leucocytes	Increased	Weak/absent on blood vessels	Increased	Strongly on all cells	No change
Treated coeliac disease	1·0† (0·25–1·5)	1·5*† (0·75–2·0)	0·0† (0·0–0·5)	1·5**† (0·75–2·0)	2·0† (1·5–2·75)	2·0† (1·25–2·75)
	Moderately on all venules	Increased	Weakly on few mononuclear cells	Increased	Strongly on all vessels	No change
	Weakly on few leucocytes	Increased	Absent on blood vessels	Weakly on blood vessels	Strongly on all cells	No change
Disease controls	1·5† (1·0–2·0)	1·5† (1·0–2·0)	0·5† (0·0–0·5)	1·0† (0·0–1·5)	2·25† (2·0–3·0)	2·25† (2·0–3·0)
	Moderately on all venules	No change	Weakly on few mononuclear cells	Moderately on mononuclear cells	Strongly on all vessels	No change
	Weakly on leucocytes	No change	Absent on blood vessles	Weakly on blood vessels	Strongly on all cells	No change

After challenge, expression of ELAM-1 and VCAM-1 was significantly increased in both untreated coeliac disease (UCD) and treated coeliac disease (TCD). ICAM-1 expression did not alter significantly.

*$P<0.05$; **$P<0.01$.

† Values expressed as median and 95% CI are given in brackets.

Table 2. Expression of adhesion molecules during 24-h rectal challenge

Time (hours)	ELAM-1 median (95% CI)	VCAM-1 median (95% CI)	ICAM-1 median (95% CI)
Prechallenge	0·75 (0·0–1·25)	0·00 (0·0–0·5)	2·25 (1·5–3·0)
4	1·25 (0·5–1·75)*	1·00 (0·5–2·0)*	2·25 (1·0–2·75)
8	1·00 (0·0–1·75)	1·75 (1·25–2·5)**	2·00 (1·0–2·5)
12	0·75 (0·0–1·5)	1·75 (1·0–2·5)***	2·00 (1·0–2·5)
24	0·38 (0·0–1·5)	2·25 (1·5–3·0)**	2·25 (1·75–2·75)

Expression of ELAM-1 increased significantly only at 4 h, whereas VCAM-1 expression continued to rise up to 24 h following rectal gluten challenge.

*$P<0.05$; **$P<0.01$; ***$P<0.005$.

molecules both on lamina propria microvasculature and the infiltrating cell types.

ELAM-1 has been proposed as a key endothelial adhesion molecule mediating neutrophil infiltration [3], with a typical time course peaking by 4 h. In our experiment, ELAM-1 expression reached maximal expression by 4 h of contact of rectal mucosa with gluten, with a slow fall-off thereafter, thus correlating roughly with the initial wave of neutrophils into the lamina propria ~4–8 h post-challenge. The number of $CD15^+$ cells in the cellular infiltrate (assumed solely to represent neutrophils) fell after 4 h, but did not return to basal levels (24 h challenge), suggesting continued low-level extravasation of neutrophils rather than persistence of the original wave of transmigrating polymorphs. Increased expression of ELAM-1 in actively inflamed colitic mucosae, particularly on venules surrounded by massive neutrophilic infiltrates, was demonstrated by Ohtani *et al.* [5] and likewise in skin [6]. ELAM-1 expression may also be increased in certain chronic inflammatory lesions, supporting the recent proposal of binding by memory T cells [4,7]. Our results suggest ELAM-1 is principally involved in neutrophil recruitment in gluten-challenged rectal mucosa; its role in lymphocyte adhesion remains to be evaluated.

VCAM-1 binds VLA-4 on lymphocytes, especially those of memory phenotype (CD45-RO) [4,8]. Expression of VCAM-1, compared with ELAM-1, is usually long-lived, as confirmed by

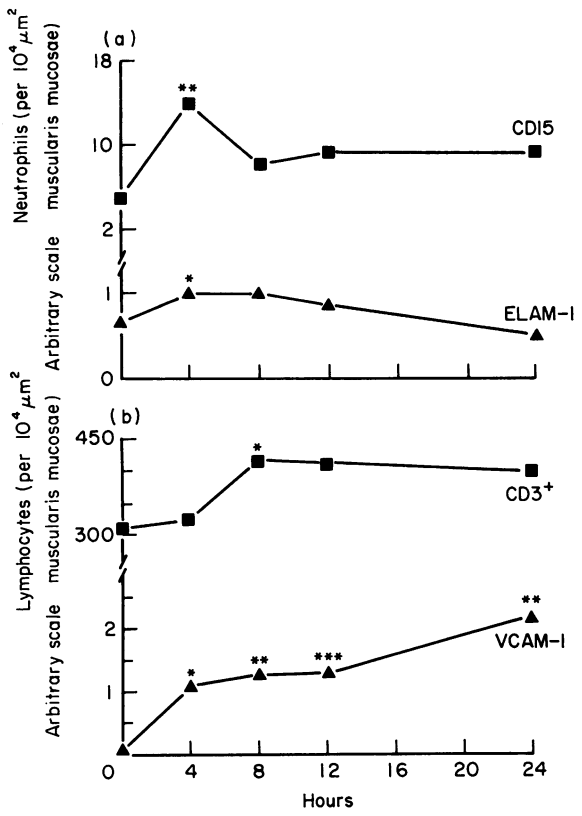

Fig. 2. Time-course expression of ELAM-1 and VCAM-1 in relation to the absolute number of T lymphocytes ($CD3^+$) and neutrophils ($CD15^+$) in coeliac rectal mucosae. (a) The 4-h rise in ELAM-1 correlates with the increase in the number of neutrophils. $^*P<0.05$; $^{**}P<0.01$. (b) Both VCAM-1 expression and the number of T lymphocytes rise at 4 h and persist for 24 h. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.005$.

our finding of elevated expression by 4 h and of it continuing to increase up to 24 h. A significant increase in VCAM-1 (8 h post-challenge) occurred with the rise in $CD3^+$ cells within lamina propria and epithelium, from this time point onwards. Increased expression of ICAM-1, ELAM-1 and VCAM-1 was similarly distributed in colonic mucosa of acute graft-*versus*-host disease [9], except for glandular epithelial staining for ICAM-1. In several studies it has been shown that VCAM-1- and/or ICAM-1-mediated stimulation (costimulation) is required for full activation, as well as adhesion of T lymphocytes, in addition to antigen/MHC recognition [10,11].

The time-course expression of VCAM-1 in experimental studies also parallels the sustained mononuclear leucocyte infiltration characteristic of classic DTH reactions: these, like the response to gluten, exemplify T cell-mediated tissue reactions. Clearly, this pathway is not unique to gluten sensitivity, and seems to be a common final pathway for a variety of responses [3,4]. That VCAM-1 was raised in untreated coeliac rectal mucosae, unlike treated coeliac and disease-control mucosae, suggested long-term antigenic stimulation in such patients, especially as expression of ELAM-1 was not markedly changed [1,2,12].

Finally, previous investigators have suggested that tissue (jejunal) damage in gluten sensitivity involves cytolysis of enterocytes by $CD3^+8^+$ $\alpha\beta$-receptor, or $CD3^+8^-\gamma\delta^+$, IEL [13], although intestinal epithelium is negative for ICAM-1 [8,14,15]. Alternatively, the prompt appearance of ELAM-1 and VCAM-1 within lamina propria, together with timed infiltrates of neutrophils and lymphocytes into this region of the mucosa, is consistent with our previous view that the initial site of inflammatory reactivity in gluten sensitivity is the lamina, so that any changes in epithelium are thus more likely to be secondary to this antigen (gluten)-induced inflammation.

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