Immunohistochemical localisation of intercellular adhesion molecule-1 in follicle associated epithelium of Peyer's patches

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Abstract
The epithelium covering domes of lymphoid follicles in Peyer's patches includes membranous cells, which are sites of entry for various macromolecules, absorptive cells, a few goblet cells, and many migrating lymphoid cells. The mechanism of migration of lymphoid cells into the follicle associated epithelium of lymphoid follicles in Peyer's patches is still unknown. This study investigated the relation between localisation of intercellular adhesion molecule-1 (ICAM-1) and the follicle associated epithelium of Peyer's patches immunohistochemically. It was found that subepithelial fibroblasts expressed ICAM beneath the follicle associated epithelium on lymphoid follicles, but not on surrounding villi. The results show that the massive lymphocytic traffic between follicle associated epithelium and lymphoid follicles may be related to ICAM-1 expression.
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Peyer's patches are important for the generation of secretory IgA by B cells, which eventually 'home' back to the lamina propria at mucosal sites. Immature B and T cells from Peyer's patches migrate by efferent lymphatics to mesenteric lymph nodes where further maturation occurs, and then by the thoracic duct into the systemic circulation. Lymphocytes home to lymphoid tissue by adhering to and tranversing postcapillary high endothelial venules. There are anti-genetically and functionally distinct lymphocyte high endothelial venules recognition systems that control the homing of lymphocytes to peripheral lymph nodes and mucosal lymphoid organs such as Peyer's patches and the appendix.[1][2] In 1964, Gowans and Knight[3] first recognised that lymphocytes migrate from blood to the lymph nodes by a morphologically distinct high endothelium of high endothelial venules. Stamper and Woodruff[4] showed that rat lymphocytes bind selectively to the endothelium of high endothelial venules exposed in frozen sections of lymphoid tissues. Jalkanen et al,[2] who adapted this high endothelial venules binding assay to mice, provided strong evidence for an important role of lymphocyte high endothelial venules interaction in determining the tissue specificity of lymphocyte migration. They proposed that this organ specificity was conveyed by interactions between specific 'homing' receptors on the lymphocyte cell surface and the tissue specific ligand of high endothelial venules. The comparative intensity with which lymphocytes bind to high endothelial venules in different lymphoid

organs varies with the lymphocyte subset and stage of differentiation and activation of the lymphocytes. Although a claim has been made that MEL-14[5] and Hermes antigen (CD44)[6] are lymphocyte 'homing receptors', they are not lymphocyte specific in their expression. Several adhesion molecules have been recognised.[7]

Owen et al[8] first found distinctive epithelial cells, which enclosed lymphocytes in attenuated cytoplasm among the follicle associated epithelial cells of Peyer's patches and solitary lymphoid follicles of the small intestine. These cells have been called 'microfold cells'[8] or 'membranous cells'[9] and are known as 'M cells'. The function of M cells has been investigated in morphological studies using various macromolecules[10-13] and organisms.[14-21] Shimazui[22] investigated the morphological relation between M cells and lymphocytes, with particular regard to whether they make contact in intercellular or intracellular space. McClugage et al[23] reported a porosity of the basal lamina covering lymphoid follicles by scanning electron microscopy after removing epithelia, and suggested these pores are an important pathway for migrating cells in the mucosal lymphoid tissue. The mechanism, however, by which immunocytes migrate to the covering epithelial layer of lymphoid follicles is still unknown. We investigated the cell to cell relation in follicle associated epithelium (FAE) of Peyer's patches using the monoclonal antibody to intercellular adhesion molecule-1 (ICAM-1).

Materials and methods
Tissue specimens of Peyer's patch were obtained from the jejunum and ileum of five week old female Wistar rats weighing about 250 g. The rats were fasted for 48 hours but had free access to water. They were then killed by inhalation anesthesia with ether. The specimens were excised immediately and processed for electron microscopic and immunohistochemical study.

LIGHT MICROSCOPY AND TRANSMISSION ELECTRON MICROSCOPY
All tissue specimens were fixed in 2·5% glutaraldehyde at 4°C for two hours. Transmission electron microscopic samples were fixed for two hours in 1% osmium tetroxide, dehydrated through an ethanol series, transferred to propylene oxide, and embedded in epoxy resin. Ultrathin sections were cut with glass knives and a Porter-Blum MT2-B ultramicrotome. For light microscopy, 1 μm sections were stained by toluidine blue. For transmission electron micro-

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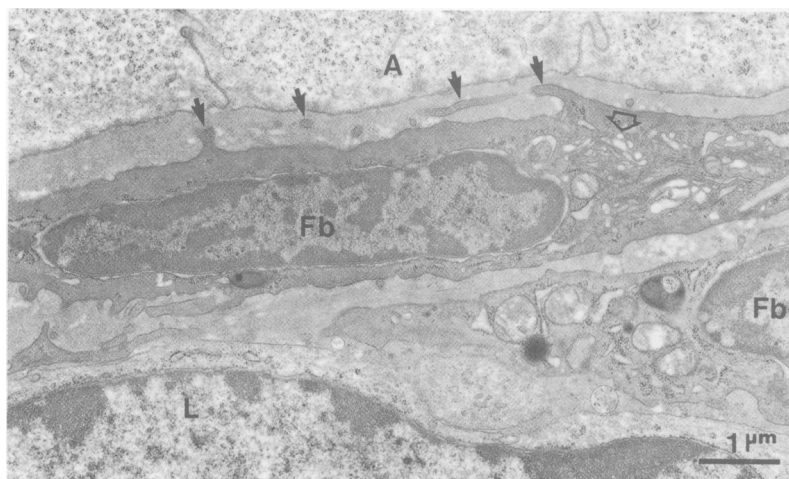Okayama, 701-01, Japan.

scopy, these sections were stained with uranyl acetate and lead citrate and viewed with a Hitachi H-500 electron microscope.

IMMUNOHISTOCHEMICAL EXAMINATION

The specimens were immediately fixed in periodate-lysine-2% paraformaldehyde solution for six hours at 4°C, rinsed in a 0·01 M phosphate buffered saline series (pH 7·6) containing graded concentrations of sucrose, and embedded in OCT compound (Miles Scientific, Elkhart, USA). They were then sectioned at 10 μm by a cryostat, mounted on poly-L-lysine coated glass slides, and air dried at room temperature for three hours.

Sections were processed for examination of ICAM-1 antigen using an avidin-biotin-peroxidase complex method.[24] After the specimens were rinsed with 0·01 M phosphate buffered saline, they were treated with 0·3% hydrogen peroxide in methanol for 10 minutes to inactivate endogenous peroxidase. The samples were then immersed in 10% normal horse serum for 30 minutes to block any non-specific reaction. Each of the above incubations specimens were then rinsed in 0·01 M phosphate buffered saline and incubated with mouse monoclonal anti-rat ICAM-1[25] (Life Science, Seikagaku Co, Tokyo, Japan) diluted 1:50 in 0·01 M phosphate buffered saline containing 10% horse serum for 12 hours in moist chambers at 4°C, and biotinylated with horse anti-mouse IgG (diluted 1:50 in 0·01 M phosphate buffered saline containing 2% horse serum, rat absorbed, Vector Laboratories, Burlingame, CA) for 60 minutes. The specimens were then rinsed for 30 minutes, in 10% sucrose at 4°C and they were incubated with the avidin-biotin complex or ABC reagent (a mixture of avidin DH solution and biotinylated enzyme, each diluted 1:50 with 0·01 M phosphate buffered saline). The horse serum, biotinylated anti-mouse IgG, and ABC complex are reagents in the Vectastain Elite ABC kit (Vectorstain; Vector Laboratories, Burlingame, CA). The incubated specimens were rinsed in 0·01% phosphate buffered saline for 10 minutes, and then fixed in 2% glutaraldehyde in 0·01 M phosphate buffered saline. The specimens were then rinsed carefully six times in 0·01 M phosphate buffered saline for five minutes.

For light microscopy, the samples were reacted with 0·02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) diluted in 0·05 M TRIS buffer, pH 7·6, for 30 minutes at room temperature and subsequently in 0·02% DAB solution containing 10 mM hydrogen peroxide and 10 mM sodium azide for five minutes.

For immunoelectron microscopy, specimens were treated as described above and then post-fixed in 2% osmium tetroxide in phosphate buffered saline for one hour. They were then dehydrated in graded ethanol solutions, embedded in epoxy resin, and allowed to stand three days for polymerisation. Ultrathin sections were cut with an MT2-B Porter-Blum ultramicrotome and examined in a Hitachi H-500 transmission electron microscope. For negative controls, non-immune mouse serum, mouse IgGl, and phosphate buffered saline were used in place of the first antibody.

Results

LIGHT MICROSCOPY AND TRANSMISSION ELECTRON MICROSCOPY

Light microscopically, subepithelial fibroblasts were recognised by toluidine blue staining as spindle shaped cells beneath epithelial cells in villi and lymphoid follicles. In lymphoid follicles, many lymphoid cells lay close to them. By transmission electron microscopy, lymphocytes were sometimes recognised in lymphoid follicles in disrupted spaces in the basal lamina. There was a well developed endoplasmic reticulum in the subepithelial fibroblasts from which cytoplasmic processes extended with sheet like and branched configuration to the reticular lamina (Fig 1).

IMMUNOHISTOCHEMICAL EXAMINATION OF ICAM-1 EXPRESSION IN PEYER'S PATCHES

ICAM-1 was expressed on the vascular endothelium of high endothelial venules (Fig 2) in interfollicular areas, in dendritic cells or macrophage like cells and in the subepithelial areas of lymphoid follicles (Fig 3A) in Peyer's patches.

Figure 1: Transmission electron micrograph shows a subepithelial fibroblast (Fb) beneath an absorptive cell (A) in a Peyer's patch. It has a well developed endoplasmic reticulum (open arrow) and cytoplasmic processes (arrows), which have a finger like or tentacle configuration in collagenous fibrils of the basal lamina. A lymphocyte (L) is closely apposed to subepithelial fibroblasts.
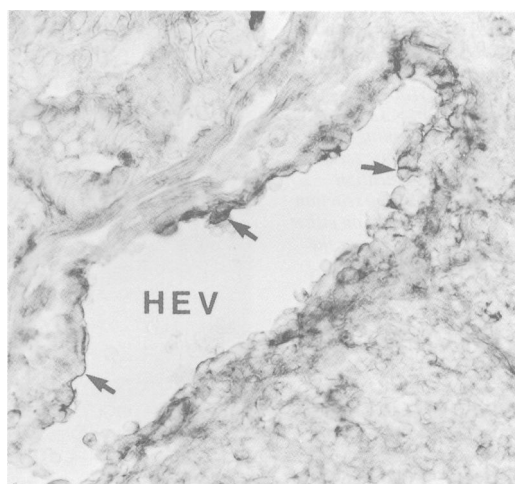

Figure 2: Immunohistochemical examination shows a high endothelial venule (HEV) of a Peyer's patch, which reacts (arrows) strongly with anti-ICAM-1 antibody (original magnification ×300).
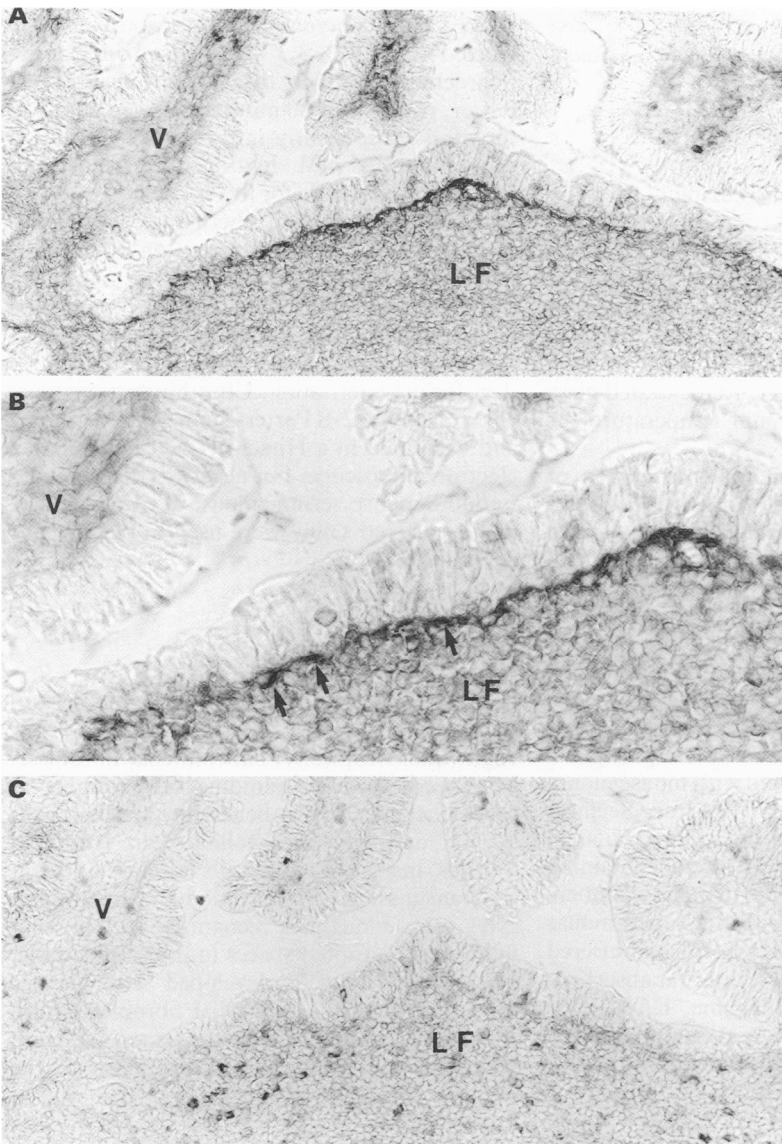
Figure 3: (A) Immunohistochemical localisation of ICAM-1 in a dome epithelium of a lymphoid follicle (LF) in a Peyer's patch. Although ICAM-1 is positive in the basal lamina on dome epithelium, it is negative on the surrounding villi (V) (original magnification ×160). (B) Enlargement of Fig 3(A) shows a positive reaction of fibroblast like cells (arrow) (original magnification ×320. (C) Control staining with non-immune mouse serum (original magnification ×160). There is no reaction in the basal lamina on either the dome epithelium or the surrounding villi.
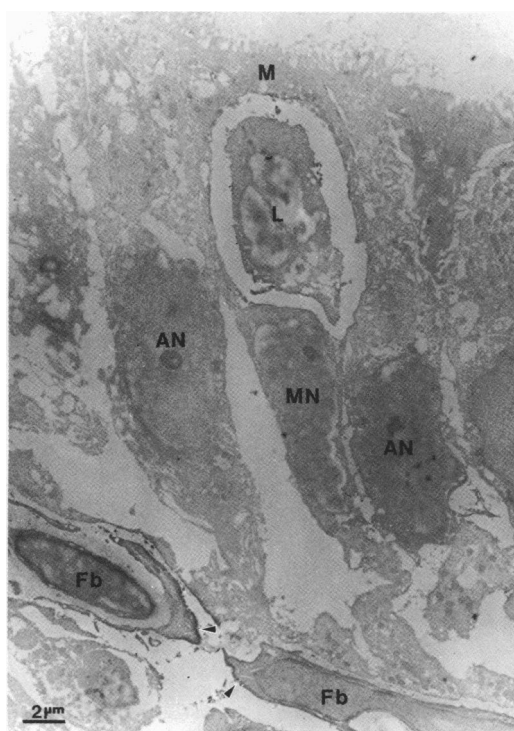

Figure 4: Immunoelectron micrograph of a section treated with anti-ICAM-1 antibody. Electron dense areas (arrows) of positive reaction are prominent on subepithelial fibroblasts (Fb), but not on an M cell (M). AN=the nucleus of an absorptive cell, MN=the nucleus of an M cell.

There was no expression of ICAM-1, however, in the subepithelial areas of surrounding villi (Fig 3A). Figure 3B is an enlarged photomicrograph of Fig 3A and shows that spindle shaped subepithelial cells with ICAM-1 expression are fibroblast like cells. There was no labelling of the subepithelial cells of Peyer's patches or surrounding villi with control stainings (Fig 3C).

IMMUNOELECTRON MICROSCOPIC EXAMINATIONS OF ICAM-1 EXPRESSION OF PEYER'S PATCHES
On ultrathin sections of Peyer's patches stained with anti-ICAM-1 antibody, ICAM-1 expression was recognised on the surface of subepithelial fibroblasts in lymphoid follicles, but there was no expression of ICAM-1 in M cells (Fig 4). Careful study, however, showed that subepithelial fibroblasts with long and sheet like cytoplasmic processes had a strong positive reaction to ICAM-1 (Fig 5A). The cytoplasmic processes of the fibroblasts extended among collagen fibrils of the basal laminae into the epithelial cells above the lymphoid follicles (Fig 5B, C). The subepithelial fibroblasts of surrounding villi showed no expression of ICAM-1.

Discussion
ICAM-1 is a cell surface glycoprotein, the ligand for LFA-1, and its expression can be induced on a variety of cell types. These include vascular endothelium,[26] dermal fibroblasts[26] and keratinocytes,[27] dendritic cells,[26] thymic epithelial cells,[26] mucosal epithelial cells in the tonsils,[28] renal tubular cells,[29] and rheumatoid synovium.[30] In vitro, the normally low basal expression of ICAM-1 on dermal fibroblasts, keratinocytes, and endothelial cells can be dramatically increased by treatment with the inflammatory mediators interferon γ, IL-1, tumour necrosis factor, or lymphotoxin. The increased ICAM-1 expression directly correlates with increased LFA-1 dependent adhesion of lymphocytes to the induced cells.[26 31 32] These findings suggest that ICAM-1 may play a part in localising the immune response to sites of inflammation. Recently, it was reported that expression of ICAM-1 positively correlated with enterocytic maturation of colon adenocarcinoma.[33] The distribution of ICAM-1 in tissue specimens is similar to that of HLA-DR.[34-36] Although M cells have been shown to express HLA-DR[37] or Ia antigen[38] on their surfaces, thus indicating that M cells are antigen presenting cells to their associated lymphocytes, our investigation showed no expression of ICAM-1 in the M cells of Peyer's patches.

The epithelium covering lymphoid follicles is specialised and differentiated epithelium, which includes M cells, a number of intraepithelial lymphocytes, and a few goblet cells. Although the origin of M cells is still unknown, there are two hypotheses: that M cells develop directly from undifferentiated crypt cells[39 40] or indirectly from mature absorptive cells.[41-43] It may be suggested that the subepithelial fibroblasts of lymphoid follicles affect the development of follicle associated epithelium including M cells. It has been shown that a cytotoxic/suppressor
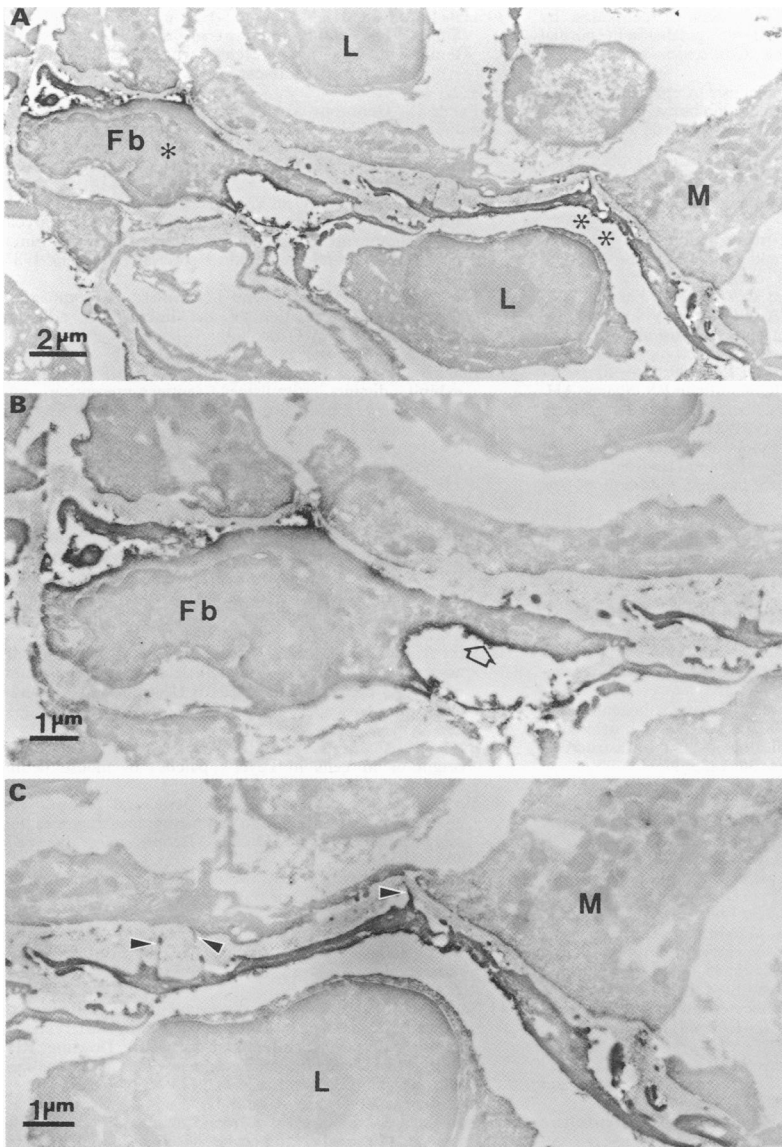
Figure 5 (A):
Immunoelectron micrograph
of a section treated with
anti-ICAM-1 antibody. An
electron dense area of strong
positive reaction in a
fibroblast (Fb) and its
cytoplasmic processes.
L=lymphocyte, M=M cell.
(B) Enlargement of the
fibroblast in Fig 5 (A) (*)
shows a strongly positive
reaction of a cytoplasmic
process (open arrow).
(C) Enlargement of the
fibroblast in Fig 5 (A) (**)
shows a strongly positive
reaction of a cytoplasmic
process. Fine processes
(arrows) from the fibroblast
extend among collagen fibrils
of the basal lamina to the
epithelial cells.
L=lymphocyte, M=M cell.

phenotype of intraepithelial T cells predominated over a helper phenotype in follicle associated epithelium,[38] and this phenotype was similar to those in villus epithelium.[44] Our study is the first to show ICAM-1 expression of subepithelial fibroblasts in the Peyer's patches of rats, but not in the surrounding villi. It may be possible that both the Ia expression of M cells and the ICAM-1 expression of subepithelial fibroblasts are induced by an interferon γ like factor emitted by T cytotoxic/suppressor lymphocytes in close contact on passage through the disrupted basal lamina.

Additionally, we found that sheet like and branched cytoplasmic processes of subepithelial fibroblasts covering lymphoid follicles closely extended to epithelial cells in the reticular lamina and the lamina densa. These findings were similar to subepithelial fibroblasts of villi.[45] Although the function of subepithelial fibroblasts is not fully known, they may give signals to epithelial cells and lymphocytes and may play a part in the differentiation and development of a variety of epithelial cell populations in Peyer's patches.

The porosity of the basal lamina of the dome epithelia of Peyer's patches has been shown.[23]

Ohtani *et al*[46] found that the sheet of collagen fibrils underlying the basal lamina also possesses morphologically heterogeneous pores corresponding to those within the basal lamina. They speculated that the pores were primarily there to provide migrating cells with passageways between the spaces formed by M cells and the reticular spaces of the dome. Our findings suggest that the ICAM-1 expression of subepithelial fibroblasts may facilitate the migration of lymphocytes into follicle associated epithelium.

Recently, Barker *et al*[47] showed that interferon γ injected into normal skin intensified the ICAM-1 expression of dermal endothelial cells and induced keratinocyte expression of ICAM-1. In addition, LFA-1 positive lymphocytes were found along the basement membrane zone close to basal keratinocytes, which have been found to be ICAM-1 positive in vivo in humans.

Interestingly, Nakamura *et al*[48] suggested that ontogenically Peyer's patches develop from aggregates of spindle cells with a morphology resembling that of endothelial cells or fibroblasts in the lamina propria under villus epithelial cells in newborn rats. Although the mechanism of generation of lymphoid follicles is still unknown, the expression of ICAM-1 by subepithelial fibroblasts, as shown in this paper, may be related to ontogenic development of the dome epithelium of lymphoid follicles.

One interesting finding in Crohn's disease has been that early aphthoid ulcers are typically seen over lymphoid follicles.[49 50] This may be related to the presence of an active immune response against a given antigen that has a predisposition for the M cell as its route of entry. Although no specific organism has been identified in a large number of previous studies, it seems that the location of aphthoid ulcers may be evidence for an infectious cause and the effect of ICAM-1 in targeting lymphoid cells to lymphoid follicles may play a part in producing aphthoid ulcers in Crohn's disease. The administration of anti-ICAM-1 may provide a new treatment for chronic inflammatory bowel disease.

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