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Increased expression of mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and lymphocyte recruitment in murine gastritis induced by *Helicobacter pylori*

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SUMMARY

Although T cell involvement in *Helicobactor pylori*-induced gastritis is known, mechanism about T cell recruitment is not understood. In this study we examined how mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is involved in lymphocyte recruitment in murine chronic gastritis induced by *H. pylori*. C57 BL/6 mice were infected with Sydney strain (SS1). Six months after infection, the stomach was removed. The expression of adhesion molecules, MAdCAM-1, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and the cell surface antigens CD4, CD8, CD45R/B220 or β7-integrin were determined by immunohistochemistry. A significant increase in CD4 lymphocytes was observed in the body portion of stomach in SS1-infected mice and most of these CD4 cells express β7-integrin, a known counter ligand for MAdCAM-1 molecule. Strong MAdCAM-1 expression was observed adjacent to these cells in the lamina propria as well as in the submucosa of SS1-infected stomach. Quantitative analysis showed that the area of MAdCAM-1 expression well correlated with the infiltration of β7-integrin positive lymphocytes. On the other hand, expression of ICAM-1 or VCAM-1 in the lamina propria was few even in the SS1-infected stomach. Increased expression of MAdCAM-1 was well correlated to the location of lymphocytes, which express CD4 and β7-integrin. These results suggest the possibility that MAdCAM-1 may be largely involved in the lymphocyte recruitment in the gastritis mucosa with *H. pylori.*

Keywords adhesion molecules β7-integrin *Helicobactor pylori* gastritis lymphocyte migration mucosal addressin cell adhesion molecule-1 (MAdCAM-1)

INTRODUCTION

Helicobacter pylori plays a causative role in the pathogenesis of gastritis, gastric atrophy and peptic and duodenal ulcer [1]. Infection by this bacterium is also associated with an increased risk of gastric adenocarcinoma, and *H. pylori* is now classified as a type I human carcinogen [2]; furthermore, a causative relationship between the presence of *H. pylori* and the occurrence of mucosa-associated lymphoid tissue (MALT) lymphoma was suggested [3]. Despite the presence of high titres of Helicobacter-specific antibodies in the serum and gastric mucosa of *H. pylori*-infected patients [4], they remain chronically infected and are unable to clear infection.

Infection with Helicobacter species results in the recruitment of $CD4^+$ and $CD8^+$ T cells in gastric tissue [5]. The accumulation of gastric Th1-type *H. pylori*-specific $CD4^+$ cells has been proposed to account for their failure to generate protective immunity and to contribute to disease pathogenesis [6]. Although many proof of T cell involvement of pathogenesis in *H. pylori*-induced gastritis, information about recruitment of T cell, including source of lymphocytes or their expression of adhesion molecules have not yet been elucidated.

Lymphocyte homing to both normal tissues and sites of inflammation is, in part, regulated by differential expression of cell surface homing receptors and their selective interactions with tissue selective vascular adhesion molecules at sites of lymphocyte recruitment from the blood [7]. In the mouse, lymphocyte homing to mucosal lymphoid tissue such as Peyer's patches and

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the intestinal lamina propria involves a single-chain 60-kDa glycoprotein, the mucosal vascular adhesion molecule MAdCAM-1 (mucosal addressin cell adhesion molecule-1) [8] and the heterodimeric alpha4beta7-integrin on leucocytes act as its ligand. Immunohistology demonstrates MAdCAM-1 expression at high levels by HEVs in mucosal-associated lymphoid tissue such as Peyer's patches but not in peripheral lymph nodes. In case of intestinal inflammation, its expression increases and is thought to be a gate of inflammatory cells to the site of inflammation [9,10]; however, it is also observed in the nonmucosal inflamed site in the chronically inflamed pancreas of non-obese diabetic (NOD) mice [11], and weakly on central nervous system venules in chronic relapsing experimental allergic encephalomyelitis [12]. Although MAdCAM-1 involves some infiltration of extra mucosal lesion, involvement to *H. pylori*-induced gastritis is not elucidated.

In this study, we investigated whether *H. pylori* infection affect localization of beta7-integrin positive CD4 cells and expression of MAdCAM-1 in the gastric mucosa by using the Sydney strain (SS1) of *H. pylori*, which has high colonizing ability for the mouse stomach [13].

MATERIALS AND METHODS

Inoculation of H. pylori

H. pylori SS1 was grown on tryptic soy agar (TSA) plates (Becton Dickinson, Cockeysville, MD, USA) containing 5% sheep blood (Remel, Leneza, KS, USA) and 100 μg of vancomycin, 3·3 μg of polymixin B, 200 μg of bacitracin, 10·7 μg of nalidixic acid and 50 μg of amphotericin B (Sigma Chemical Co., St Louis, MO, USA) per ml. The plates were incubated for 72–80 h at 37°C in 10% CO_2 and 5% O_2 in a Trigas incubator (Queue Systems, Ashville, NC, USA). Female 6-week-old specific pathogen-free C57BL/6 mice (Nihon CLEA, Yokohama, Japan) were housed under conventional conditions in our animal facilities. The animals were handled according to the guidelines of Animal Research Committee of National Defense Medical College. The mice had free access to food and water. Mice were inoculated with a bacterial suspension obtained from 2-day liquid cultures of SS1. After overnight fasting, the animals were dosed twice in a 5-day period with 0·5 ml of bacterial suspension (approximately 5×10^8 cfu/ml) using a stomach tube ($n = 8$). As controls mice were given suspension buffer solution alone ($n = 8$). After 6 months, the stomach was removed and the excised stomachs were cut along the greater curvature and rinsed with physiological saline.

Blood samples were collected from the left ventricle. Sera were used to determine the titre of anti-*H. pylori* IgG antibody by enzyme-linked immunosorbent assay (ELISA) (HEL-p Test II, Amrad Operation Pty Ltd, Melbourne, Australia) with the change of the second antibody to antimouse IgG. The antibody titre was expressed by way of an arbitrary index; values >1·5 were accepted as indicating positive detection of *H. pylori*.

Histological examination and immunohistochemistry for MAdCAM-1

Tissue specimens of gastric mucosa were fixed in 10% buffered formalin and embedded in paraffin after hydration. Consecutive 4 μm sections were stained with haematoxylin and eosin or Giemsa. Degree of mucosal inflammation was assessed in haematoxylin–eosin sections, and the presence of bacteria was confirmed by Giemsa sections. Immunohistochemical study was performed using LSAB (labelled streptavidin–biotin) method. Specimens were fixed in PLP (periodate, lysine-paraformaldehyde) solution, and were vertically embedded carefully in OCT compound (Sakura Fineteck Inc., Tokyo, Japan). Well-orientated 6 μm of cryostat sections were transferred to APS-coated slides and air dried for 1 h at 20°C. After they were washed in phosphate-buffered saline (pH 7·4) containing 1% Triton X for 5 min, sections were incubated in 10% normal goat serum in phosphate buffered saline (PBS). Monoclonal antibodies used in this study and the dilutions were follows. Anti-mouse CD4 antibody (L3T4, PharMingen:10 μg/ml), antimouse CD8 antibody (Ly-2, PharMingen: 10 μg/ml), antimouse CD45R/B220 antibody (RA3–6B2 PharMingen: 10 μg/ml), antimouse β7-integrin (M293, PharMingen: 10 μg/ml), antimouse MAdCAM-1 (MECA-367, PharMingen: 5 μg/ml), antimouse intercellular adhesion molecule-1 (ICAM-1) (3E2, PharMingen: 5 μg/ml), and antimouse and vascular cell adhesion molecule-1 (VCAM-1) (429, PharMingen: 5 μg/ml). Isotype and species matched IgG was used for control study. After overnight incubation at 4°C, sections were treated with subclass- and host-matched biotinylated antibodies for 1 h at room temperature. They were visualized by streptavidin–FITC. Rinsing with PBS containing 1% bovine serum albumin was performed along each step. A cover slip was applied using glycerol jelly. These sections were observed under a fluorescent microscope (BX60, Olympus, Tokyo). In order to stain MPO positive cells, serial sections were incubated for 15–20 min with 0·0125 g/100 ml 3,3′-diaminobenzidine (Sigma) and 0·03% (v/v) hydrogen peroxide in 0·02 mol/l PBS. Methyl-green was used as a counterstain. These sections were observed under a light microscope (BX60, Olympus, Tokyo). In all experiments, pictures were captured in computer, and 5 mm length of muscularis mucosa was analysed using image analyser (NIH Image) as described previously [9,10] (control: $n = 8$, SS-1 infected: $n = 8$). The MAdCAM-1 positive vessels in lamina propria were calculated using image analyser and quantified as length of positively stained vessel walls per mm muscularis mucosa. All of the infiltrated cells (CD4, CD8, B cell or MPO positive cells) in the lamina propria and in the submucosa were counted in the section and expressed as the number of cells per mm muscularis mucosa.

Double immunolabelling and laser scanning confocal microscopy

For double staining of CD4 and β7, essentially the same immunohistochemistry procedure was used as for normal fluorescent microscopy. Briefly, sections were incubated with both primary antibodies against biotinylated antiβ7-integrin and FITC-conjugated anti-CD4 antibody overnight. In a second step, after rinsing with PBS, sections were incubated with rhodamine-conjugated streptavidin (streptavidin–rhodamine) (Amersham International plc, Buckinghamshire, UK) for 30 min at room temperature. Fluorescent preparations were examined using a Carl Zeiss laser scan microscope equipped with an argon laser (488 nm excitation for FITC), and rhodamine fluorescence was examined with the 543 nm laser. An appropriate emission filter system was used, and scanning with the 543 and 488 nm laser was performed sequentially (Carl Zeiss, Jena, Germany).

Statistics

Results are expressed as median and range. Data were statistically analysed by Kruskal–Wallis and Scheffé's *F*-test (among subsets of infiltrating cells) or Mann–Whitney test (between control

group and SS1 infection group). *P*-values of 0·05 or less were considered to be statistically significant. Association among parameters was assessed by use of the Spearman rank-correlation technique.

RESULTS

The serum antibody (IgG) against *H. pylori* was positive for all mice and was negative for all animals of control groups. As the gastric histological specimens revealed the presence of the bacterial body of *H. pylori* bacteria in all stomachs in the SS1-inoculated group, persistent infection was confirmed in the SS1 group during the observation period. A significant cell infiltration was observed not only in the submucosa but extended to the upper part of the mucosa of SS1-infected mice compared with non-infected control mice by H&E staining (Fig. 1a,b).

Serial stomach sections of control and infected mice were investigated for the distribution of MPO-positive cells, CD4 T

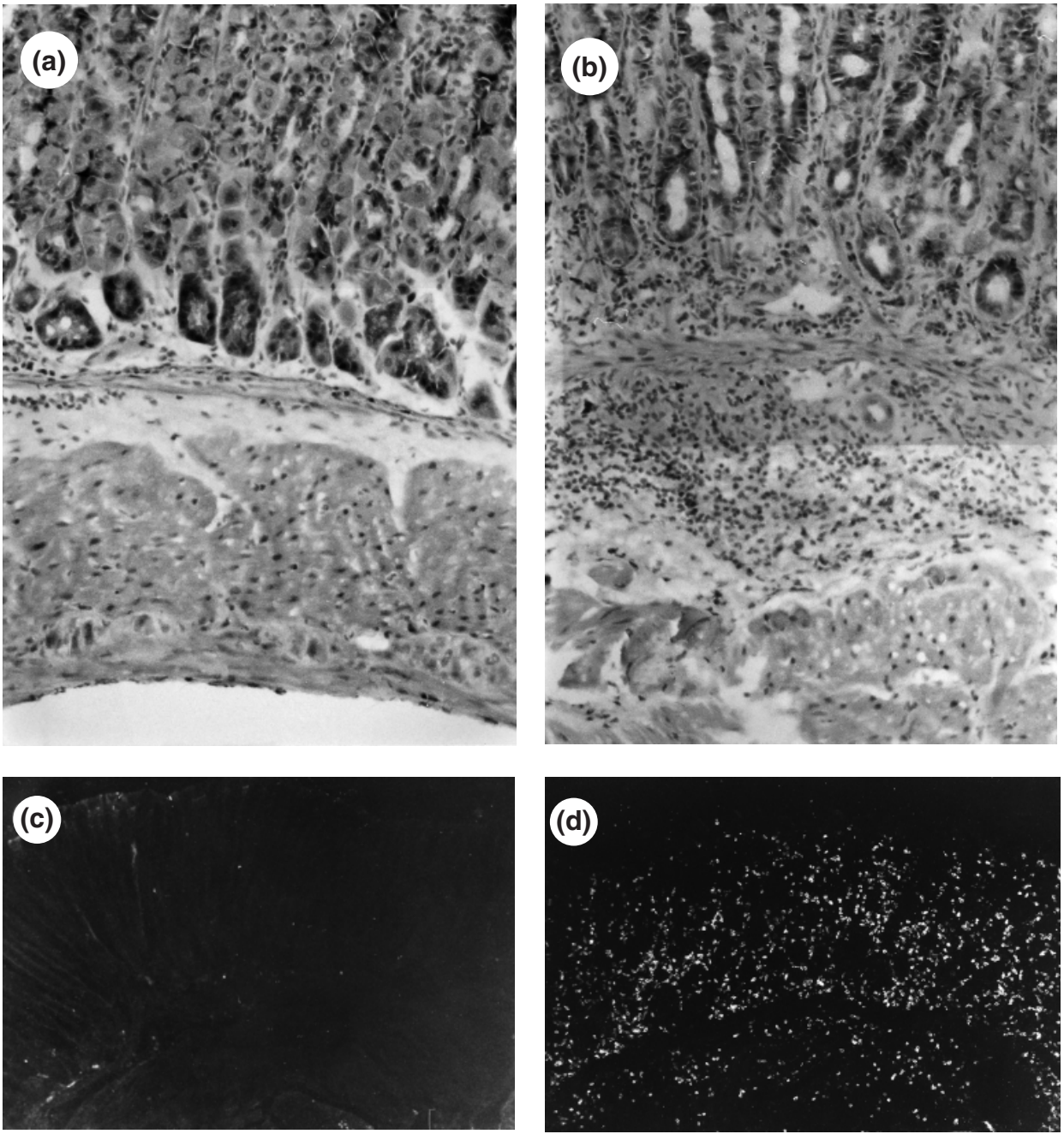

Fig. 1. Microscopic pictures of the gastric mucosa of SS1-infected mice compared with noninfected control mice. (a) Control mice (H&E staining, ×100). (b) SS1-infected mice (H&E staining, ×100). (c) Immunohistochemical study of CD4 positive cells in the stomach of control mice. (d) Immunohistochemical study of CD4-positive cells in the stomach of SS1-infected mice. The same specimens as (a) and (b) were observed.

cells, CD8 T cells, B cells and for the expression of cell adhesion molecules such as β7-integrin and vascular endothelial cell adhesion molecules. Figure 2 shows accumulation of leucocytes after SS-1 infection and compared the number of infiltrated cells among different subsets in the lamina propria (Fig. 2a) and in the submucosa (Fig. 2b). In control mice, there were a few CD4 T cells in the gastric mucosa. On the other hand, in SS1-infected mice, there was a marked infiltration of CD4 lymphocytes not only in the lamina propria (control, 1·14/mm *versus* SS1, 288·2/mm; $P < 0·01$), but also in the submucosa (control, 1·31/mm *versus* SS1145·2/mm; $P < 0·01$) extending to the upper part of the gastric mucosa (Fig. 1c,d). A significant increase in MPO positive cells (mainly consisting of neutrophils) was also demonstrated in the lamina propria (control, 1·12/mm *versus* SS1, 75·1/mm SS1; $P < 0·01$) and in the submucosa (control, 1·52/mm *versus* SS1, 94·5/mm; $P < 0·01$) of infected mice. In control mice some B cells were observed, mainly in the lamina propria (3·12/mm), but B cells were observed mainly in the submucosa around the large vessels of SS1-infected mice and a significant increase in B cell accumulation was observed in the submucosa (control, 3·1/mm *versus* SS1, 21·5/mm SS1; $P < 0·01$). There were a few CD8 (1·06/mm) T cells in the gastric mucosa of control mucosa, but this subset did not increase significantly in the infected mice. Therefore, the dominantly infiltrated cells were CD4 positive cells in the SS1-infected gastric mucosa, and their number was markedly greater than those of CD8 T cells or B cells ($P < 0·01$) and MPO-positive cells ($P < 0·05$).

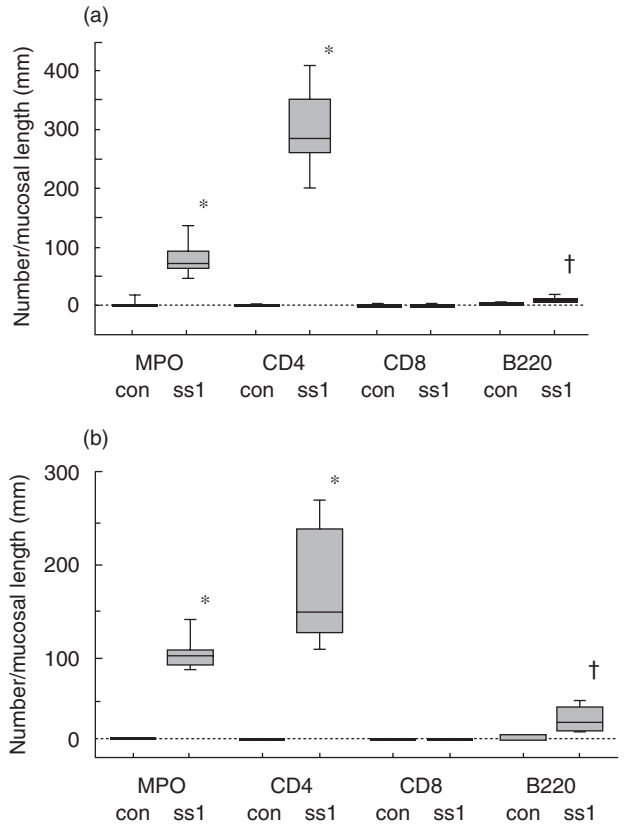

Fig. 2. The number of infiltrated cells of different subsets in the lamina propria (a) and submucosa (b) in control mice (con) and SS1-infected mice (SS1). The number of MPO positive cells, CD4, CD8 and B220 positive cells were determined as cells per mm of mucosal length. Values are expressed median and range (box) and minimal and vertical values (vertical line) from eight animals. *$P < 0·01$ compared with control groups. †$P < 0·05$ compared with control groups.

Next we investigated the expression of adhesion molecule β7-integrin in infiltrating lymphocytes. In control mice, there were few β7-integrin-positive cells in the gastric mucosa. On the other hand, we observed that the markedly infiltrating cells both in the gastric mucosa (control, 1·41/mm *versus* SS1, 270·6/mm; $P < 0·01$) and in the submucosa (control, 1·64/mm *versus* SS1, 167·2/mm; $P < 0·01$) expressed β7-integrin, a known marker for mucosal homing lymphocytes. In order to examine whether the infiltrating lymphocytes, which consisted of mainly CD4 cells, co-express β7-integrin molecules, dual labelling for CD4 and β7-integrin was performed in the gastric mucosa of SS1-infected mice. A confocal microscopic image demonstrated that there were many FITC positive CD4-positive cells in the lamina propria (Fig. 3a) and that the rhodamine-positive–β7-integrin-positive cells were also seen in the same area (Fig. 3b). The cells shown in yellow in Fig. 3c indicated that the almost all of CD4 lymphocytes co-expressed β7-integrin.

To investigate which adhesion molecules are involved when cells are infiltrated from the microvascular beds to the gastric tissue, the expression of vascular adhesion molecules, ICAM-1, VCAM-1 and MAdCAM-1 in the gastric mucosa was determined by immunohistochemistry. The specificity of antibodies was verified by the absence of non-specific staining in negative controls. In control mice, there was no detectable expression of ICAM-1 or VCAM-1 in the gastric tissue. In SS1-infected mice, expression of ICAM-1 was observed mainly in the vascular endothelium in the submucosa, and a weak expression of ICAM-1 was also shown in the vessels of lamina propria as well as on the surface of infiltrating cells in the inflamed mucosa (Fig. 4a). On the other hand, a strong expression of VCAM-1 was observed on venular endothelium in the submucosa (Fig. 4b), although no expression was observed in the mucosal area. In addition to venular endothelium, VCAM-1 was also observed on the cells of lamina propria in the infected mucosa. MAdCAM-1, a known counter ligand for β7-integrin, was not observed in control non-infected mice, either in the submucosa or lamina propria (Fig. 5a). A strong expression of MAdCAM-1 was observed clearly both in the lamina propria and in the submucosa of infected stomach (Fig. 5b), and the length of positively stained vessels reached $4·2 \pm 1·2 \times 10^2$ μm/mm muscularis mucosae (control $0·0 \times 10^2$ μm/mm; $P < 0·01$). These significant expressions of MAdCAM-1 in the lamina propria were usually seen around the infiltrating cells expressing β7-integrin (Fig. 6). The correlation between the number of infiltrating cells and expression of MAdCAM-1 were determined from serial sections using NIH image and plotted. A statistically significant correlation could be found between the length of MAdCAM-1 positive vessels and the number of β7-positive infiltrating lymphocytes in the infected gastric mucosa ($r = 0·915$, $P < 0·01$).

DISCUSSION

Although there are many reports of CD4 lymphocytes participating in cell impairment and in the formation of *H. pylori*-induced gastritis [5,14,15], they do not fully explain the origin of lymphocytes or from which vascular addressin these cells were recruited to the gastric mucosa. Recently, Michetti *et al*. reported that gastric infection with *H. felis* induced a recruitment of CD4 T cells

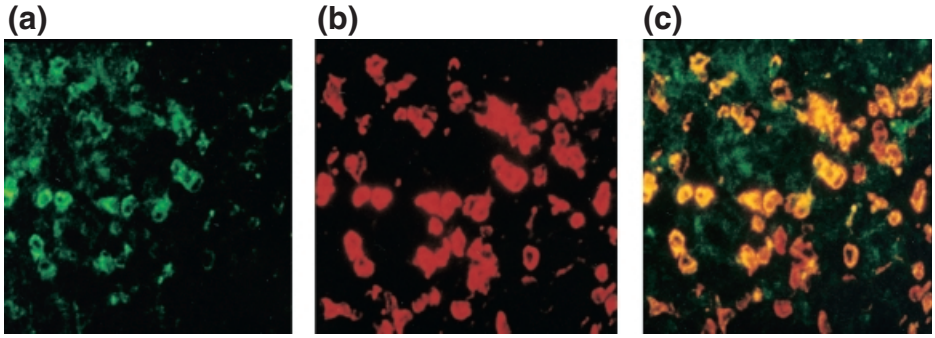

Fig. 3. Expression of CD4 and β7 in the lamina propria of gastric mucosa of SS1-infected mice observed by double immunolabelling (×400). A confocal microscopic image demonstrates the FITC-positive CD4 positive cells (a) and the rhodamine positive–β7-integrin-positive cells in the same area (b). The CD4 lymphocytes co-express β7-integrin were indicated in yellow (c).

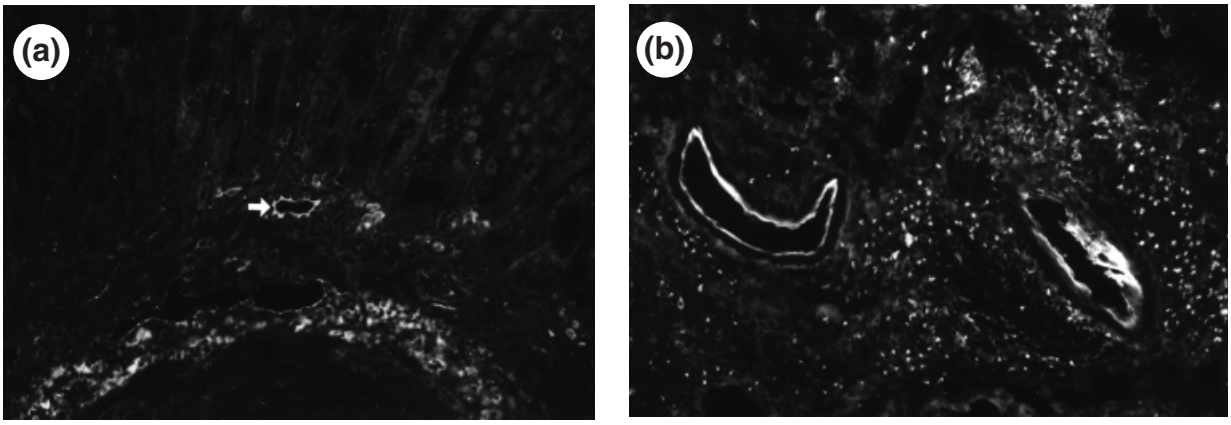

Fig. 4. Expression of vascular adhesion molecules, ICAM-1 (a) (×100, arrow) and VCAM-1 (b) (×200) in the gastric mucosa of SS1-infected mice determined by immunohistochemistry.

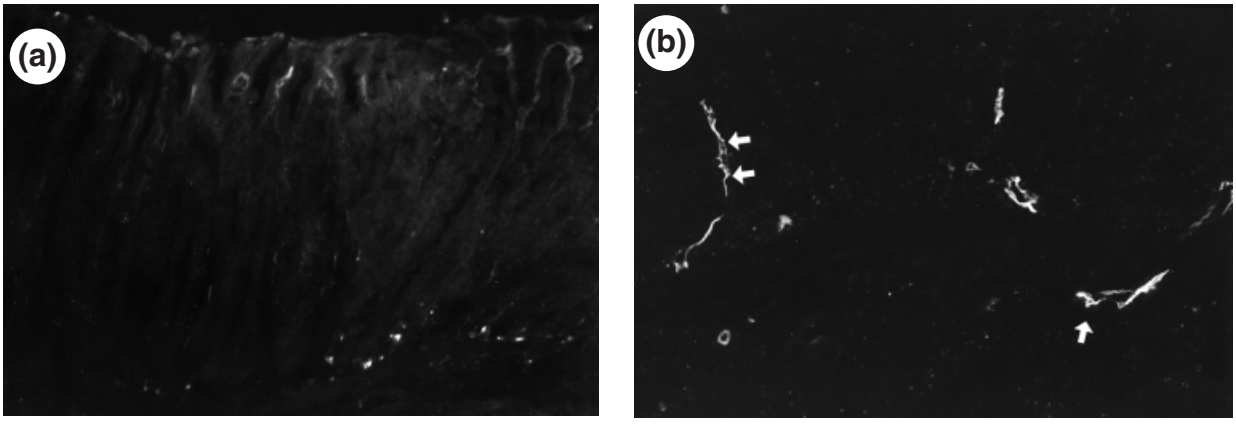

Fig. 5. Expression of vascular adhesion molecule, MAdCAM-1 in the gastric mucosa of control mice (a) and SS1-infected mice (b) determined by immunohistochemistry (×200). MAdCAM-1 expression is observed in the lamina propria (left arrows) and in the submucosa (right arrow) of infected mucosa (b).

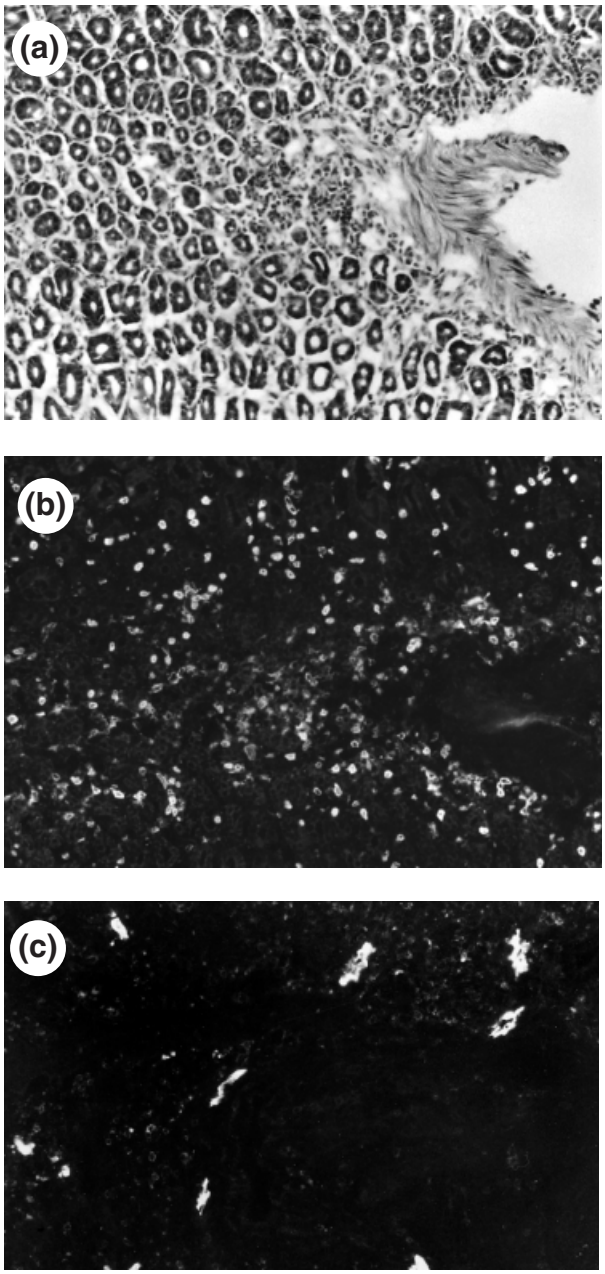

Fig. 6. Localization of MAdCAM-1 expression and β7-integrin-positive cells in the lamina propria of SS1-infected mice using serial section of gastric mucosa (×200). (a) H&E staining. (b) β7-integrin-positive cells determined by immunohistochemistry of the serial section of the H&E picture. (c) MAdCAM-1 expression in the lamina propria determined by immunohistochemistry using the serial section.

into gastric lamina propria and that a high proportion of them expressed alpha4beta7 integrin [16], although those authors did not analyse these cells quantitatively in histological sections. In this study, using well-orientated specimens, we studied the location and number of infiltrating cells *in situ* by immunohistochemistry. The present finding of a significant increase in CD4 T cell infiltration after infection into both the lamina propria and submucosa is consistent with their study, but the significant increase in B cell infiltration into the submucosa, which was not observed in the previous study, might result from the difference in the infection period (6 months *versus* 8 weeks) or a difference in Helicobacter species.

In our study most of the CD4 lymphocytes in the gastric mucosa of SS1-infected mice expressed β7-integrin, a lymphocyte marker originating from gut-associated mucosa. The critical role of β7-integrin in lymphocyte recruitment for the formation of the gut-associated lymphoid tissue has been proposed [17]. In the inflamed tissue, a significant number of mucosal lymphocytes could migrate aberrantly to non-originating organs, where these cells are seldom seen under non-inflamed conditions [18]. Dogan *et al*. examined the expression of $\alpha 4\beta 7$ or B cells in the lymphoid follicle of the stomach induced by *H. pylori* infection [19]. It might be considered that *H. pylori* infection induces a host environment favourable for the accumulation of these lymphocytes as an autoimmune-like condition.

MAdCAM-1 is an immunogloblin superfamily adhesion molecule expressed on mucosal endothelium and a counter ligand for integrin $\alpha 4\beta 7$ [8]. It should be noted that in this study increased expression of MAdCAM-1 was seen not only in the submucosa, but also in the lamina propria of the infected mucosa where $\beta 7$ positive cells were infiltrated densely, whereas almost no MAdCAM-1 expression was seen in control stomach. So, it is considered that in *H. pylori*-gastritis inflammatory cells preferentially migrated into the gastric mucosa through venular endothelium which expresses MAdCAM-1. In this study up-regulation of VCAM-1 expression was limited only on venular endothelium in the submucosa, not in the mucosal area. Since VCAM-1 binds lymphocytes which express $\alpha 4\beta 1$, but not $\alpha 4\beta 7$ [20], we speculate that VCAM-1 plays little role in lymphocyte infiltration to the mucosal site. The differences in lymphocyte subsets binding to either MAdCAM-1 or VCAM-1 may be correlated to the delineation between mucosal and non-mucosal trafficking compartments, allowing for separation of specialized immune responses. In this study there were also VCAM-1-positive cells in the lamina propria of inflamed mucosa. Although we could not identify these cells, they may possibly include dendritic cells [21], fibroblast-like cells [22] or T cells [23]. In the *H. pylori*-infected stomach, ICAM-1 was observed in the submucosa and only a weak expression of ICAM-1 was observed in the lamina propria. However, because it is considered that ICAM-1 has a role in neutrophil infiltration in gastric epithelial cells [24], this adhesion molecule may not be involved actively in the attraction of CD4-$\beta 7$ positive lymphocytes to the mucosal site.

In *H. pylori*-induced gastritis it is uncertain how MAdCAM-1 expressed aberrantly. Sikorski *et al*. investigated inducer of MAdCAM-1 in mouse venular endothelium [25]. They proved that LPS strongly induce MAdCAM-1 expression as well as TNF-α or IL-1β. LPS exists also in *H. pylori*, although it is considered to have less toxicity than that of *Esherichia coli* [26]. There is a possibility that *H. pylori* LPS itself can induce MAdCAM-1 expression in gastric mucosal venules, causing gastritis by aberrantly migrated $\beta 7$-positive cells.

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