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ORIGINAL ARTICLE

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SS1 *Helicobacter pylori* disrupts the paracellular barrier of the gastric mucosa and leads to neutrophilic gastritis in mice

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Abstract *Helicobacter pylori* induces severe neutrophilic infiltration in the lamina propria of the stomach, which leads to gastritis in humans. The possible involvement of a paracellular route for bacterial nutrients and etiologic agents that may play an important role in colonization of the bacteria and cause gastritis has been suggested. To study the functions of the paracellular barrier of gastric surface epithelium, SS1, a strain of *H. pylori* adapted to the murine stomach, was inoculated into the stomachs of C57BL/6 mice. At 4 months after inoculation, SS1 had achieved a high level of colonization (10^6–10^7 colony-forming units/g tissue) associated with neutrophilic infiltration in the lamina propria of the junctional zone. Disruption of the paracellular barrier was observed in the SS1-infected stomachs, as revealed by the invasion of a lanthanum tracer into the paracellular space of the surface epithelium. Only 2% of junctions were permeable in control stomachs, whereas 72% of the paracellular barrier was disrupted in the SS1-infected gastric epithelia. Furthermore, distribution of tight junction-related molecules such as 7H6 antigen, occludin, and cortical actin was affected in the surface epithelium by SS1 infection. The linear expression pattern of occludin was disrupted and became irregular or punctuated. The 7H6 antigen accumulated as aggregates in the apical portion of the surface epithelium and cortical actin became irregular and punctuated. Taken together, these results indicate that infection by SS1 directly or indirectly caused an increase in paracellular permeability and altered the localization of tight junction-related molecules of the gastric surface epithelium. This observation suggests that the paracellular pathway may play a significant role in establishing *H. pylori*-induced gastritis in the clinical setting.

Keywords *Helicobacter pylori* · SS1 · Tight junction · Occludin · 7H6 antigen

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Introduction

There is a growing body of evidence that the gram-negative bacterium *Helicobacter pylori* is a pathogen of gastritis and gastric ulcers [5, 11,17]. The inflammatory reaction of host tissues to *H. pylori* is considered to be initiated by a number of soluble factors released from the bacteria [8,9]. *H. pylori* also requires a flow of ions and nutrients from the host mucosa for growth and differentiation [5]. The paracellular pathway of gastric epithelium has been considered an important route for bacterial derivatives in the inflammatory host reaction and the flow of nutrients from the host tissue needed for fully developing pathogenic infection.

The tight junction localizes the most apical of the apposing epithelial cells and acts as a physiological barrier for paracellular transport of water, ions, and nonionic small molecules [1,4]. However, little is known about the involvement of the paracellular pathway of gastric mucosa in this particular infection. Moreover, there is no in vivo study testing the paracellular barrier function using *H. pylori*. This is partly because of the lack of in vivo animal model for *H. pylori* infection [18].

It is agreed that *H. pylori* models are needed for vaccine development and study of the pathogenesis of *H. pylori* in the murine system. However, mouse adaptation of *H. pylori* is a random event and there is extreme variability in colonization [7, 10,15]. Criteria for a mouse

model should be (1) colonization and pathology in the antrum and body, (2) a high level of colonization, and (3) the presence of adhesion to the gastric epithelium [18]. Sydney strain 1 (SS1), is a CagA- and VacA-positive strain of *H. pylori* [16] which achieves a high level of colonization in C57BL/6 mice. Furthermore, the bacteria are reported to be visible at the transitional zone between the antrum and the body of the stomach, with adhesion to the epithelial surface. This culture is available to all, providing a useful murine model for studying the pathogenesis of *H. pylori* infection [16].

In this report, we study changes of tight junctions of gastric mucosa using C57BL/BL6 mice infected with SS1. A high level of colonization was observed, with infiltration of neutrophils into the lamina propria of the transitional zone between the antrum and the body. An apparent increase in paracellular permeability and changes of tight junction-related molecules such as 7H6 antigen, occludin, and actin were observed. These observations indicated that disruption of the paracellular barrier, with altered molecular structures of tight junctions, could be an important event for the type B gastritis caused by *H. pylori*.

Materials and methods

Bacterial growth media, cultures, and reagents

The SS1 was kindly provided by Dr. A Lee [16]. All bacteria were grown on *Campylobacter*-selective agar (CSA) consisting of sterile horse blood (5% vol/vol) in blood agar base no. 2 (Oxoid, Basingstoke, U.K.) containing Skirrow's supplement: 10 mg/l vancomycin (Sigma, St. Louis, Mo., USA), 5 mg/l trimethoprim lactate (Sigma), 2500 IU/l polymyxin B (Sigma), and 5 mg/l amphotericin B (Squibb, Princeton, N.J., USA). The plates were incubated in an incubator set at 10% CO_2 and 95% humidity. For titration of *H. pylori*, a gastric biopsy obtained from the transitional part of each stomach was weighed (about 100 mg) and homogenized with a Dounce homogenizer (Eyela, Tokyo, Japan). These homogenates were serially diluted with brain heart infusion (BHI) broth (Oxoid) and plated on the plates mentioned above. All other reagents were purchased from Sigma unless otherwise stated.

Histologic examination

The stomachs were gently dissected out with a portion of the proximal duodenum and distal esophagus, then placed on a plastic platform and fixed in 10% formalin/PBS. Samples were cut into slices longitudinally, embedded in appropriate orientation in paraffin, and stained with hematoxylin-eosin or Giemsa stain.

Paracellular permeability

Stomach samples were fixed in ice-cold 0.05 M cacodylate buffer (pH 7.4) containing 4% lanthanum nitrate and 2.5% glutaraldehyde for 30 min with gentle agitation and then in ice-cold 0.05% cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde overnight with several changes of fixatives without lanthanum nitrate [27]. This procedure minimized lateral invasion of lanthanum nitrate between the basement membrane and basolateral space of epithelial cells, which hampers estimation of paracellular permeability by electron microscopy. After fixation, a portion of stomach including the junctional zone between the antrum and body was selected. These samples were cut into pieces and postfixed with 1% osmic acid and 1% potassium ferrocyanide in H_2O for 2 h at 4°C, dehydrated, and embedded in Epon 812 resin (Dupon, Calif., USA). Semi-thin (1 µm) sections were stained with toluidine blue and specimens that included surface epithelium of the junctional zone were selected. Ultra-thin sections were prepared and examined by electron microscopy (JOEL, Tokyo, Japan). The frequency of paracellular invasion of lanthanum nitrate was calculated as the permeability index and expressed in percent: permeability index=(number of paracellular labels beyond the tight junction/number of junctions between apposing surface epithelia)×100. In all samples, at least 100 junctions were examined. Six mice were inoculated with either medium only or bacteria. Three independent experiments were conducted.

Immunohistochemistry

Samples were snap-frozen and 5-µm-thick frozen sections were fixed with methanol/acetone (1:1 vol/vol) at –20°C for 10 min. Fixed samples were washed with PBS several times and incubated with the first antibody as described [25]. Antibodies used were rabbit antioccludin [2,3], rabbit anti-7H6 antigen [23,28], and rabbit anti-ZO-1 (Zymed, San Francisco, Calif., USA) at dilutions of 1:20, 1:20, and 1:100, respectively. Immunostaining was analyzed using an MRC-1024 confocal laser scanning microscope (Bio-Rad, Tokyo, Japan).

Animals

All animals were specific pathogen-free, inbred female C57BL/6 mice 8–10 weeks old (CLEA, Tokyo, Japan). All were housed in the Microbiology Animal House, Health Sciences University of Hokkaido under clean conditions for the duration of the experiments. The animals were fed a commercial diet (CLE-3, CLEA) and given water ad libitum.

Statistical analysis

At least six mice were examined in each experiment and identical protocols were performed three times. The mean response of each experimental group was compared with its simultaneous control using the unpaired Student's *t*-test. Analysis of variance was used to compare the mean responses of experimental and control groups.

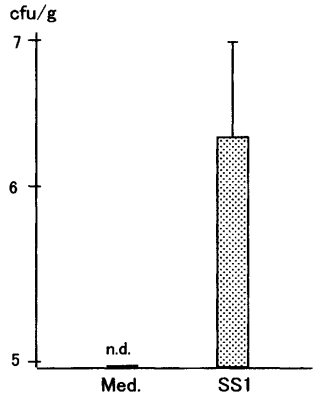

Fig. 1 Colonization of SS1 in the stomach in C57BL/6 mice. 1×10^9 CFU of SS1 in 100 µl of BHI broth was directly inoculated into the stomach. After 4 months, the CFU of each stomach was estimated as described in "Materials and methods". A high level of SS1 colonization (mean 3.8×10^6 CFU/g tissue) was observed. The logarithmic value is shown. The CFU of SS1 was not detectable in control mice (*n.d.*)

Results

Colonization of SS1 in the stomachs of C57BL/6 mice

A total of 1×10^9 colony-forming units (CFU) of SS1 in 100 µl of BHI broth were inoculated into the stomach directly using a blunt-ended 22-gauge needle. For the control, BHI broth only was given. After 4 months, the mice were killed and the CFU of each stomach was calculated. The incubation period was determined by preliminary experiments and the earliest time that gave the most reproducible colonization of SS1 was chosen. As shown in Fig. 1 A, a high level of colonization of SS1 (mean±SD $3.8\pm2.7)\times10^6$ CFU/g of tissue was observed at 4 months after inoculation. Histologic examination with Giemsa staining revealed many characteristic S-shaped bacteria overlying the surface at the junctional zone between the antrum and the body of the stomach (Fig. 2).

Increase of paracellular permeability of the surface epithelium of SS1-colonized mucosa

Paracellular permeability of surface epithelium was assessed by degree of invasion of lanthanum nitrate into the paracellular space of the surface epithelium (Fig. 3). The paracellular space of the surface epithelium of the SS1-colonized stomachs showed labeling of the paracellular space of the apposing epithelial cells by lanthanum nitrate (Fig. 3B), whereas in control mice the invasion of lanthanum nitrate was minimal and stopped at the apical site of the paracellular space (Fig. 3A). The frequency of lateral invasion (beyond tight junctions) was calculated as the permeability index (see Materials and methods section). It was found that this index increased more than 30-fold in SS1-infected mice (72±18%) over control mice (2±2%) (Fig. 4).

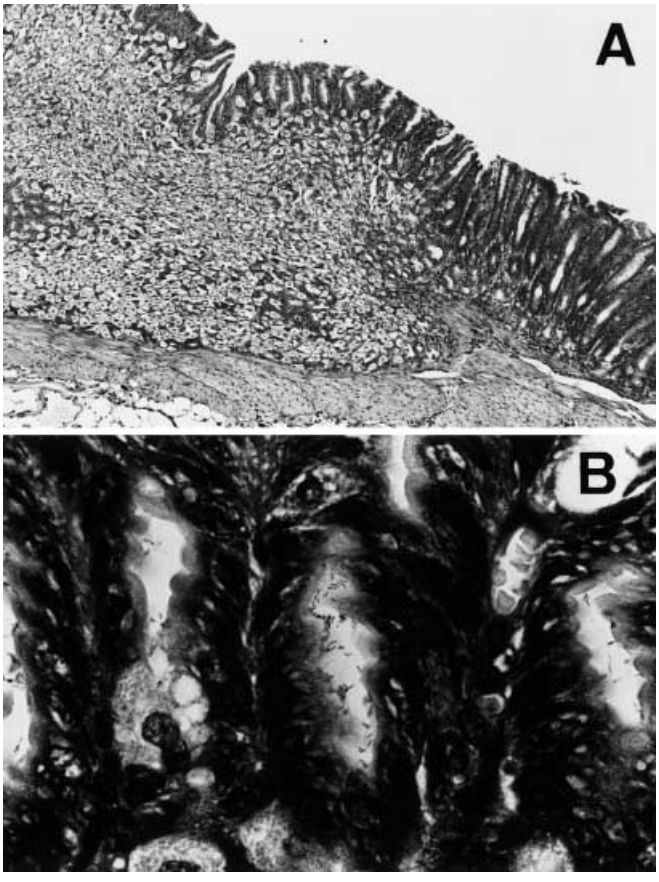

Fig. 2A,B Colonization of SS1 in the transitional zone of the murine stomach. At the junctional zone between the antrum and the body (**A**), histologic examination with Giemsa staining revealed many characteristic S-shaped bacteria overlying the surface (**B**), with neutrophilic infiltration in the lamina propria. Original magnification (**A** ×100, **B** ×400)

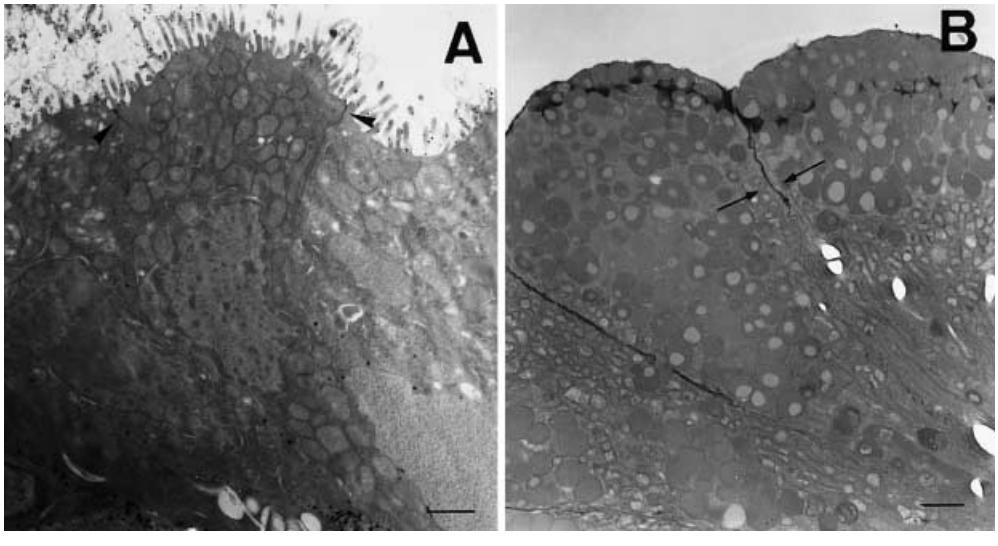

Fig. 3A,B Disruption of the paracellular barrier of the surface epithelium of the SS1-infected murine stomach. Whereas the invasion of lanthanum nitrate was minimal and stopped at the apical site of the paracellular space in control mice (**A,** *arrow*), paracellular spaces of the surface epithelium of the SS1-colonized stomach showed labeling of the paracellular space of apposing epithelial cells by lanthanum nitrate (**B,** *arrows*). *Bar*=1 µm

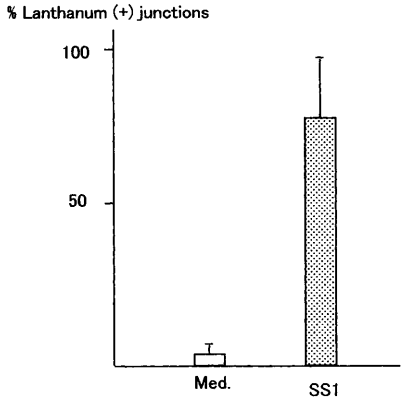

Fig. 4 Increase in paracellular permeability in the SS1-infected murine stomach. The frequency of lateral invasion (beyond tight junctions) shown in Fig. 3 was calculated as the permeability index. This index increased more than 30-fold in the SS1-infected mice (72±18%) over controls (2±2%) ($P<0.01$)

Changes of 7H6 antigen, occludin, and cortical actin in SS1-infected surface epithelium

The distribution of the tight junction-related molecules 7H6 antigen, occludin, and cortical actin was examined by immunohistochemistry. In the control surface epithelial cells, both 7H6 antigen and occludin were concentrated at the most apical of the apposing cells, consistent with the distribution of tight junctions. In clear contrast, both 7H6 antigen and occludin showed clearly distinct distribution patterns in the SS1-infected mice (Fig. 5). Although the immunolocalization of occludin was found still to be at the apex, the staining pattern was irregular and punctuated. The 7H6 antigen showed more evident changes. It failed to localize at the most apical apposing cells and accumulated in the cytoplasm. Cortical actin in the SS1-infected mice was also shown to be changed. It became fainter and a punctuated pattern was observed. Of interest, immunostaining of these molecules on the epithelium rather than the transitional zone of SS1-infected stomach showed little change compared to those of control mice (data not shown).

Transitional zone-specific gastritis in the SS1 infected mice

Histologic examination revealed that infiltration of neutrophils was concentrated in the lamina propria of the SS1-infected stomach with a lower amount of mononuclear cells, indicating transitional zone-specific infiltration of neutrophils (Fig. 6 D). In the control mice, almost no cellular infiltration was observed (Fig. 6B).

Discussion

Using biopsy specimens from human gastric mucosa, structural alterations of tight junctions were observed to occur in close correlation to *H. pylori* infection [13, 19,20]. The inflammatory reaction of host tissues to *H. pylori* is thought to be initiated by soluble factors released from the bacteria [5]. *H. pylori* also require a flow of ions and nutrients from the host mucosa for their growth and differentiation [5]. Because *H. pylori* never invaded the lamina propria, a paracellular pathway in gastric epithelium as a route for the bacterial derivatives might be important for the inflammatory host reaction and flow of nutrients from the host tissue in the development of a fully pathogenic infection. However, human biopsy specimens are not appropriate for the study of paracellular barrier function. With them, very small, basolateral surfaces of specimens are exposed to tracers for the study of barrier functions. This results in nonspecific invasion of the electron-dense tracer and hampers estimation of the paracellular barrier. The murine-adapted *H. pylori* strain SS1 provided the important model for studying the possible involvement of the paracellular barrier in this important infectious disease. A high level of SS1 colonization resulted in impairment of the paracellular barrier function of surface gastric epithelium with changes in tight junction-associated molecules. Disruption of the paracellular barrier was quite evident, as shown by the more than 30-fold increase in lateral invasion of the tracer compared to control mice. The data required careful interpretation, since the present study does not directly specify bacterial factors, which down-regulates paracellular barrier function in the present model. However, massive infiltration of neutrophils in the lamina propria of the transitional zone correlated with the dysfunction of the paracellular barrier, indicating a close relation of the paracellular barrier and neutrophilic gastritis. This is the first demonstration that the paracellular pathway is actually affected in stomachs infected by *H. pylori*.

Although disruption of the paracellular barrier was evident, accompanying alterations of tight junction-associated molecules, the molecular mechanism of the impairment of the paracellular barrier by *H. pylori* is still largely unknown. The present in vivo model may provide several useful experimental systems for understanding the mechanism inhibiting the barrier function of gastric mucosa. It has been reported that *H. pylori* sonicates disrupt the epithelial barrier of T84, a human colon cancer cell line, in a process inhibited by protein kinase C activator [26]. Furthermore, a recent report using heterologous epithelial cell lines suggested that VacA toxin indeed down-regulated the paracellular barrier function of this model epithelium [21]. It should be examined with an in vivo model whether this activity of VacA is actually involved in the development of neutrophilic gastritis. Using a mutant strain derived from SS1, many of these questions could be addressed.

H. pylori infection is highly disseminated in human beings and is strongly linked to gastritis and gastric ulcers. Prolonged infection by *H. pylori* is associated with increased risks of developing gastric adenocarcinoma [6] and mucosa-associated lymphoid tissue (MALT)

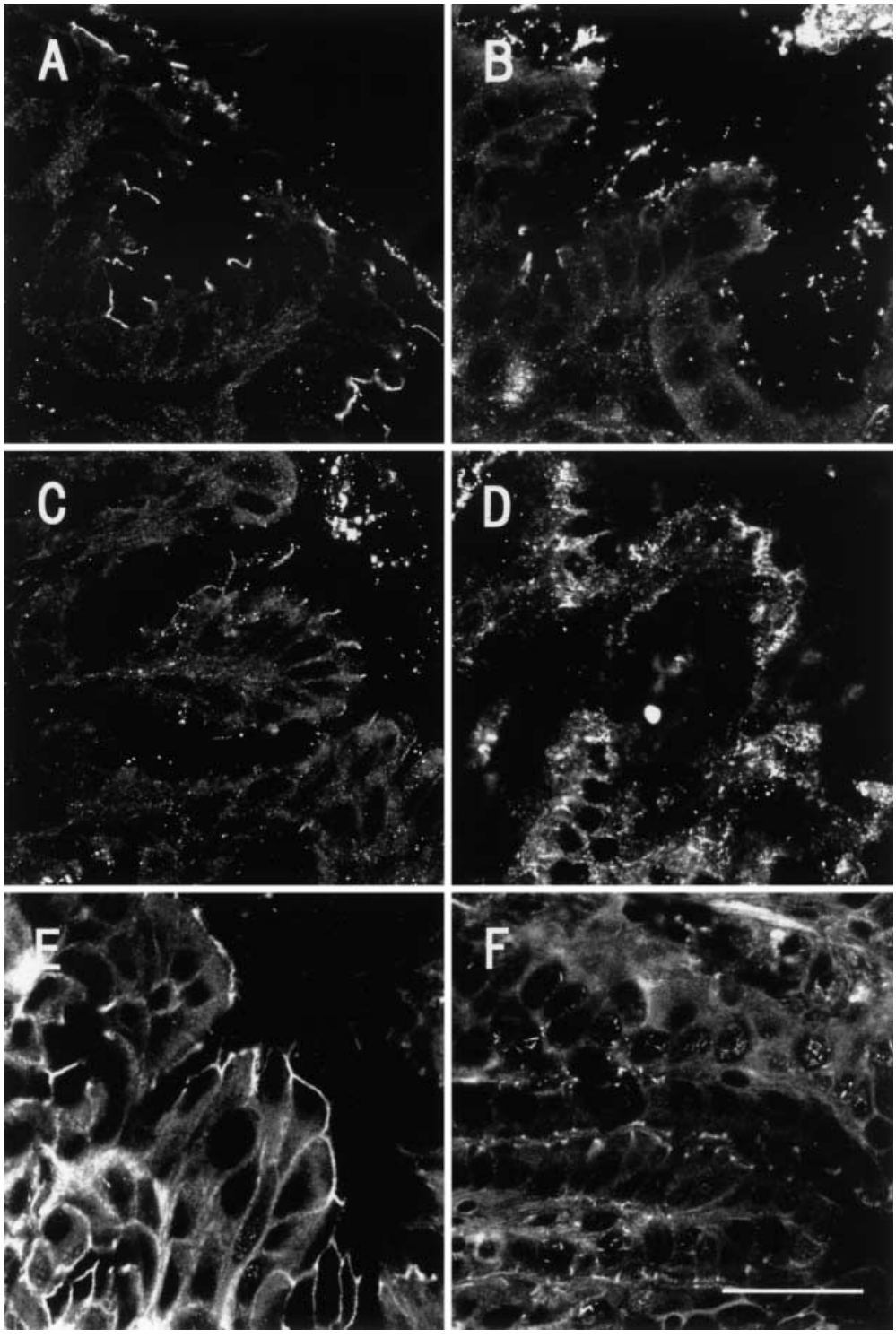

Fig. 5A–F Changes of tight junction-related molecules in the SS1-infected murine stomach. The distribution of the tight junction-related molecules occludin (**A, B**), 7H6 antigen (**C, D**), and cortical actin (**E, F**) was examined in the surface epithelium of control (**A, C, D**) and SS1-infected mice (**B, D, F**). In the control surface epithelial cells, both 7H6 antigen and occludin were concentrated at the most apical of the apposing cells. In the SS1-infected mice, in contrast to the control, the immunolocalization of occludin was found to be irregular and punctuated (**B, A**). The 7H6 antigen (barmotin) failed to localize at the most apical of the apposing cells and accumulated in the cytoplasm (**D, C**). Cortical actin in SS1-infected mice was also shown to become fainter and punctuated (**F, E**). *Bar*=32 µm

lymphomas [22]. As described in the present study, the paracellular barrier was impaired by *H. pylori* infection. This observation suggests the possibility of drug design with the aim of rebuilding the paracellular barrier of gastric mucosa. Since *H. pylori* requires ions and nutrients from the host mucosa, shutting off the route should prevent further colonization by this particular microorganism.

A possible role of paracellular barriers has been suggested in a number of gastrointestinal diseases[14]. Furthermore, adherens and tight junctions have been reported to be altered in several malabsorption diseases. In celiac disease, malabsorption is secondary to the impaired transport of nutrients though the damaged enterocytes. A close link to some type of histocompatibility antigens suggests that a certain antigen evokes antibodies to glu-

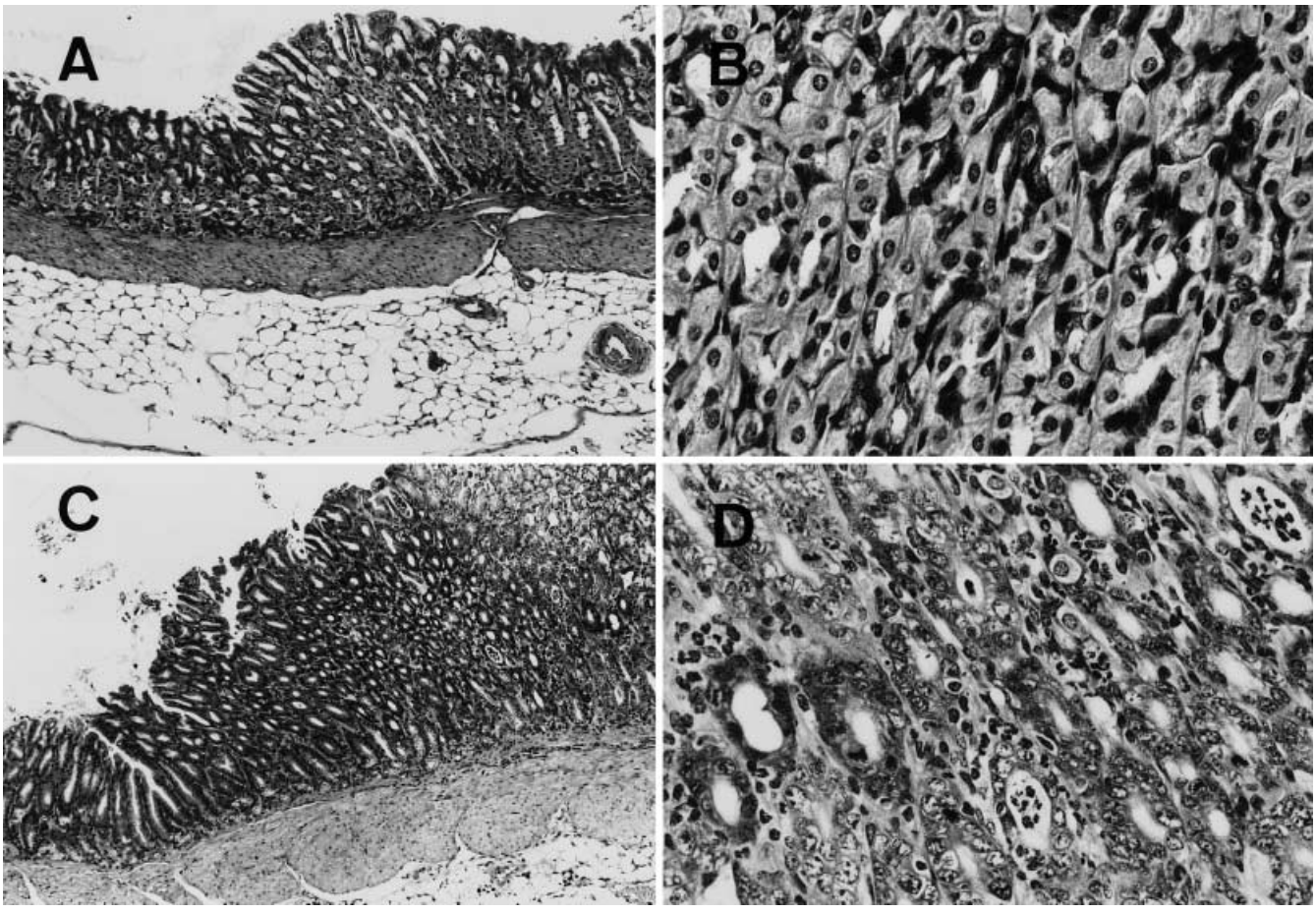

Fig. 6A–D SS1-induced transitional zone-specific neutrophilic gastritis. Histologic examination of the transitional zone of control (**A**, **B**) and SS1-infected mice (**C**, **D**) revealed neutrophilic gastritis in SS1-infected mice but not in controls. Original magnification (**A** and **C** ×100, **B** and **D** ×400)

ten, which results in the binding of gluten to the enterocyte, with subsequent mucosal damage. In ulcerative colitis and Crohn's disease, genetic factors and enterobacterial antigens provoke mucosal injury. Collectively, host immune responses induced by bacterial antigens have been suggested to be central in these inflammatory conditions, which in turn increase paracellular permeability of gastrointestinal epithelium. Indeed, it was shown recently that interleukin-10 is effective for treatment of inflammatory bowel disease in murine models [24]. Interleukin-8, a cytokine secreted by *H. pylori*-infected gastric mucosa, stimulated an impairment of the gastric epithelial barrier and led to leukocyte migration across gastric epithelial cells [12]. Subsequent events of SS1 colonization, such as cytokine secretion, should play an important role for the regulation of paracellular permeability. Considering these host reactions is critical for interpretation of the current result.

In summary, colonization by the *H. pylori* strain SS1 resulted in disruption of the tight paracellular barrier of gastric surface epithelium closely correlated with transitional zone-neutrophilic gastritis. This model system that demonstrates the molecular aspects of the paracellular barrier in relation to the development of neutrophilic gastritis may help us develop ways to cure and prevent *H. pylori* infection and its successive disorders.

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