Helicobacter pylori and two ultrastructurally distinct layers of gastric mucous cell mucins in the surface mucous gel layer

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Abstract

***Background and aims*—*Helicobacter pylori* locate not only on the apical surface of surface mucous cells but also in the mucous gel layer covering the gastric mucosa. The present study was undertaken to observe the mucous gel layer itself and any *H pylori* in this layer at the electron microscopic level, and to determine whether *H pylori* proliferate in this layer.**

***Methods*—We examined resected human stomachs (five cases, fixed in Carnoy's solution, paraffin embedded) under the light microscope, and gastric biopsy specimens (10 cases, fixed in glutaraldehyde with or without osmium, epoxy embedded) under the electron microscope. We performed histochemical staining for gastric mucins and immunostaining for *H pylori*, gastric gland mucous type mucins, and intestinal mucins.**

***Results*—Under the electron microscope, surface mucous cell type mucins and gland mucous cell type mucins in the mucous gel layer covering gastric mucosa without intestinal metaplasia showed reticular and band like structures, respectively. *H pylori* were frequently found as small aggregates within the mucous gel layer of surface mucous cell type mucins, and *H pylori* within these aggregates were seen dividing. *H pylori* were frequently found in the mucous gel layer of the surface mucous cell type mucins along the border with the layer of gland mucous cell mucins. Occasionally, *H pylori* were trapped by frayed thin threads of the gland mucous cell type mucins.**

***Conclusions*—The two types of gastric mucins in the mucous gel layer differ in ultrastructure. *H pylori* preferentially colonise and form microcolonies within the mucous gel layer of surface mucous cell type mucins. Mucins from gland mucous cells may disturb the movement of *H pylori* within the mucous gel layer.**

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Keywords: gastric mucin; *Helicobacter pylori*; immunohistochemistry; surface mucous gel layer

The surface of the gastric mucosa is covered by the surface mucous gel layer (SMGL). Ota and Katsuyama[1] revealed that the SMGL has a multilaminated structure formed by two types of layers overlapping each other. One is formed by mucins derived from the surface mucous cells and the other by mucins derived from the gland mucous cells (cardiac gland cells, mucous neck cells, and pyloric gland cells). More recently, Shimizu and colleagues[2] demonstrated that *Helicobacter pylori* were localised not only on the apical surface of the surface mucous cells but also within the SMGL, preferentially in the layers of surface mucous cell type mucins.

The present study was undertaken to examine the structure of the SMGL at the electron microscopic level, to observe *H pylori* in the SMGL, and to determine whether *H pylori* colonise the SMGL or whether the organism in the SMGL is a non-viable form. Our results indicated that these two types of mucins exist in different ultrastructural arrangements and that *H pylori* proliferate in the SMGL and form microcolonies. Furthermore, our results suggested that mucins from gland mucous cells may disturb the movement of *H pylori* within the SMGL.

Methods

For the light microscopic study, five human stomachs resected for gastric carcinoma at Shinshu University Hospital were used (two men, three women; age range 49–60 years, mean 54.2). They were immediately fixed in freshly prepared Carnoy's solution following the method described previously[1] and cut into rectangular sections (5 mm×25 mm) with the long axis parallel to the lesser curvature. For histological and histochemical examination, 3 μm serial paraffin sections were prepared which were then either (i) immunostained for *H pylori* (rabbit anti-*H pylori* polyclonal antibody, 1:10; Dako, Carpenteria, California, USA) using an indirect immunoalkaline phosphatase method or (ii) stained using galactose oxidase/thionine Schiff reaction/paradoxical concanavalin A staining (GOTS-PCS) to enable identification of the surface mucous cell mucins and gland mucous cell mucins.[3–5] In addition, immunostaining for sialosyl-Tn (1:100; Dako) was performed to identify intestinal mucins in metaplastic goblet cells and in the SMGL covering the gastric mucosa.[6]

For the ultrastructural study, two biopsy specimens taken from the antrum (greater curvature, approximately 3 cm from the pyloric channel) and two from the body (greater

Abbreviations used in this paper: GOTS-PCS, galactose oxidase/thionine Schiff reaction/paradoxical concanavalin A staining; SMGL, surface mucous gel layer.

Table 1 Reagents used in this study

Antibody	*Purpose or localisation of antigen*	*Dilution*	*Source*
Anti-*H pylori* (rabbit polyclonal antibody)	To identify *H pylori*	1:10	Dako
Antisialosyl-Tn (mouse monoclonal antibody, IgG)	To identify intestinal mucins from metaplastic goblet cells	1:100	Dako
HIK1083 (M-GGMC-1) (mouse monoclonal antibody, IgM)	To identify gastric gland mucous cell mucins	1:10	Kanto Chemicals
Alkaline phosphatase labelled antirabbit immunoglobulin	Second antibody for immunostaining for *H pylori*	1:50	Dako
Horseradish peroxidase labelled antimouse immunoglobulin	Second antibody for immunostaining for sialosy-Tn	1:50	Dako
Colloidal gold (15 nm) labelled antimouse IgM	Second antibody for electron immunohistochemistry with HIK1083	1:20	EY Laboratories
Colloidal gold (15 nm) labelled antimouse IgG	Second antibody for electron immunohistochemistry for sialosyl-Tn	1:20	EY Laboratories

curvature of the upper body) of the stomach in 10 patients with chronic active gastritis (six men, four women; age range 27–71 years; mean 52.0) at the time of routine upper gastrointestinal endoscopy were used. These 10 patients presented at our university hospital for dyspeptic symptoms and were positive for *H pylori* by histological examination and bacterial culture. It was confirmed that none of these patients had undergone previous eradication therapy for *H pylori* and none had been using medications for gastrointestinal disease for at least one month. In addition, using an identical biopsy protocol, we studied five non-infected

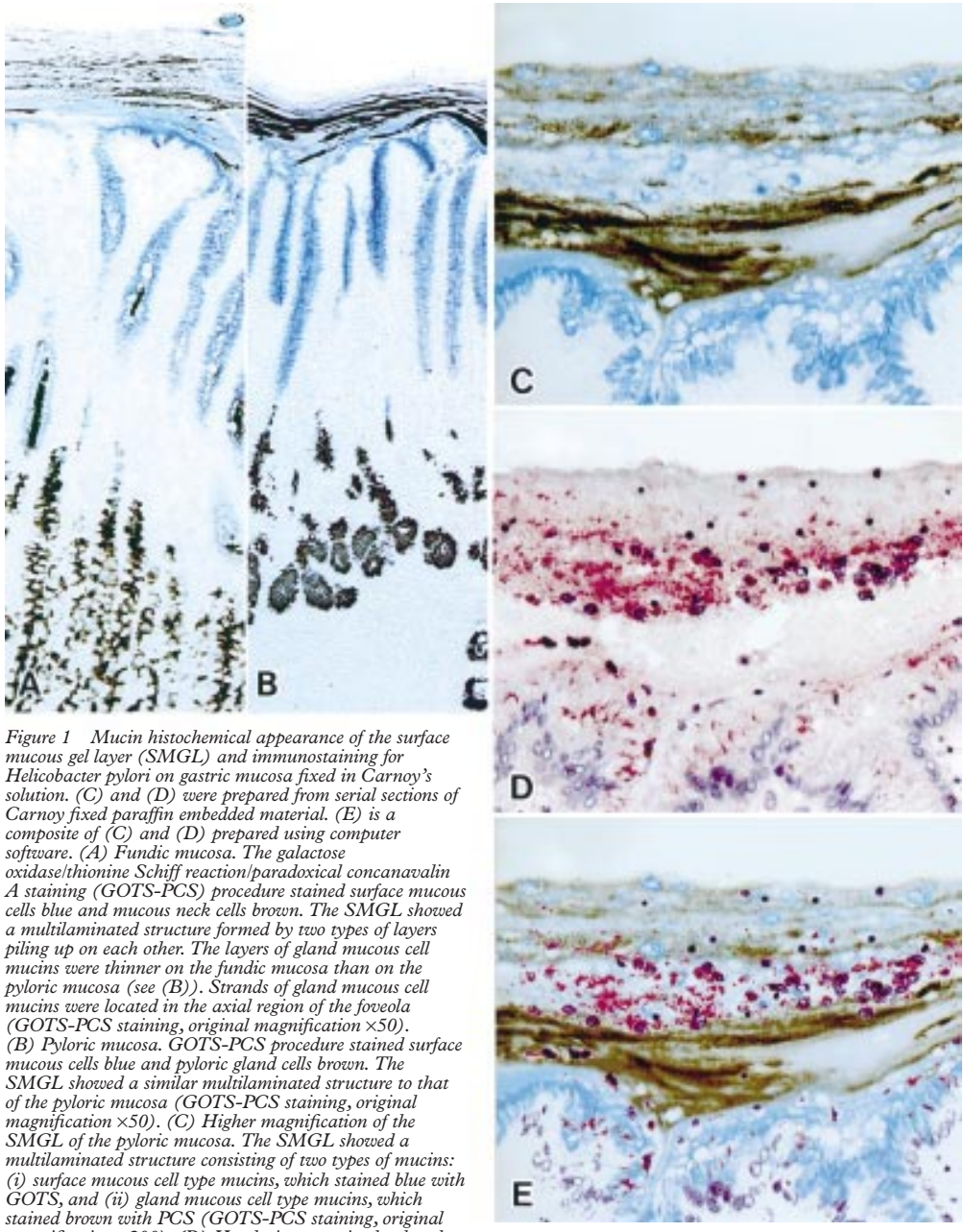

Figure 1 Mucin histochemical appearance of the surface mucous gel layer (SMGL) and immunostaining for Helicobacter pylori on gastric mucosa fixed in Carnoy's solution. (C) and (D) were prepared from serial sections of Carnoy fixed paraffin embedded material. (E) is a composite of (C) and (D) prepared using computer software. (A) Fundic mucosa. The galactose oxidase/thionine Schiff reaction/paradoxical concanavalin A staining (GOTS-PCS) procedure stained surface mucous cells blue and mucous neck cells brown. The SMGL showed a multilaminated structure formed by two types of layers piling up on each other. The layers of gland mucous cell mucins were thinner on the fundic mucosa than on the pyloric mucosa (see (B)). Strands of gland mucous cell mucins were located in the axial region of the foveola (GOTS-PCS staining, original magnification ×50). (B) Pyloric mucosa. GOTS-PCS procedure stained surface mucous cells blue and pyloric gland cells brown. The SMGL showed a similar multilaminated structure to that of the pyloric mucosa (GOTS-PCS staining, original magnification ×50). (C) Higher magnification of the SMGL of the pyloric mucosa. The SMGL showed a multilaminated structure consisting of two types of mucins: (i) surface mucous cell type mucins, which stained blue with GOTS, and (ii) gland mucous cell type mucins, which stained brown with PCS (GOTS-PCS staining, original magnification ×200). (D) H pylori were stained red and were demonstrated not only on the apical and/or lateral surface of the surface mucous cells but also within the SMGL in which H pylori were seen as small aggregates with detached epithelial cells in their centre. Aggregations of H pylori were distributed in layers (immunoalkaline phosphatase staining for H pylori, original magnification ×200). (E) Aggregations of H pylori were mostly distributed in the layers containing mucins derived from surface mucous cells.

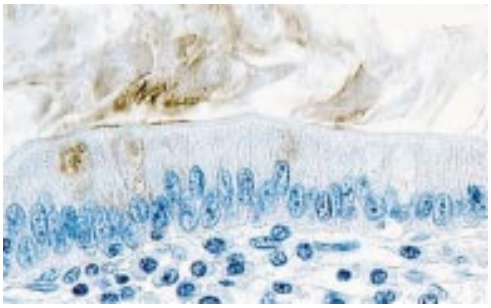

Figure 2 Pyloric mucosa fixed in Carnoy's solution. Sialosyl-Tn was demonstrated in metaplastic goblet cells and in the surface mucous gel layer covering the area of intestinal metaplasia (immunoperoxidase staining for sialosyl-Tn, original magnification ×200).

volunteers (four men, one woman; age range 29–44 years; mean 34.6) with no subjective or objective evidence of gastrointestinal disease, with negative serology for anti-*H pylori* antibody, and with no visible organisms in any of the biopsy specimens. The biopsy specimens were taken from the gastric mucosa well away from any visible lesions, such as intestinal metaplasia or apparent erosive lesions. The specimens were fixed for 24 hours in phosphate buffered 2.5% glutaraldehyde solution at 4 °C, care being taken not to lose the SMGL (that is, tissue samples in the forceps of the endoscope were gently removed using wooden toothpicks and dropped into the fixatives) and we were extremely careful not to agitate the container. Then, for each patient, one specimen from the antrum and one from the body were further fixed in phosphate buffered 1% osmium tetroxide solution for two hours at 4°C. The fixed specimens were dehydrated through graded alcohols, immersed in propylene oxide, and embedded in epoxy resin. Semi thin sections prepared from these tissue blocks were stained with toluidine blue. The specimens showing the best preservation of the SMGL were selected for further study. Ultrathin sections for morphological observation were mounted on single slot copper grids, and stained with uranyl acetate and lead citrate. Immunostaining with (i) HIK1083 (1:10; Kanto Chemical, Tokyo, Japan), which reacts with gastric gland mucous cell type mucins[7–11] and (ii) with antisialosyl-Tn (1:100; Dako) was carried out as previously described.[10] The epitope structure of HIK 1083 was elucidated to consist of an oligosaccharide bearing peripheral α linked GlcNAc.[7 12] HIK1083 has been demonstrated to label gastric gland mucous cell type mucins and cells producing these mucins in vertebrate and human normal gastrointestinal tracts and in human metaplastic or neoplastic tissues.[8–11] Ultrathin sections thus prepared were observed using a JEOL JEM1010 transmission electron microscope at 80 kV accelerating voltage.

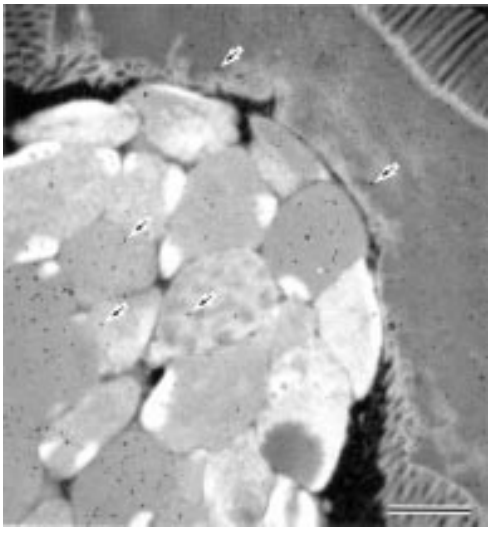

Figure 3 Immunogold staining with antisialosyl-Tn antibody of the area of the intestinal metaplasia. Sialosyl-Tn was demonstrated in the metaplastic goblet cells and in the surface mucous gel layer covering the area of the intestinal metaplasia (arrows) (Immunogold staining with antisialosyl-Tn antibody; bar=1 μm).

The reagents used in this study are summarised in table 1.

The tissues were used with the approval of the ethics committee of Shinshu University, Japan, and only after obtaining written consent from patients.

Results

LIGHT MICROSCOPIC OBSERVATIONS

Gastric mucosa away from carcinoma tissue showed chronic active gastritis with various degrees of intestinal metaplasia. In haematoxylin-eosin preparations of Carnoy fixed paraffin embedded materials, the SMGL was well preserved in all cases and appeared as an eosinophilic band covering the gastric mucosa. The SMGL of the fundic mucosa was thicker than that of the pyloric mucosa. For gastric mucosa showing intestinal metaplasia, the SMGL was thinner than on the surrounding mucosa without intestinal metaplasia.

Histochemistry and immunohistochemistry

The GOTS-PCS procedure stained surface mucous cells blue and gland mucous cells brown (fig 1). The SMGL on gastric mucosa without intestinal metaplasia consistently showed a multilaminated structure formed by two types of layers piling up on each other. One of these was formed out of surface mucous cell mucins and the other out of gland mucous cell mucins (fig 1). The SMGL did not contain sialosyl-Tn positive mucins. This laminated array within the SMGL was confirmed in both the fundic and pyloric gland areas (fig 1A, B). The layers of gland mucous cell mucins were thinner on the fundic mucosa than on the pyloric mucosa (fig 1A, B). The luminal surface of the surface mucous cells was consistently covered by the former type of layer (that is, surface mucous cell mucins) (fig 1). The foveolar lumen was filled with surface mucous cell mucins (fig 1) and with thin strands of gland mucous cell mucins located in the axial region of the foveola (fig 1).

Metaplastic goblet cells showed immunoreactivity for sialosyl-Tn antigen (fig 2). On gastric mucosa showing intestinal metaplasia, the luminal surface of the intestinal metaplastic cells was covered by sialosyl-Tn positive mucins (fig 2).

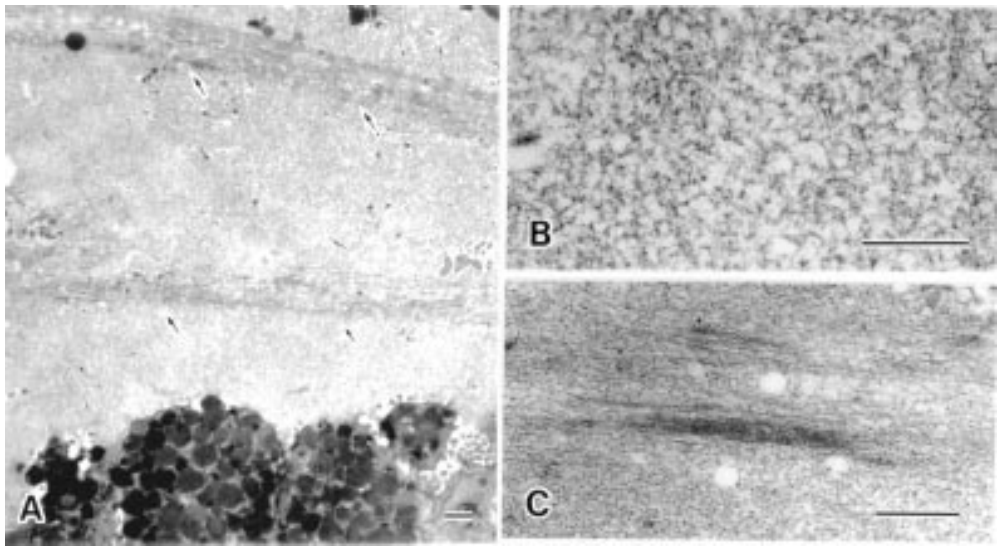

Figure 4 Electron microscopic appearance of the surface mucous gel layer (SMGL). (A) Two structural patterns were observed in the SMGL: a reticular pattern (see (B)) and a thick band like pattern (arrows) (see (C)). In this particular view, thick bands running parallel to each other can be seen passing among the mucin threads marking up the reticular pattern. The thickness of the bands varied considerably (bar=1 μm). (B) At higher magnification, mucins with a reticular pattern can be seen to consist of thin threads crossing each other; their diameter was 1.67 (0.69) nm (bar=0.5 μm). (C) At higher magnification, the thick band like mucins appear as an aggregation of thin filamentous threads, approximately 1.07 (0.32) nm in diameter (bar=1 μm).

H pylori and its distribution, with reference to the SMGL

In all five cases examined, *H pylori* were demonstrated immunohistochemically. This organism was not only attached to the apical and/or lateral surface of the surface mucous cells but also scattered within the SMGL as small aggregates with detached epithelial cells in the centre (fig 1D). These aggregations were not distributed randomly but rather arranged in layers (fig 1D). Comparison with the adjacent sections stained by GOTS-PCS disclosed that the aggregations were mostly distributed within the layers containing mucins derived from surface mucous cells (fig 1E). In the foveolae, attachment of *H pylori* to the surface mucous cells decreased gradually towards the deeper region and almost none was seen around the glandular neck. *H pylori* did not adhere to intestinal metaplastic cells.

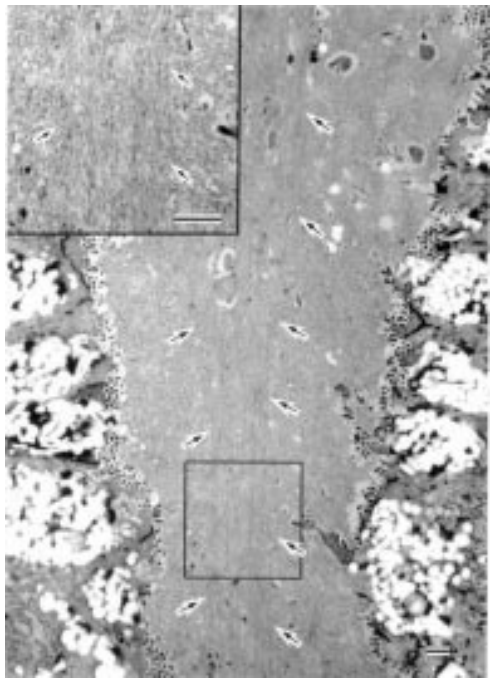

Figure 5 Electron microscopic appearance of mucins in the foveolar lumen. The axial region of the foveolar lumen was occasionally occupied by mucins showing thick threads running parallel with each other (arrows) (bar=1 μm).

ELECTRON MICROSCOPIC OBSERVATIONS

The SMGL was observed in three of five cases with a normal stomach and in seven of 10 cases with *H pylori* infection, although even in those preservation of the SMGL was not as complete as in tissue preparations used for light microscopy.

In one of seven cases with *H pylori* infection, intestinal metaplasia was found in the antral biopsy. Electron immunohistochemically, immunoreactivity for sialosyl-Tn, was found in secretory granules of metaplastic goblet cells and also in the SMGL covering the area of intestinal metaplasia (fig 3). In other cases, immunoreactivity for sialosyl-Tn was not found in either gastric mucous cells or the SMGL. For the following observations we used serial tissue sections of the specimens (three of five cases of normal stomach and six of 10 cases with *H pylori* infection) in which the SMGL was preserved and in which there was no intestinal metaplasia and no immunoreactivity for sialosyl-Tn.

Ultrastructure of the SMGL of the normal gastric mucosa

The mucus forming the SMGL exhibited two structural patterns (fig 4A). There was no significant difference in ultrastructural pattern between the SMGL on the pyloric mucosa and that on the fundic mucosa. The predominant pattern was a reticular one; this consisted of thin threads of mucin crossing each other. Their diameter was 1.67 (0.69) nm (fig 4B). The other type was a thick band like pattern.

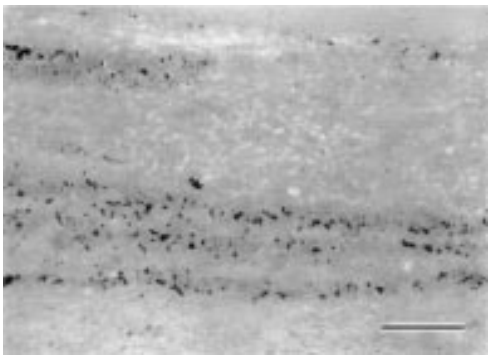

Figure 6 Surface mucous gel layer Immunogold staining with HIK1083. Mucins of the thick band like pattern were selectively labelled with HIK1083 (Immunogold staining with HIK1083; bar=1 μm).

The thickness of the bands varied considerably. These bands ran parallel to each other among the mucin threads making up the reticular pattern layer (fig 4A). The thick bands occasionally frayed into thinner bands. At higher magnification, the bands appeared as an aggregation of thin filamentous threads approximately 1.07 (0.32) nm in diameter (fig 4C). The SMGL adjacent to the apical surface of the surface mucous cells always showed the reticular pattern. In the foveolar lumen, the axial region was occasionally occupied by mucins showing up as thick threads running parallel to each other (fig 5). These mucin threads were gradually transformed into the mucins of the thick band like pattern in the SMGL. In the secretory granules, neither the mucins of surface mucous cells nor those of gland mucous cells exhibited a reticular or filamentous pattern.

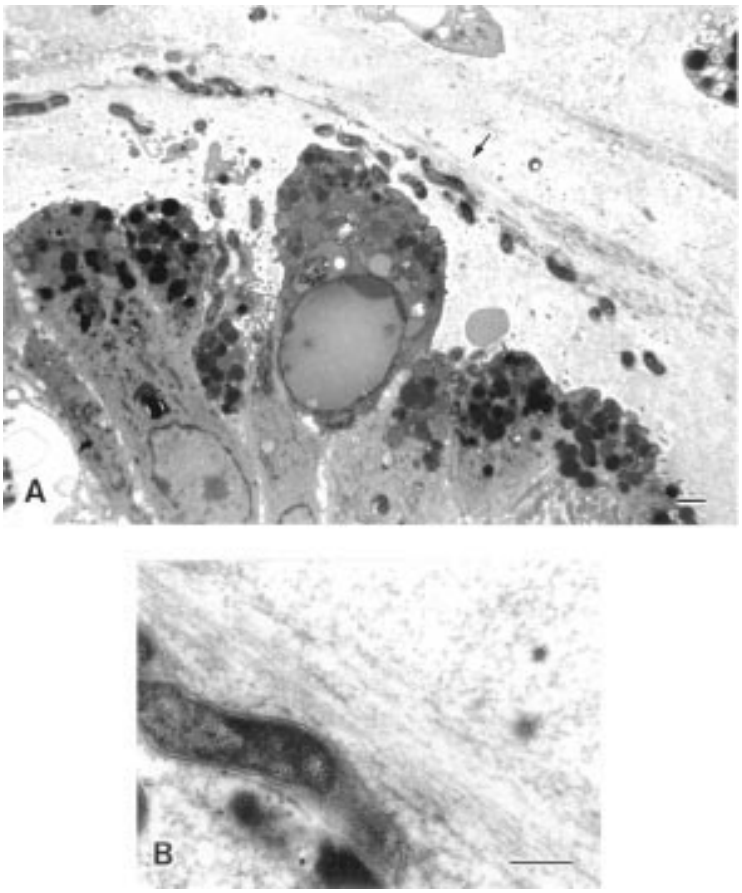

Figure 7 Electron microscopic appearance of Helicobacter pylori infected gastric surface mucous cells. (A) H pylori were also demonstrated on the apical plasma membrane and/or between the lateral plasma membranes of the surface mucous cells. Detaching cells also had H pylori attached to their surface. H pylori located in the reticular pattern layer (arrows) frequently ran parallel to the mucosal surface or to the mucins of the thick band like pattern (bar=1 μm). (B) Higher magnification of H pylori identified with arrows in the surface mucous gel layer in (A) (bar=0.5 μm).

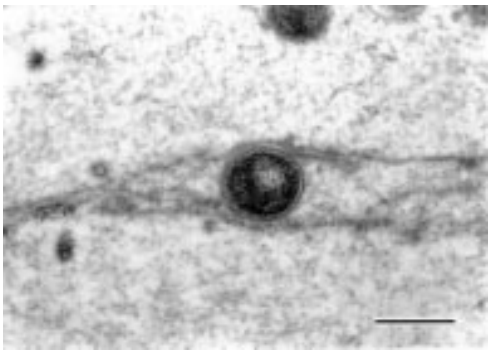

Figure 8 Electron microscopic appearance of Helicobacter pylori within the surface mucous gel layer. H pylori were trapped by frayed thin threads of mucins of the thick band like pattern (bar=0.5 μm).

Electron microscopic immunohistochemistry

Immunostaining with HIK1083 revealed a highly specific affinity (i) to mucin granules within mucous neck cells and pyloric gland cells, (ii) to the mucins of the thick band like pattern within the SMGL (fig 6), and (iii) to the thick mucin threads in the axis of the foveolae.

H pylori and its distribution, with reference to the SMGL

In six cases with *H pylori* infection, *H pylori* were demonstrated at the electron microscopic level on the apical plasma membrane and/or between the lateral plasma membranes of the surface mucous cells (fig 7A). Detaching cells also had *H pylori* attached to their surface (fig 7A).

H pylori were consistently located within the SMGL showing the reticular structure, especially along the border of the SMGL with the thick band like structure (fig 7). In the SMGL, *H pylori* frequently lay parallel to the mucosal surface or to the mucins of the thick band like structure (fig 7) but *H pylori* lying perpendicular to or at an oblique angle either to the mucosal surface or to the mucins of the thick band like structure were not found in this study. *H pylori* were only rarely found within the SMGL of the thick band like pattern. Occasionally, *H pylori* were trapped by frayed thin threads of the mucins of the thick band like structure (fig 8).

Microcolonies of *H pylori* were frequently seen within the SMGL where they were accompanied by degenerated epithelial cells in the centre of the colony (fig 9A). At higher magnification, *H pylori* in the microcolonies showed the typical spiral form with well developed flagellae. Division of the organisms was frequently seen with these microcolonies (fig 9B).

The mucus forming the SMGL exhibited two ultrastructural patterns similar to those

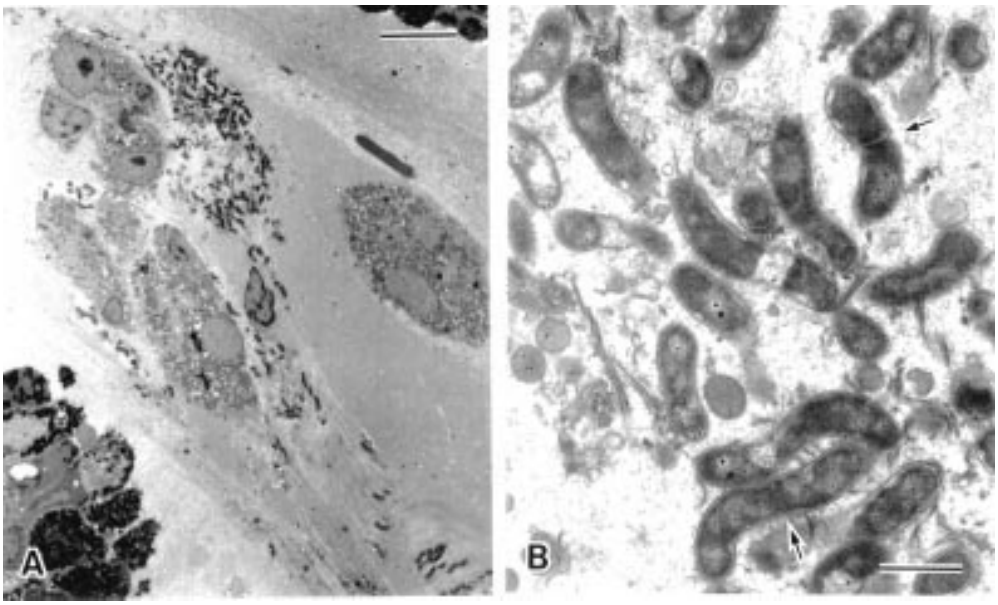

Figure 9 Electron microscopic appearance of the aggregates of Helicobacter pylori within the surface mucous gel layer (SMGL). (A) The aggregates of H pylori located in the reticular pattern layer were accompanied by degenerated epithelial cells (bar=10 μm). (B) At higher magnification H pylori showed the typical spiral form with well developed flagellae. Division of the organisms was seen (arrows). Around the microcolonies, the electron density of the SMGL was considerably decreased and the reticular pattern became obscured (see fig 4) (bar=1 μm).

seen in the SMGL of the normal gastric mucosa, except that around the microcolonies the electron density of the SMGL was considerably decreased and the reticular pattern became obscured (fig 9).

Discussion

The present study confirmed and extended our previous reports (based on light microscopic observations) that the SMGL shows an alternating laminated array of two types of mucins[1] and that *H pylori* are found distributed not only on the mucosal surface but also within the SMGL, preferentially in the layer of surface mucous cell mucins.[2] In addition, we have demonstrated that *H pylori* divide in the SMGL and form microcolonies.

There are two types of mucous layers in the SMGL, apparently formed separately by mucins derived from surface mucous cells or gland mucous cells. These two types of mucin layers showed fundamentally different ultrastructural features: a reticular pattern or a thick band like pattern. In the SMGL, the surface mucous cell type mucins are assumed always to show the reticular pattern and the gland mucous cell type mucins the thick band like pattern. Histochemical and molecular biological studies suggest that the gastric surface mucous cell type and gland mucous cell type mucins differ from each other both in the core peptides and oligosaccharides.[5 10 12 13] However, the three dimensional structure of neither the mucins nor the mucus has yet been clarified and, moreover, the mechanisms causing gastric mucins to form such different arrangements remain to be identified. This is the first paper to report an ultrastructural difference in the way surface mucous cell type mucins and gland mucous cell type mucins are arranged within the SMGL. In the secretory granules, neither the mucins of surface mucous cells nor those of gland mucous cells ever exhibit a reticular or band like pattern.[10] The processes leading to formation of a reticular or band like arrangement of mucins within the SMGL after their secretion from the mucin granules could not be deduced from this study however.

Aggregations of *H pylori* found within the SMGL showed several characteristic features: (1) the organisms forming the aggregations revealed a spiral form and evidence of division, (2) the aggregations were frequently accompanied by degenerated epithelial cells, and (3) around the aggregations the SMGL was less electron dense and the reticular pattern of the mucins was obscured. These findings most likely indicate that the *H pylori* forming the aggregations are proliferating actively and that the aggregations actually represent microcolonies. Possibly, degenerated epithelial cells might detach from the superficial region of the mucosa with *H pylori* attached. Changes in the SMGL around the microcolonies may be caused by degradation of mucin molecules as *H pylori* secrete both protease and phospholipase.[14 15] Shimizu and colleagues[2] demonstrated histochemically that the alternating laminated structure of the SMGL deteriorated markedly when colonised by *H pylori* and that after eradication of *H pylori* the SMGL showed an alternating laminated structure.

Within the SMGL, *H pylori* seem preferentially to locate in the layer containing mucins derived from surface mucous cells. This finding could be explained in two ways. One is that this layer may contain substances that induce chemotaxis of *H pylori*. The other is that the physicochemical properties of the gland mucous cell mucins might inhibit the movement of *H pylori*. The latter seems the more plausible as *H pylori* were frequently found along the border of the layer of gland mucous cell mucins. The fact that within the SMGL *H*

pylori were only rarely found at an oblique angle to or perpendicular to the mucosal surface suggests that it may not be easy for *H pylori* to move within the SMGL.

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