Gastric mucosal barrier: evidence for *Helicobacter pylori* ingesting gastric surfactant and deriving protection from it

B A Hills

Abstract

Ultrastructural examination by electron microscopy has been undertaken on human oxyntic mucosa from biopsy specimens obtained during diagnostic endoscopy from patients in whom infection by *Helicobacter pylori* was subsequently confirmed. A novel fixation procedure was used that avoided conventional fixatives based upon glutaraldehyde, which can destroy the hydrophobic lining of surfaces such as gastric mucosa. The resulting electron micrographs show densely osmophilic inclusions of varying sizes in *Helicobacter*, some of which can be resolved and identified as lamellar bodies and their partially digested states. This finding indicates that *Helicobacter* may act as an aggressive agent by ingesting a gastric mucosal barrier of gastric surfactant, exposing the surface to attack by acid while simultaneously rendering it less hydrophobic. There is also evidence that *Helicobacter pylori* avoid their own digestion by coating themselves with essentially the same barrier of gastric surfactant, probably derived from the host. This is a possible explanation for the apparent absence of these bacteria in the duodenum.

(Gut 1993; 34: 588-593)

contain SAPL.^{6,7} Moreover, the hydrophobicity of the gastric mucosa could be eliminated by common 'barrier breakers,' each of which either reacts with SAPL or dissolves it.⁵ It has recently been confirmed that SAPL is a barrier to hydrogen ions in vitro⁸ and in vivo⁹ and, when administered to the stomach as various forms of exogenous surfactant, it clearly imparts a protective effect against ulceration.⁹⁻¹³

The acquisition of ultrastructural evidence for a physical barrier of SAPL may have been thwarted by the composition of conventional fixatives and the well known tendency for aldehydes to destroy hydrophobic surfaces.¹⁴ When substituting tannic acid for most of the glutaraldehyde used traditionally,¹⁵ an oligolamellar layer of SAPL has been demonstrated, not only on the mucosal surface,^{16,17} but also lining the oxyntic ducts and other mucus free surfaces exposed to highly corrosive environments within the stomach wall.¹⁸ SAPL was also demonstrated as the oligolamellar matrix material of gastric mucus where it could be considered to provide many hydrophobic mini-barriers in series to the back diffusing hydrated hydrogen ions $H(H_2O)_3$.⁸ Surface active phospholipid has also been demonstrated as lamellar bodies in parietal cells^{16,17} and these and other

The gastric mucosal barrier has come to be regarded more as a physiological concept than the physical reality envisaged when the term was first put forward by Davenport¹ as a result of his many fundamental studies. Many alternative theories of mucosal protection have been proposed but the evidence still favours a physical barrier to the back diffusion of hydrogen ions.² The only obvious layer interposed between the mucosal surface and the corrosive contents of the stomach is the mucous lining, or 'mucous-bicarbonate blanket,' as it is often termed.³ Estimates that its resistance to acid transmission is only one quarter of that needed, together with the absence of mucus on epithelial surfaces within the ducts of the stomach wall, however, have led some to consider other structures for the elusive physical barrier.

One such physical structure was indicated by the molecular similarity between the highly surface active phospholipid (SAPL) found in vivo and surfactants widely used to protect a variety of surfaces against corrosion in vitro,⁴ when they act by adsorption. An incidental finding was that they also render the surface hydrophobic.⁴ It could therefore be considered pertinent that the gastric mucosa is hydrophobic,⁵ and gastric juice and wall 'scrapings'

Figure 1: Typical low magnification electron micrograph of the luminal lining of the human stomach showing three bacteria located beneath the mucous layer but above the surface of surface mucous cells. Bar represents 1 μ m.

Department of
Physiology, University of
New England, Armidale,
Australia
B A Hills

Professor B A Hills,
Department of Physiology,
University of New England,
Armidale, NSW 2350,
Australia.

Accepted for publication
22 September 1992

Figure 2: *Helicobacter pylori* identified by its characteristic spiral configuration and displaying a larger number of very small yet densely osmiophilic inclusions. Bar represents 100 μm .

lamellated forms have been found in surface mucous cells.^{18 19}

If oligolamellar SAPL – or SAPL in any other physical form – is really providing the gastric mucosal barrier, then it raises the question of *Helicobacter pylori* and its role in inducing gastric ulcers.²⁰ More specifically, why this bacterium should only induce ulcers under acidic conditions and why it can reduce the hydrophobicity

Figure 3: *Helicobacter pylori* identified by its characteristic spiral configuration containing fewer very small osmiophilic inclusions than shown in Fig 2 but also some larger ones. Bar represents 100 μm .

Figure 4: Cross section of a *Helicobacter pylori* displaying much the same range of densely osmiophilic inclusions as shown in an axial section in Fig 3. Bar represents 100 μm .

of the gastric mucosa.²¹ One factor in these deliberations is clearly the recently demonstrated production of phospholipase A₂ by *Helicobacter pylori*^{22 23} with obvious implications for the digestion of SAPL, but it would seem highly desirable to see what ultrastructural changes might occur in the presence of these bacteria.

Another question that can be considered simultaneously concerns the reason why gastric juice is effective in digesting most bacteria reaching the stomach and yet it does not digest *Helicobacter pylori*. This raises the possibility that there might also be a physical barrier protecting these particular bacteria.

This study has been designed to observe *Helicobacter pylori* in relation to the gastric mucosa, avoiding conventional fixatives that could destroy SAPL or compromise any hydrophobic lining.

Materials

Fresh biopsy specimens were obtained from 10 patients with abdominal pain or dyspepsia undergoing diagnostic upper gastrointestinal endoscopy. Within five minutes of excision small samples from each specimen were placed in a fixative for electron microscopy and the remainder was used for routine histological examination. The prefixation was continued to postfixation in 12 samples from patients in which the presence of *Helicobacter pylori* was confirmed by the urease test (CLO test)²⁴ performed on an additional antral biopsy specimen.

Methods

Two blocks of oxyntic mucosa from each sample were fixed for 72 hours in 3% tannic acid plus 1%

Figure 5: Lamellar body located adjacent to *Helicobacter pylori* at the interface between gastric mucus and the mucosa. This is remarkably similar to lamellar bodies studied in the lung.²⁹ Bar represents 50 μm .

glutaraldehyde buffered at a pH of 7.4 with 0.1M sodium cacodylate at 4°C and rendered isotonic (320 mM) with sodium chloride. The glutaraldehyde was greatly reduced from conventional concentrations of around 5%¹⁵ to avoid the propensity for aldehydes to destroy hydrophobic surfaces,¹⁴ substituting tannic acid known for its ability to resolve any lamellated phospholipid structures such as those found in preliminary studies on rat gastric mucosa.¹⁷ The long fixation times were adopted to allow the water soluble fixatives to penetrate a barrier characterised by its impermeability to water soluble solutes.¹ Postfixation was effected with 1% osmium tetroxide buffered at a pH of 7.4 with embedding in resin (Spurr mix 'A': Probing and Structure, Kirwan, QLD) polymerised at 60°C. Thin sections (60 nm) were cut to facilitate resolution of any lamellated structures.

Results

Many *Helicobacter pylori* were seen below the mucous layer but above the gastric mucosa (see Fig 1). At higher magnification, the characteristic spiral curvature of the bacteria was evident (Figs 2 and 3). A total of 50 *Helicobacter* were randomly selected for detailed examination at higher magnification. Within each bacterium were densely osmiophilic areas, ranging from a large number of small inclusions (Fig 2) to fewer but larger areas (Fig 3) which were also clearly discernible in cross section (Fig 4). On enlargement, with much reduction in contrast on film development, these densely osmiophilic inclusions – and many of those external to the bacterium – were found to be lamellated structures of the form shown in Fig 5.

Most of the very small osmiophilic points such as those shown in Fig 2 proved too dense to

Figure 6: Enlargement of dense osmiophilic inclusions in Fig 4. The smaller one is typical of the very dense dots seen in Figs 2–4 which can be resolved into a lamellated structure at higher magnification. The larger lamellar body can be seen to be similar to lamellar bodies found external to the bacterium (Fig 5), the large reduction in density of the outer (arrowed) lamellae indicating partial digestion by phospholipase within the *Helicobacter pylori*. Bar represents 50 μm .

resolve but others were resolved as shown in Fig 6. The small lamellar body (arrowed) is close to a much larger osmiophilic body that would seem to be in the process of digestion as indicated by the much lower density of the outer lamellae. Although no other bacteria were found in the

Figure 7: Another cross section of a *Helicobacter* this time closer to the pole of the bacterium than shown in Fig 4. Note the oligolamellar coating of highly osmiophilic material, presumably SAPL. Bar represents 50 μm .

Figure 8: Enlargement of the wall of the bacterium shown in Fig 4. Note the distinct third line (arrowed) running parallel and of comparable spacing to the lipid bilayer of the outer membrane. Bar represents 25 μm .

samples to act as suitable controls, the lamellated bodies found differed from the densely osmiophilic mesosomes demonstrated in such bacteria as *Escherichia coli*²⁶ because they have solid cores (Figs 5 and 6) whereas those in *Escherichia coli* are vesicular in structure.

The bacterial wall seemed densely osmiophilic (see Fig 4), such structures having been previously reported as a glycocalyx.²⁰ In some very thin sections, however, the densely osmiophilic

Figure 9: Enlargement of the cell wall of the bacterium shown in Fig 3. Note that the outer rail (arrowed) of the tramlines representing the cell membrane is much denser than that on the cisternal side. Bar represents 25 μm .

wall proved to be multilamellar on the external side (Fig 7), with a true glycocalyx much thinner than indicated from those previous studies. At least, this was observed towards the pole of the organism, whereas over other areas of the surface where the wall was thinner, there was either an additional bilayer adjacent to that of the cell membrane (Fig 8) or the two 'tramlines' of the cell membrane were of widely differing density. Figure 9 shows this more clearly; that the cisternal monolayer of the lipid bilayer is much less dense than the exterior layer adjacent to the true glycocalyx.

Discussion

The formulation of the fixative to preserve any lamellated structure has led to a slight loss in cellular detail in the electron micrographs consistent with reducing to 1% the glutaraldehyde content normally adopted in conventional studies for its ability to fix proteins.¹⁵ This loss, however, seems no worse than that in previous electron micrographs of *Helicobacter* at comparable magnification²⁰ with more conventional fixation procedures²⁷ and the lamellated structures seen in Figs 5–7 seem well preserved by the increased tannic acid, as predicted.²⁵ Moreover these structures would not seem to be artefacts because flat lamellated structures (Fig 7) are not shown in atlases of known artefacts in biological electron microscopy,²⁸ nor are true lamellar bodies (Figs 5 and 6) – that is, multilamellar vesicles *provided* the core is solid – as is the case in these micrographs. Thus there is a high degree of confidence that the novel fixation procedure did not introduce artefacts.

The very dense osmiophilic regions in the characteristically spiral *Helicobacter* seen in Figs 2 and 3 immediately attract attention because SAPL is so highly osmiophilic.²⁵ It would seem that many of the dense osmiophilic inclusions seen in Figs 3 and 4 are indeed phospholipid when enlarged (Fig 5). This view is much more forcefully substantiated by the highly lamellated 'whorls' with the solid cores, which are the hallmark of SAPL as it is produced in the alveolar type II cell and secreted on to the alveolar surface.²⁹ These highly characteristic forms of alveolar surfactant/SAPL have been much studied in the lung for two decades³⁰ and, more recently, have been reported in parietal cells^{16 17} and elsewhere in the stomach wall.¹⁷ Their presence is consistent with the high surface activity of the gastric lining.⁵

The finding that these lamellar bodies are found *within* the *Helicobacter* indicates that they have been ingested by the bacteria as similar bodies are found in the surrounding medium. The number of densely osmiophilic inclusions (Fig 2) indicates that the rate of ingestion could be high. There is also evidence of digestion of the ingested lamellar bodies as seen in the large lamellar bodies in Fig 6, which could be facilitated by the known production of phospholipase A₂ by *Helicobacter pylori*.^{22 23} It could be argued from the electron micrographs alone, however, that the bacteria could be synthesising the lamellar bodies and even secreting them; although this explanation seems unlikely for

Figure 10: Substitution of osmium atoms for the polar head groups of phospholipid molecules³⁵ to offer (A) the popular explanation for four lines (two bilayers) joining to give three lines and (B) indicating how the adsorption of a monolayer to one side of the bilayer could increase the density of that line without showing a third line.

several reasons. Firstly, a search of published work did not reveal evidence of lamellar bodies in bacteria, and the conventional fixatives are capable of demonstrating lamellated phospholipid in mesosomes in *Escherichia coli* as mentioned earlier. Secondly, it seems unlikely that *Helicobacter pylori* would be synthesising phospholipids when these bacteria are already loaded with phospholipase A₂,^{22,23} which digests such substances. This would apply to the synthesis of lamellar bodies in particular.

This capability of *Helicobacter pylori* to ingest phospholipid could be especially relevant to mucosal protection if the gastric mucosal barrier is indeed an oligolamellar lining of SAPL as predicted from basic studies of the hydrophobicity of the gastric mucosa.^{5,31,32} Further evidence to support this model of a physical barrier includes the nature of common barrier breakers which dissolve or react with SAPL,⁵ the loss of hydrophobicity that occurs when the barrier is broken,^{5,21,31} and the explanation it offers for the protective action of prostaglandins³³ and exogenous SAPL.¹⁰⁻¹³ Moreover, the gastric mucosal barrier has been visualised as an oligolamellar lining on the mucus free surfaces of oxyntic ducts^{16,17} and a similar structure for the intergranular matrix material could impart the barrier properties to unrefined gastric mucus as a number of minibarriers in series.¹⁸ Thus by ingesting SAPL or secreting phospholipase A₂, or both, the *Helicobacter* could be digesting the gastric mucosal barrier to allow acid to induce ulcers, while simultaneously reducing the hydrophobicity of the mucosa.²¹ This would explain the need for acid to induce ulcers, even in the presence of *Helicobacter pylori*. The ingestion of oligolamellar SAPL as the intergranular matrix material could also explain the apparent reduction in viscosity caused by these bacteria.³⁴

Another issue on which this study sheds some

light is why *Helicobacter pylori* do not get digested in the highly corrosive environment where they are found in the stomach (Fig 1). It was therefore interesting to find an oligolamellar lining on portions of the bacterial membrane (Fig 7) that closely resembled that found on the gastric mucosa and deeper epithelial surfaces of the stomach wall.¹⁷ Over the rest of the surface, there was either one additional line or a much denser 'outer rail' to the 'tramlines'. The first could indicate two bilayers (four monolayers) as osmium ions replacing the polar headgroups of one bilayer came so close to those of the nearside monolayer of the adjacent bilayer that they could not be distinguished.³⁵ Figure 10A depicts this. Figure 10B shows how the adsorption of a monolayer of SAPL to the outer layer of the lipid bilayer could result in the unequal density found in Fig 9. This corresponds closely to the common use of surfactants in the physical sciences where adsorbed monolayers provide protection against acids and other noxious chemicals.³⁵ Hence it is tempting to speculate that the *Helicobacter pylori* adopt much the same protection mechanism as the host and, maybe, derive that protection from the host. This has interesting clinical implications for the role of 'barrier breakers', especially bile, which could explain the apparent absence of *Helicobacter pylori* in the duodenum.^{20,24}

Of course, any secretion of phospholipase A₂ by the *Helicobacter pylori* would also act to digest their own protective mechanism but the state of the lamellar bodies – for example, the larger one in Fig 6 – would indicate that more digestion of SAPL occurs within the bacterium.

Thus *Helicobacter pylori* have a most interesting ultrastructure compatible with the hypothesis that they can break a physical gastric mucosal barrier of SAPL by ingesting it, while coating themselves with much the same barrier derived from the host to prevent their own digestion in the stomach.

This work was funded by the NH and MRC of Australia.

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