Rapid Interaction of *Helicobacter pylori* With Microvilli of the Polar Human Gastric Epithelial Cell Line NCI-N87

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ABSTRACT

Infection with *Helicobacter pylori* results often in chronic gastritis, gastric ulcers or even gastric tumor development. Little is known about the initial interaction between gastric epithelial cells and *H. pylori*. The aim of the present study was to analyze the initial host contact to the bacteria. Monolayers of the human gastric epithelial cell line NCI-N87 grown on porous membranes were used and the apical side of the epithelium was exposed to the *H. pylori* wild-type strain P1 for 1 hr. Many epithelial cells were colonized by bacteria within the period of 60 min. Using scanning electron microscopy we detected that the bacteria were in close contact with the epithelia via microvilli. Further, transmission electron microscopy of the contact sites revealed no difference in the morphology of the microvilli in comparison to those not attached to the bacteria. The present study demonstrates the importance of microvilli on apical epithelial cells during the initial contact of the host by colonizing *H. pylori*. Anat Rec, 296:1800–1805, 2013. © 2013 The Authors. The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology published by Wiley Periodicals, Inc. on behalf of the American Association of Anatomists.

**Key words:** gastric epithelial cells; scanning electron microscopy; tight junction; trans epithelial electrical resistance

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*H. pylori*, the bacteria were closely located at the epithelial cell membrane, in part microvilli were attached to the bacteria (Smoot et al., 1993; Shaffer et al., 2011; Johnson et al., 2012). In biopsies from patients suffering from *H. pylori* infection the gastric cells develop protrusions directly in the neighborhood of the bacteria (Smoot et al., 1993). So far it is not known at what time point these morphological changes occur after the exposition of the epithelium with *H. pylori*. The results from *in vitro* experiments were often studied 4 h after bacterial exposition (Johnson et al., 2012), in biopsies from *H. pylori* infected patients the time point and the morphology of the initial contact of host epithelium and bacteria can not be determined. As recent studies demonstrate that isoenzymes of the protein kinase C are already phosphorylated beginning 30 min after *H. pylori* exposition *in vitro* (Sokolova et al., 2013), our study aimed at an early morphological characterization of the contact of bacteria with the epithelium 60 min after exposure.

The interaction of host and *H. pylori* is dependent on the presence of important proteins of the apical cell membrane and the tight junctions (Oliveira et al., 2009). Therefore the human gastric epithelial cell line NCI-N87 was used as it forms a tight monolayer and exhibits gastric epithelial characteristics like ZO-1 and E-cadherin expression (Chailler and Menard, 2005). The morphology of the host/bacterium interface was studied using scanning as well as transmission electron microscopy techniques.

**MATERIAL AND METHODS**

**Cell Culture**

The human NCI-N87 gastric cell line (American Type Culture Collection, Manassas, VA, ATCC CRL 5822) was cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS, heat inactivated; both PAN-Biotech, Aidenbach, Germany) and incubated in a humid atmosphere at 37°C and 5% CO2 (referred as normal cell culture conditions).

To determine their generation time the cells were cultured in 96-well plates (culture area per well: 0.28 cm², volume 100 μL; NUNC, Wiesbaden, Germany), various seeding densities were used (1 × 10⁴, 3 × 10⁴, and 6 × 10⁴ per 100 μL). Cells were allowed to adhere for 6 hr. At this time point all wells were washed with PBS. Cells of two wells of the 96-well-plate were harvested with Trypsin/EDTA (Sigma, Taufkirchen, Germany) for cell count. The cell number of 6 hr refers to the cell number at the beginning of the experiment (N₀). Fresh medium was applied to all other wells of the 96-well-plate. Further on, the medium was aspirated and the cells were washed and harvested with Trypsin/EDTA at the time points of 24, 48, and 72 hr (N) to calculate the cell number. In the experiment (n = 3) cell count was examined in duplicates for each time point. The generation time (tg) was calculated after the following equation:

\[
tg = \frac{\log 2 \times dt}{\log N \times \log N_0}
\]

For all other experiments the cells were seeded in a density of 2 × 10⁵ cells mL⁻¹ on porous 12-well inserts (area 1.12 cm², pore size 1 μm; ThinCerts™ Cell Culture Inserts, Greiner-bio one, Frickenhausen, Germany) and monolayers were grown for 7 days. Cell culture was regularly tested and found to be free of *Mycoplasma Spp.* (Venor® GeM Mycoplasma detection Kit; Minerva Biolabs, Berlin, Germany). One day before infection cells were washed with PBS and serum-free medium was added.

**TEER Measurement**

Measurement of transepithelial electrical resistance (TEER) was performed using a Millicell electrical resistance system (Millipore, Molsheim, France). Cells were deemed to be confluent at a TEER value of ≥0.90 kΩcm⁻² (corresponding to a TEER value of 1 kΩm per 12-well insert), which was obtained after 7 days.

**Bacteria Strains and Growth Conditions**

*H. pylori* wild-type strain P1 was cultured on agar plates containing 10% horse serum under micro-
aerophilic conditions at 37°C for 48 hr. Bacteria were harvested from agar plate in serum-free cell culture medium (RPMI) and added to the serum-starved NCI-N87 cells (grown on 12-well inserts) in a ratio of 50:1 multiplicity of infection (MOI), meaning 50 bacteria per cell. This MOI is in the order of magnitude used in earlier studies (Sokolova et al., 2013), and results in final bacterial numbers suitable for morphological evaluation.

Transmission Electron Microscopy (TEM)

Confluent, polar grown NCI-N87 cells on insert membranes at the age of 7 days were incubated with a suspension of *H. pylori* cells in a ratio of 50:1 MOI for 1 hr. After this period membranes were detached from inserts and fixed for 30 min in 4% PFA. Membranes were then treated with fixation solution containing 0.5% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) over night. After fixation the membranes were washed in 0.1 M PB. Subsequently, samples were treated with 1% osmium tetroxide (Science Services, Munich, Germany) in 0.1 M PB for 1 hr. Samples were then rinsed in buffer, dehydrated and block contrasted for 1 hr with 1% uranyl acetate in 70% ethanol. Finally, the membranes were infiltrated with Durcupan ACM (Fluka, Buchs, Switzerland) and flat embedded between plastic foils. The resin polymerized in an oven at 70°C for 2 days. Regions of interest were cut out and glued vertically onto a blank block of resin with a small groove. With an Ultracut S ultramicrotome (Reichert, Leica AG, Vienna, Austria) 1 μm semi-thin sections were cut and counterstained with toluidine blue for light microscopic measurements on slides. Ultrathin sections (50–70 nm) were collected on Formvar-coated slot grids of copper. They were further contrasted with 2% uranyl acetate and 0.1% lead citrate and examined in a LEO 906 E transmission electron microscope of Zeiss (Jena, Germany) equipped with a digital 1K BioScan camera (Gatan, Pleasanton, USA).

Fig. 2. (A) Trans epithelial electrical resistance (TEER) measurement was carried out on day 4, 7, 11, 14, 18, and 21. On day 7 the TEER was higher than 0.90 kOhm × cm². (B) Tight junction connected monolayers with many microvilli on day 7—bar 10 μm; insert: details of tight junctions (white arrow)—bar 500 nm (TEM). (C) Two areas of non-*H. pylori* exposed NCI-N87 cells—the length of microvilli and there density per area is variable—bar 500 nm (SEM).

Fig. 3. Scanning electron microscopy of an epithelial monolayer of NCI-N87 cells, apical view, 1 hr after the exposition with bacteria (MOI 50:1). *H. pylori* are located on the epithelium, within this short period close attachments of microvilli/bacteria have been established. (A) overview—bar 1 μm, (B) budding of microvilli toward the bacteria cell wall—bar 200 nm, (C) some bacteria have few if any microvillus attachments—bar 250 nm.
Scanning Electron Microscopy (SEM) of H. pylori in Contact With NCI-N87 Cells

Procedure of incubation and fixation of NCI-N87 cells and bacteria was the same as described before in section TEM. Beside this, the fixed membranes were kept in 0.1 M cacodyl buffer until post-fixation with 1 % osmium tetroxide (Science Services, Munich, Germany) in H2O. After three times washing in H2O samples were dehydrated in a series of ethanol and then dried by an automated critical point dryer (Leica CDP300, Wetzlar, Germany) using liquid CO2 substituting ethanol prior to evaporation at 31°C. SEM inspection of samples was performed by a Hitachi S-4800 SEM following Gold coating with a sputter coater (Bal-Tec, Pfaffikon, Switzerland).

RESULTS

Human gastric tumor NCI-N87 cells (1 × 10⁴, 3 × 10⁴, and 6 × 10⁴) were seeded on 96-well-culture plates (culture area per well 0.28 cm²) and the growth of the cells until a confluent monolayer was studied for 7 days (Fig. 1A). At the time points of 24, 48, and 72 hr after seeding, the cell number was quantified. The cell number at the time point of 6 hr was used as starting cell number (N₀). Herein, only 33 % of the seeded NCI-N87 cells were adherent on the cell culture plates. The generation time (tg) was calculated in the log phase at the time point of 72 h (see section Material and Methods). Interestingly, the time for reduplication of the NCI-N87 cells was quite long and took 42 hr (Fig. 1B).

For electron microscopical studies the cells were grown on porous membrane inserts (12 well; culture area 1.12 cm²) in a monolayer. The tightness of the monolayer was determined by measuring the transepithelial electrical resistance (TEER). The measurement was performed within a period of 21 days (Fig. 2A). The cells reached the state of complete tightness with a TEER value of 0.96 kOhm × cm² at around 7 days of cell culture. Therefore this time point was used for further experiments. NCI-N87 cells developed tight junction connected monolayers with a number of microvilli on the apical side after 7 days of cultivation (Fig. 2B; TEM). SEM showed at this time many microvilli of variable length on the surface of the cells, the density of microvilli per area was different (Fig. 2C).
**DISCUSSION**

In the present study, the human gastric epithelial cell line NCI-N87 was used as polar monolayer in order to analyze the first contact of H. pylori at the cellular surface. This cell line has its origin from an adenocarcinome (Chailler and Menard, 2005). Based on the population doubling time of 42 hr (Fig. 1A) and using an adequate seeding density (see Fig. 1B) monolayers were obtained for the exposition with H. pylori. In contrast to other tumor-derived cells, NCI-N87 cells obviously have a replication time more comparable to epithelial cells (Chailler and Menard, 2005). NCI-N87 develop tight junctions and a brush border with numerous microvilli (Fig. 2B,C), a prerequisite for H. pylori exposition studies. Oliveira et al. have shown that structural proteins for tight-junctions such as ZO1 and E-cadherin and other apical cell membrane proteins (e.g. Ezrin) are essential for establishing a bacteria/host interaction comparable to the in vivo situation (Oliveira et al., 2009). After 1 hr of H. pylori exposition of the NCI-N87 cells bacteria were located at the tips of the microvilli, interestingly a certain budding of the microvillus tips had occurred already, and neighboring cells had entrapped the bacteria.

The interaction of H. pylori with gastric epithelia has been studied earlier - in cell lines, primary epithelial cells (Hemalatha et al., 1991; Corthesy-Theulaz et al., 1996; Heczko et al., 2000; Shaffer et al., 2011; Johnson et al., 2012) and human gastric biopsies (Smoot et al., 1993; Chun et al., 2002; Bimczok et al., 2010). In the human AGS tumor cell line, the interaction with bacteria was studied 4 hr after exposing the cells to H. pylori (Shaffer et al., 2011). However, AGS-cells develop few and very short microvilli and lack other structures of the apical cell membrane (Oliveira et al., 2009)—resulting in a different morphology in comparison to our experiments (Shaffer et al., 2011). Our TEM results are comparable to studies in human biopsies—but in patients the infection is longer lasting and time point of the initial contact is unknown (Chun et al., 2002). Thus, NCI-N87 cells represent a suitable cell model to study the pathology of H. pylori infection, especially as comparable morphology was observed as in human biopsies, and as they provide the apical cell environment necessary for effective colonization by H. pylori (Chailler and Menard, 2005; Oliveira et al., 2009).

The entrapping of H. pylori by microvilli of neighboring cells may reflect an important first sign for the initiation of the immune response against H. pylori. Recently it has been reported that gastric dendritic cells obtained from patients suffering from H. pylori infection induce CD4+ Th1 cells (Bimczok et al., 2010). Thus, stimulation of dendritic cells by H. pylori may lead to the permeation of proinflammatory cytokines within the inflamed tissue in chronic H. pylori infections (Drakes et al., 2006). The tight junction associated protein tricellulin is expressed between neighboring cells and enables stomach dendritic cells to interact with the epithelial surface (Mariano et al., 2011). Thus, our observed entrapment of H. pylori by neighboring cells is possibly a prerequisite of the interaction between the bacteria and dendritic cells.

Future experiments are necessary to explain whether the early contact between microvilli and H. pylori is sufficient to induce NF-kB activation in gastric epithelial cells (Sokolova et al., 2013). Further, it has to be clarified whether the initial physical microvillus/H. pylori interaction already results in H. pylori-induced maturation of dendritic cells (Guiney et al., 2003; Kranzer et al., 2004) and in the recently described association of CagA with e-Met, E-cadherin, and p120-catenin that regulates the invasion of H. pylori (Oliveira et al., 2009).

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