Microaerophilic Conditions Permit to Mimic in Vitro Events Occurring during in Vivo Helicobacter pylori Infection and to Identify Rho/Ras-associated Proteins in Cellular Signaling*

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Molecular dissection of the mechanisms underlying Helicobacter pylori infection suffers from the lack of in vitro systems mimicking in vivo observations. A system was developed whereby human epithelial cells (Caco-2) grown as polarized monolayers and bacteria can communicate with each other under culture conditions optimal for each partner. Caco-2 cells grown on filter supports were inserted in a vertical position into diffusion chambers equilibrated with air and 5% CO2 at their basal surface (aerophilic conditions) and 5% CO2, 90% N2 (microaerophilic conditions) in the apical compartment. Remarkably, the epithelial polarized layer was stable under these asymmetric culture conditions for at least 24 h, and the presence of Caco-2 cells was necessary to maintain H. pylori growth. In contrast to previous studies conducted with non-polarized Caco-2 cells and other cell lines kept under aerophilic conditions, we found H. pylori-dependent stimulation of cytokine secretion (MCP-1 (monocyte chemotactrant protein-1), GRO-α (growth-regulated oncogene-α), RANTES (regulated on activation normal T cell expressed and secreted)). This correlated with nuclear translocation of NF-κB p50 and p65 subunits. Tyrosine phosphorylation of nine cellular proteins was induced or enhanced; we identified p120RasGAP, p190RhoGAP, p62dok (downstream of tyrosine kinases), and cortactin as H. pylori-inducible targets. Moreover, reduction of H. pylori urease expression was observed in adherent bacteria as compared with bacteria in suspension. In addition to mimicking several observations seen in the inflamed gastric mucosa, the novel in vitro system was allowed to underscore complex cellular events not seen in classical in vitro analyses of microaerophilic bacteria-epithelial cell cross-talk.

Upon infection of the host, enteric pathogens first encounter the host’s mucosal surfaces lined by epithelial cells (1). The function of these cells in host defense goes far beyond the mere mechanical barrier separating the external environment from the internal milieu of the host. Epithelial cells have to be considered as an integral component of the mucosal immune system, as they are capable of providing the underlying mucosa with the message that an infection occurs (2). This is accomplished by the release of molecular messengers including cytokines and chemokines by the epithelial cells that orchestrate initial phases of the immune response. Given the molecular complexity of these events, their dissection requires the availability of in vitro systems, which mimics the in vivo situation. To fulfill this requirement, viability of all cell partners has to be guaranteed, and biologic read-outs should resemble those established in vivo.

Although in vivo studies have contributed to important breakthroughs regarding the morphological and physiological events resulting from the infection by Helicobacter pylori, the major causative agent of chronic gastritis, peptic ulcer, gastric adenocarcinoma, and mucosa-associated lymphoreticular tissue lymphoma (3, 4), in vitro approaches are important to refine the study at the molecular and cellular levels. So far, the design of such a robust in vitro system has not been reported for the study of H. pylori-epithelial cell interactions. Two major difficulties have to be overcome: 1) gastric cell lines cannot be grown as polarized epithelia mimicking the gastric mucosa (5–8), 2) or if so (9) do not stand the microaerophilic conditions needed for the particular metabolism of H. pylori. The low oxygen conditions prevailing in the gastric environment are important in the activation of H. pylori virulence genes (10). Among them, urease is required for initial gastric colonization (11), allowing the bacterium to maintain its periplasmic pH near neutrality (12, 13). The expression of H. pylori CagA protein, strongly associated with duodenal ulceration, is influenced through the culture medium pH in vitro (14). These examples indicate that neglecting crucial bacterial growth parameters could preclude the refined study of H. pylori-epithelial cell interactions. Moreover, all studies have been performed under aerophilic conditions whereas a restricted oxygen atmosphere is required for proper H. pylori metabolic and growth functions (15).

For these important reasons, we designed a novel in vitro system maintaining optimal culture conditions for H. pylori added to polarized epithelial cells serving as an interface between the apical and basolateral compartments of diffusion chambers. Bacteria in BHI,1 pH 5.5, 5 mM urea, 5% O2, 5% CO2,
90% N₂ kept dividing up only in the presence of the polarized Caco-2 monolayer. Bacterium-cell contact resulted in pedestal formation and brush border disruption as observed in vivo. Tyrosine phosphorylation of proteins including p120GAP, p190GAP, p62dok, and cortactin was observed for the first time. In addition to NF-κB nuclear translocation and IL-8 secretion, MCP-1, GRO-α, and RANTES induction detected in biopsies could be reproduced under microaerophilic conditions only. In comparison with H. pylori floating in suspension, bacteria associated with Caco-2 cells exhibited decreased urease A and B subunits expression, such as the bacterial virulence program, the mechanism of adherence and the contribution of epithelial cell to bacterial colonization, and the cross-talk with inflammatory cells.

**EXPERIMENTAL PROCEDURES**

**Caco-2 Cell Culture Conditions**—Human colonic adenocarcinoma epithelial Caco-2 cells (HTB 37; American Type Tissue Collection) were grown in C-DMEM consisting of Dulbecco’s modified Eagle’s medium-Glutamax (Invitrogen) supplemented with 10% fetal calf serum (Seromed), 1% non essential amino acids (Seromed), 10 mg MEPS (In-vitrogen), 0.1% transferrin (Invitrogen), and 1% streptomycin/penicillin (Seromed) and used between passages 23 and 37. Cells cultivated to 80% confluency were seeded on Snapwell filters (12 mm; pore size, 0.4 μm; Corning Costar) at a density of 0.8 × 10⁶ cells/cm². The formation of a polarized Caco-2 cell monolayer at week 3 was established by morphology and monitoring of the TER (200—400 ohm cm²) using a MilliCell-ERS apparatus (Millipore).

**Helicobacter pylori Culture Conditions**—The H. pylori CagA strain ATCC 43504 was grown on agar plates made of 36 g/liter GC agar base (Oxoid AG) containing 12.5% heat-inactivated horse serum (In-vitrogen) and 1% IsoVitale X (Baltimore Biological Laboratories) in a microaerophilic atmosphere (90% N₂, 5% CO₂, 5% O₂) for 2 days before harvest into either plain BHI (BioMérieux) or into BHI supplemented with 0.25% yeast extract (BHI-C; Difco Laboratories) and 10% fetal calf serum (Seromed). The total number of bacteria was determined by measuring the A₆₀₀ of the bacterial suspension, 1.0 optical density unit corresponding to 10⁶ bacteria.

The Asymmetrical Culture System—The diffusion chamber device developed by Grass and Sweetana (16) was modified to host H. pylori and the polarized Caco-2 cells under optimal growth conditions. Caco-2 cells were seeded onto Snapwell filter (Costar) and allowed to form a tight, polarized monolayer (17). The filters carrying the cell monolayers were inserted between the two chambers, thus resulting in the physical separation into apical and basolateral compartments containing different culture media (Fig. 1). Up to six devices could be mounted serially on a support. Caco-2 cells were fed through the basolateral compartments containing C-DMEM perfused with air and 5% CO₂. The apical compartments containing BHI-C, BHI, or BHI-U (BHI medium containing 5 mM urea, pH 5.5) were perfused continuously using a Millicell-ERS apparatus (Millipore). Oxygenated media containing different culture media were perfused with the microaerophilic gas mixture (90% N₂, 5% CO₂, 5% O₂). Polarized Caco-2 cell monolayers on Snapwell filters inserted between the apical and the basolateral compartments are cultured in C-DMEM and perfused with air and 5% CO₂. Note that the Caco-2 cell apical surface lies in a vertical position once mounted in the diffusion chamber device.

**Immunoprecipitation and Immunoblotting**—Caco-2 cells on Snapwell filters were lysed by adding a buffer containing 25 mM HEPES, pH 7.2, 300 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 0.1 mM EDTA, 0.1 mM Na₂VO₄, and 1 mM phenylmethylsulfonyl fluoride and rocking on ice for 30 min. Cells were then scraped into 1.5 ml centrifuge tubes, and the lysates were centrifuged at 1,000 × g for 5 min. The supernatant was removed, and the pellets were washed two times with lysis buffer. The pellets were resuspended in lysis buffer containing 1% Triton X-100. Following SDS-PAGE under reducing conditions, and transfer to nitrocellulose membranes (Bio-Rad), immunodetection of proteins was performed with rabbit antiserum against human p120GAP (1:250; Transduction Laboratories), mAb against human p190GAP (1:500; Transduction Laboratories), mAb against human p62dok (1:250; Transduction Laboratories), and mAb against human cortactin (1:1,000; Transduction Laboratories). Immunoblotting of tyrosine-phosphorylated proteins recovered from whole Caco-2 cell extracts was performed with biotinylated mAb 4G10 (1:1,000; Upstate Biotechnology). Urease in H. pylori lysates was detected by immunoblotting using rabbit antiserum against urease apoenzyme (1:1,000).²

² B. Cortésy, unpublished observations.
Fig. 2. Integrity of the TER of polarized Caco-2 cell monolayer in the asymmetrical culture system. A, effects of the culture medium on the TER. The polarized Caco-2 cell monolayer was kept in C-DMEM (D) both apically (Ap) and basolaterally (Ba) or with BHI-C (B) apically. The basolateral compartments were maintained under the aerophilic atmosphere, whereas the apical compartments were perfused with the aerobic (A/A) or the microaerophilic (A/M) gas mixture. B, stability of the Caco-2 monolayer TER exposed to H. pylori in basolateral C-DMEM and apical microaerophilic BHI, pH 7.3. C, stability of the Caco-2 monolayer TER exposed to H. pylori in basolateral C-DMEM and apical microaerophilic BHI, pH 5.5 and 4.0, complemented with 5 mM urea.

RESULTS

Culture Conditions Optimized for H. pylori Growth Do Not Alter the Polarized Caco-2 Monolayer—Increased oxygen tension (21), alkaline or neutral pH (22), and prolonged incubation (23) prompt formation of coccoid forms of H. pylori no longer able to adhere and signal like spiral forms (24). We thus sought to establish conditions optimal for H. pylori growth and not deleterious to epithelial Caco-2 cells grown as polarized monolayers. A microaerophilic gas mixture (5% O₂, 5% CO₂, 90% N₂) was applied to the apical compartment, whereas the basolateral surface of cells was exposed to air and 5% CO₂. TER (a measure of the integrity of the Caco-2 cell polarized monolayer) resulting from polarized Caco-2 cells was stable either when BHI replaced C-DMEM or when the microaerophilic conditions were applied to the apical compartment (Fig. 2A). In microaerophilic apical BHI, a multiplicity of infection of 10, respectively, 100 did not affect TER for up to 48 h (Fig. 2B). TER was preserved down to pH 5.0 (a pH value resembling that reported in the stomach antrum) when aerophilic or microaerophilic BHI was present in the apical compartment.3 We found no drop in TER value when 5 mM urea, a substrate favoring H. pylori viability at low pH, was added (Fig. 2C). This defined that culture conditions ensuring optimal growth and adhesion (see below) of H. pylori, namely plain BHI, pH 5.5, 5 mM urea, and a microaerophilic gas mixture, are well tolerated by polarized Caco-2 monolayers.

The Presence of Caco-2 Cells Ensures Not Only Survival but Growth of H. pylori in the Asymmetrical Culture System—The definition of optimal culture conditions for H. pylori in the apical compartment is summarized in Table I. Under aerobic conditions, bacterial counts (c.f.u.) in the medium dropped rapidly within 4 h in C-DMEM (column 1); in BHI-U, the drop in bacterial counts showed a lag phase, eventually resulting in poor survival at 24 h (column 2). In contrast, we found that continued growth of H. pylori over a 24-h period was observed in microaerophilic BHI-U (column 3). Determination of the number of live bacteria in the apical compartment at different
times revealed that growth resumed between 8 and 12 h. Remarkably, the effect was dependent on the presence of polarized Caco-2 cells at the interface between the apical and basolateral compartment (compare columns 3 and 4). The data demonstrate that maintenance of live \textit{H. pylori} under non-physiological conditions depends on parameters, of which ignorance is likely to seriously affect its growth, and thus adhesion and resulting signaling properties (see below).

Because the microaerophilic gas conditions led to a much better bacterial growth capacity in the apical compartment, we then examined, using c.f.u. counting, whether this consistently improved adhesion of \textit{H. pylori} to Caco-2 cells. At 4 h, >20 times more adhesive bacteria were recovered when microaerophilic BHI and BHI-C media were used as compared with aerophilic conditions (Fig. 3A). In BHI-U, this factor raised up to 100-fold in favor of the microaerophilic environment (Fig. 3A). Under microaerophilic conditions at 24 h, the capacity of \textit{H. pylori} to adhere to Caco-2 cells was similar in all three media examined, whereas c.f.u. counts remained very low under aerophilic conditions (Fig. 3A). Adhesion is therefore linked closely with the preserved ability of \textit{H. pylori} to multiply (Table I, column 3). \textit{H. pylori} laid down over Caco-2 or AGS cells grown as non-polarized monolayers on plastic in C-DMEM yielded c.f.u. counts as low as in aerophilic BHI media. We observed the average binding of seven bacteria per Caco-2 cell (5 × 10^5 \textit{H. pylori} per filter carrying 7 × 10^9 Caco-2), and found that 10-fold differences in the initial bacterial load did not modify this ratio, thus arguing for specific association (Fig. 3B). Together, this indicates that optimized culture conditions for \textit{H. pylori} and Caco-2 cells avoid possible nonspecific effects including epithelial cell apoptosis resulting from the addition of dead bacteria, bacterial debris (25, 26), or excessive amounts of bacteria.

\textbf{The Interaction of \textit{H. pylori} with Polarized Caco-2 Resembles That Seen in Vivo—} Caco-2 cells simply grown on plastic miss both brush border and tight junctions (27). In contrast, Caco-2 cells seeded on Snapwell filters exhibit such features, with expression of brush border sucrose isomaltase and basolateral polymeric Ig receptor (28). Given that Caco-2 cells maintained high TER values, and \textit{H. pylori} showed much improved viability (see Figs. 2 and 3), we thus examined by electron microscopy what Caco-2 and \textit{H. pylori} looked like in the novel system described herein. Similar to gastric biopsies, the association between Caco-2 epithelial cells and \textit{H. pylori} triggers morphological changes including brush border effacement and pedestal formation (Fig. 4, A and B). The pictures obtained after 24 h of incubation showed the expected rod shape of \textit{H. pylori}, as well as the presence of non-aggregated bacteria sitting at the Caco-2 surface (Fig. 4A). Under these conditions, no coccoid \textit{H. pylori} could be observed at 24 h, yet this has been observed upon exposure to non-polarized epithelial cells maintained under non-physiological conditions.

\begin{figure}
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\includegraphics[width=\textwidth]{figure3}
\caption{Attachment characteristics of \textit{H. pylori} to polarized Caco-2 cells in the asymmetrical culture system. \textbf{A}, adhesion capacity of \textit{H. pylori} (1–3 × 10^9 bacteria per ml) as a function of time, medium, and gas conditions (A, aerophilic; M, microaerophilic). At the indicated times, filters carrying the Caco-2 cells and adherent bacteria were recovered, and the number of live bacteria was determined. \textit{p} values ranging between 0.008 and 0.0002 indicate statistically significant differences between aerophilic and microaerophilic conditions. \textbf{B}, saturation of \textit{H. pylori} binding sites on the surface of Caco-2 cells measured after 24 h in microaerophilic BHI-U. Incubation of 6 × 10^6 (filled square) or 40 × 10^6 (open square) \textit{H. pylori} in the apical compartment resulted in similar attachment to polarized Caco-2 cells. exp., two independent experiments (n = 3 filters/group).}
\end{figure}

\begin{table}
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\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Apical medium} & \textbf{1} & \textbf{2} & \textbf{3} & \textbf{4} \\
\hline
Caco-2 Gas & C-DMEM & BHI-U & BHI-U & BHI-U \\
\hline
\textbf{Time} & \textbf{h} & \textbf{c.f.u.} \\
\hline
0 & 8.6 & 8.6 & 8.6 ± 3.0 & 8.6 ± 3.0 \\
4 & 0.0005 & 1.4 & 3.6 ± 1.2 & 3.5 ± 1.6 \\
8 & 0 & 2.3 & 5.2 ± 1.6 & 3.5 ± 1.8 \\
12 & ND & ND & 8.9 ± 2.6 & 1.5 ± 0.091 \\
24 & 0 & 0.65 & 37 ± 5.4 & 0.54 ± 0.16 \\
\hline
\end{tabular}
\caption{Determination of the number of \textit{H. pylori} in various media bathing the apical compartment.}
\end{table}

\textsuperscript{4} S. Cottet, unpublished observations.
aerophilic conditions (8). Actin rearrangement within the host cell occurred directly beneath the site of attachment of \textit{H. pylori}, forming a very fine condensed structure concentric to the bacterium (Fig. 4B). No such alterations were seen in the absence of \textit{H. pylori} or under aerophilic conditions not ensuring bacterial viability (Table I). In contrast to previous data showing no pedestal formation and actin filament rearrangement using non-gastric cells (29), the establishment satisfied this requirement, thus suggesting optimized cross-talk between \textit{H. pylori} and Caco-2 cells.

\textit{H. pylori}-induced Activation of NF-\kappa B and Phosphorylation of p120\textsuperscript{RasGAP}, p190\textsuperscript{RhoGAP}, p62dok, and Cortactin in Polarized Caco-2 Cells—Epithelial cells grown on plastic and exposed to \textit{H. pylori} for 1–6 h have been shown to trigger NF-\kappa B activation (30, 31). Using polarized Caco-2 cells, and thanks to the potential of the culture system to preserve \textit{H. pylori} viability (Table I), this enabled us to extend the analysis to 24 h. We first compared the translocation properties of NF-\kappa B p50 and p65 subunits induced by \textit{H. pylori} in aerophilic and microaerophilic BHI-U (Fig. 5A). The latter gave rise to a much more pronounced nuclear accumulation of both subunits (p50, 5.5-fold; p65, 3-fold). Nuclear translocation was strictly dependent on the incubation of Caco-2 cells with \textit{H. pylori} and was sustained for up to 24 h. The level of p65 kept increasing between 2 and 24 h, whereas that of p50 reached steady-state after 2 h. Consistent with this, the higher amount of translocated p50 and p65 resulted in the formation of more DNA-NF-\kappa B complexes in EMSA (Fig. 5B, compare lanes 1 and 2 with lanes 5 and 6). Antisera to either subunit abolished (lanes 3 and 4) or reduced (lanes 7 and 8) the formation of the DNA-NF-\kappa B complex, indicating that the p50-p65 heterodimer represents the DNA binding form of NF-\kappa B activated after exposure to \textit{H. pylori}. Competition experiments confirmed the specificity of binding to the consensus NF-\kappa B DNA probe (lanes 9–12). Under identical EMSA conditions, barely detectable DNA-NF-\kappa B complexes were obtained with nuclear extracts from AGS cells grown on plastic\(^5\); in agreement with Table I, dead or coccoid bacteria can trigger NF-\kappa B activation only to low levels.

Although \textit{H. pylori} triggers protein phosphorylation in AGS cells (8), only scarce information exists as to the nature of these activities. We thus examined the pattern of phosphorylation of Caco-2 cytoplasmic proteins after incubation with \textit{H. pylori} (Fig. 5C). In microaerophilic BHI-U, we found that ten protein bands were tyrosine-phosphorylated \textit{de novo}, with apparent molecular weights (\(M_\text{r}\)) ranging from 47,000 to 184,000. Under aerophilic conditions, only doublets at 136 kDa yielded a signal\(^7\). The pattern was the same at 4 and 24 h, indicating that the interaction between the epithelial cells and the bacterium led to prolonged stimulation of pathways supposed to turn off rapidly. To shed light on the identity of protein triggered by \textit{H. pylori}, we performed immunoprecipitation on Caco-2 lysates using anti-phosphotyrosine mAb, followed by Western blot analysis. Specific signals were obtained with antibodies against p120\textsuperscript{RasGAP}, p190\textsuperscript{RhoGAP}, p62dok, and cortactin. No equivalent signals were obtained in the absence of \textit{H. pylori} or in aerophilic BHI-U (lanes 6 and 7). Likewise, no detection occurred after exposure of immunoblots with antibodies against ezrin and p130\textsuperscript{cas} \textit{H. pylori} infection resulted in the production in the stomach antrum of IL-8, MCP-1, GRO-\(\alpha\), and RANTES (35). However, with the exception of IL-8, no induction of other chemokines has been reported using classical \textit{in vitro} systems. Because RT-PCR analysis of cellular transcripts does not reflect strictly the protein production in the supernatant (36), we examined expression at the protein level using sandwich enzyme-linked immunosor-

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\(^5\) B. Cortheys, unpublished observations.

\(^6\) S. Cottet, unpublished observations.

\(^7\) S. Cottet and B. Cortheys, unpublished observations.

**Fig. 4.** Transmission electron microscopy of Caco-2 cell monolayer exposed to \textit{H. pylori} for 24 h. A, the cell-cell contact between \textit{H. pylori} and the Caco-2 cell triggers disruption of the Caco-2 brush border at the apical side. Round structures are spiral forms cut transversally. B, formation of characteristic pedestal protuberances at the site of bacterial adhesion. White bars correspond to 1 \(\mu\)m.
FIG. 5. Cellular events induced by the contact between *H. pylori* and Caco-2 cells. A, *H. pylori* (H.p.)-dependent nuclear translocation of NF-κB subunits p50 and p65 as a function of time and gaseous conditions (Cond.) (A, aerophilic conditions; M, microaerophilic conditions). Immunoblotting was performed on Caco-2 cell nuclear extracts (N.E.). B, EMSA performed with N.E. from Caco-2 cells incubated with *H. pylori* under various conditions. Comp., competition with a 50-fold molar excess of mutated (m) or increasing molar excess (5, 20, and 50) of consensus NF-κB binding site oligonucleotide; α-p50, antiserum to the p50 subunit; α-p65, antiserum to the p65 subunit. C, *H. pylori*-mediated tyrosine phosphorylation detected in Caco-2 cell lysates. Incubation was for 2 (lane 2) or without *H. pylori* for 24 h (lane 3). Bands appearing upon contact with *H. pylori* are marked by an arrow. Short exposure revealed *H. pylori*-mediated phosphorylation of the doublet at 130/136 kDa. Western blot analysis of anti-phosphotyrosine immunoprecipitates using anti-p190 RhoGAP, anti-p120, anti-p62, and anti-cortactin antisera identified these proteins under microaerophilic conditions and in the presence of *H. pylori* solely (compare lanes 4 and 5 with lanes 6 and 7).

bent assay. Major differences in chemokine secretion were observed as a function of gaseous and medium conditions (Fig. 6). Rise in IL-8 level occurred under aerophilic conditions in BHI-U and C-DMEM, with a lag time of 8 h in BHI-U; this resembles the situation seen with non-polarized cells incubated with *H. pylori* (37). Under microaerophilic conditions, detection of IL-8 was significant at 24 h in BHI-U (p < 0.002), whereas incubation in C-DMEM or without bacteria led to reduced chemokine release by Caco-2 cells (Fig. 6A). No specific changes because of exposure to *H. pylori* was observed for MCP-1 under aerophilic conditions; similar to IL-8, only at 24 h was the level of MCP-1 enhanced 3-fold (p < 0.008) in microaerophilic BHI-U as compared with controls (Fig. 6B). The RANTES profile exhibited the same kinetics and aspects, with a 2.5-fold up-regulation (p < 0.001) occurring at 24 h in microaerophilic BHI-U (Fig. 6C). Finally, to underscore weak activation of GRO-α (p < 0.02), microaerophilic BHI-U was required (Fig. 6D). *H. pylori* killed with gentamicin or 4% formalin did not induce production of chemokines above levels seen in the absence of bacteria under any gas conditions. Extrapolation of these data to Table 1 suggests that *H. pylori* in growing phase provide optimal cross-talk to induce expression and secretion of proinflammatory mediators by epithelial cells.

**Discussion**

The lack of an *in vitro* model has considerably impeded the study of *H. pylori*-host cell interaction at the cellular and molecular levels. Numerous data that have been obtained to date suffer from the limitation that polarized monolayers were not used. Furthermore, incubation of *H. pylori* and epithelial cells was performed under aerophilic conditions, which are not appropriate to ensure sustained bacterial growth for more than a few hours. Moreover, no data were obtained on the role of epithelial cells in *H. pylori* viability/growth or on modulation of *H. pylori* virulence factors. This can undoubtedly interfere with the underscoring of complex signal transduction pathways that require optimal bacteria-host cell interaction.

We have therefore designed an *in vitro* system based on the use of a *H. pylori* strain encoding the cag pathogenicity island and polarized intestinal Caco-2 cells, which approached the optimal culture conditions for both the bacteria and epithelial cells. The choice of the bacterial strain was based on the prevalent role of the cag pathogenicity island in gastric diseases and induction of initial events necessary for interaction with epithelial cells (40) and subsequent gene activation (41). Although not a strict equivalent to gastric cell lines, Hep-2, HT-29, T-84, Madin-Darby canine kidney cells, or Caco-2 cells have been used consistently to study IL-8 release, *H. pylori*-host interaction, permeability increase, and polymorphonuclear leukocyte migration (42–46). Moreover, the finding that *H. pylori* can associate with the duodenal and colonic mucosa (47–49) makes the approach of using an intestinal cell line valid. Several new molecular data could be obtained that remained elusive to date in other systems used previously to study *H. pylori*-host cell interaction. Experimental read-outs

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*a* S. Cottet and B. Corthézy, unpublished observations.
identical or close to physiological observations make it relevant to the refined dissection of the complex consequences of *H. pylori* infection.

The diffusion chamber we used offers the flexibility to select for different culture media, pH settings, and gas mixtures in either the apical or the basolateral compartment. The integrity of the Caco-2 monolayer was maintained using BHI at pH values between 5 and 7.3, not affected by the presence of up to $10^8$ bacteria for 48 h, not sensitive to the addition of 5 mM urea for 24 h, and preserved using microaerophilic gas conditions in the apical medium. Together, the data indicate that the unusual conditions Caco-2 monolayers can stand at the apical membrane permitted exposure to *H. pylori* kept under ideal culture conditions without deleterious morphological consequences that might affect the interaction (50) and resulting signal transduction pathways.

Following the binding of *H. pylori* to polarized Caco-2 cells, we observed inducible tyrosine phosphorylation of ten cellular proteins, with apparent $M_r$ ranging from 47,000 to 184,000. In the absence of *H. pylori* genes homologous to eukaryotic or bacterial tyrosine kinases (51, 52), phosphorylation has to be because of Caco-2 cell activities (53). The 151-kDa band might correspond to the 145-kDa band observed in gastric AGS cells (54). This protein was identified as *H. pylori* CagA antigen translocated into epithelial cells upon *H. pylori* attachment (55–57). In AGS cells again, the moderately increasing tyrosine phosphorylation of a protein with a $M_r$ of 105,000 was reported (8), which might correspond to the 104–107-kDa doublet observed similarly in Caco-2 cell lysate.

Reactivity of tyrosine-phosphorylated proteins with specific antiserum and mAb to Ras/Rho-associated proteins indicates that a novel pathway of cellular signaling by *H. pylori* has been
identified. Phosphorylated p62dok binds to p120RhoGAP and down-regulates its Ral GTPase activity. This observation contributes to link proteins of the Ras superfamily involved in the control of normal and neoplastic proliferation and the role played by *H. pylori* in gastric adenocarcinoma. Further, p120RhoGAP associates with p190RhoGAP to regulate actin dynamics and cytoskeleton rearrangement (58). This suggests the notion that p120RhoGAP connects the Rho and Ras pathways (52) through mechanisms involving *H. pylori*-regulated tyrosine-phosphorylated proteins. Detection of *H. pylori*-triggered phosphorylation of cortactin provides a clue for cytoskeletal reorganization seen upon bacterial binding. The perversion of cellular proteins appears as a paradigm of host-pathogen interactions. For example, enteropathogenic *Escherichia coli* induces tyrosine phosphorylation of three eukaryotic proteins, all apparently cytoskeletal-associated (59). Along the same line, *Listeria monocytogenes* induces the tyrosine phosphorylation of two isozymes (42 and 44 kDa) of the mitogen-activated protein kinase (60) found downstream of the ras-raf-mitogen-activated protein kinase/extracellular signal-regulated kinase pathway.

Intracellular signaling is required for short term activation of chemokine transcription by epithelial cells. We have shown in our system that inflammatory chemokines are produced in response to *H. pylori*. How does it compare with *in vitro* and *in vivo* gastric profiles? In KATO-III and MKN 45 gastric cells, IL-8 transcription induced by *H. pylori* was dependent on protein-tyrosine kinases (61–63). Effect of *H. pylori* on NF-kB activation in gastric (KATO III, MKN 45) and colonic (IIT-29) cell lines is well documented (50). While confirming these observations, our system brought up information on activation of chemokines such as MCP-1, GRO-a, and RANTES known to be involved in gastric inflammation *in vivo* (55). Although produced at relatively low levels, the chemokines were expressed with delayed kinetics by Caco-2 cells. Differences in the kinetics of production, coupled with quantitative differences in their production, suggest that epithelial cells may play a regulatory role in mucosal inflammation by influencing temporal and spatial recruitment of leukocytes within the mucosa. Features like late secretion of chemokines and concomitant sustained NF-kB activation most likely reflect that only *H. pylori* maintained under microaerophilic conditions “communicates” adequately with Caco-2 cells to which it is attached. It remains to be appreciated whether the sustained cellular signals observed in this study with a 1:5 ratio of Caco-2 cells to *H. pylori* might somehow reflect the chronicity of the infection as occurring in the gastric mucosa.

Urease activity is essential for initiating colonization of the stomach of animal models by *Helicobacter* sp. (64). Our data support the notion that urease *per se* does not function as an adhesin (4), because adherent *H. pylori* express three to four times less urease subunits than their suspension counterpart. It might well be that the urease signal contributed by adherent *H. pylori* corresponds to the cytoplasmic form, which appears essential to recurrence of infection after treatment with urease inhibitors (65). Thus, the system described herein could serve to screen for such novel inhibitors (66) at reduced costs. In addition, the advantage of using polarized cells responding *in vitro* like gastric cells should permit the future study of transmigration of polymorpho-nuclear leukocytes as a function of the *H. pylori* strain and possibly assay modulators of inflammation including IgA under well controlled experimental conditions.

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