Production of ammonium by *Helicobacter pylori* mediates occludin processing and disruption of tight junctions in Caco-2 cells

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Tight junctions, paracellular permeability barriers that define epithelial cell polarity, play an essential role in transepithelial transport, cell–cell adhesion and lymphocyte transmigration. They are also important for the maintenance of innate immune defence and intestinal antigen uptake. Ammonium (NH_4^+) is elevated in the gastric aspirates of *Helicobacter pylori*-infected patients and has been implicated in the disruption of tight-junction functional integrity and the induction of gastric mucosal damage during *H. pylori* infection. The precise mechanism of the effect of ammonium and the molecular targets of ammonium in host tissue are not yet identified. To study the effects of ammonium on epithelial tight junctions, the human colon carcinoma cell line Caco-2 was cultured on permeable supports and the transepithelial resistance (TER) was measured at different time intervals following exposure to ammonium salts or *H. pylori*-derived ammonium. A biphasic response to treatment with ammonium was found. Acute exposure to ammonium salts or $\text{NH}_3/\text{NH}_4^+$ derived from urea metabolism by wild-type *H. pylori* resulted in a 20–30% decrease in TER. After 24 h, the NH_4Cl -treated cells showed a partial recovery of TER. In contrast, the control culture, or cultures that were exposed to supernatants derived from urease-deficient *H. pylori*, showed no significant decrease in TER. Occludin-specific immunoblots revealed the expression of a low-molecular-weight form of occludin of 42 kDa upon $\text{NH}_3/\text{NH}_4^+$ exposure. The results indicate that modulation of tight-junction function by *H. pylori* is ammonium-dependent and linked to the accumulation of a low-molecular-weight and detergent-soluble form of occludin.

Received 17 March 2005

Revised 10 June 2005

Accepted 4 July 2005

INTRODUCTION

The breakdown of epithelial tight-junction integrity is likely to be an early event in triggering intestinal malfunction and subsequent inflammation and mucosal injury. Animal models of gastrointestinal barrier function and various studies on epithelial cells confirm that ammonium exerts cytotoxic effects and induces apoptotic cell death in gastric mucosa (Suzuki *et al.*, 2000, 2002; Smoot *et al.*, 1990; Hagen *et al.*, 2000). As an uncharged small molecule, ammonia (NH_3) permeates the luminal side of membranes (Suzuki *et al.*, 2000; Lichtenberger & Romero, 1994; Kleiner, 1981), and the charged ammonium ion (NH_4^+) passes through cation channels located mainly on the basolateral cell

membrane of epithelial cells (Handlogten *et al.*, 2004). $\text{NH}_3/\text{NH}_4^+$ has a pK of 9.0 and predominates as NH_4^+ within the acidic gastric lumen under physiological conditions. It is well known that the strong urease activity of *H. pylori* helps the bacterium to maintain cytoplasmic pH homeostasis and to ensure its survival in the acidic environment of the stomach (Eaton *et al.*, 1991; Stingl *et al.*, 2002; Sidebotham *et al.*, 2003). Since *H. pylori* resides largely within the surface mucous layer and adjacent to the gastric epithelial cells, the luminal surface might be exposed to considerable concentrations of $\text{NH}_3/\text{NH}_4^+$. This microenvironment has a higher pH than the gastric lumen and thereby permits gradient diffusion of NH_3 . The ammonium concentration in the gastric juice of *H. pylori*-infected subjects has been shown to reach 30 mM (Triebling *et al.*, 1991). Clinical studies indicate that acid-suppression therapy by proton-pump inhibitors exacerbates the progression of gastritis in *H. pylori*-infected patients (Gillen *et al.*, 1999). This adverse effect is consistent with the finding that elevation of the pH at the gastric mucosal surface enhances the generation of NH_3 and accelerates ammonium-induced apoptosis (Suzuki

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Abbreviations: a.u., arbitrary units; ccs, co-culture supernatant; LMW occludin, low-molecular-weight occludin; LY, lucifer yellow; MDCK cells, Madin-Darby canine kidney cells; TER, transepithelial resistance; wt, wild-type.

et al., 2002; Sidebotham *et al.*, 2003). The local elevation of mucosal surface pH during *H. pylori* infection can lead to ammonium-induced gastric injury, which may contribute to the opening of tight junctions that has been ascribed to virulence factors such as the vacuolating cytotoxin VacA and the cytotoxin-associated antigen CagA (Papini *et al.*, 1998; Amieva *et al.*, 2003; Blaser & Atherton, 2004). Therefore, intervention in *H. pylori*-derived ammonium levels should be considered as an additional therapeutic target.

In this study, we set out to investigate if the exposure of epithelial cells to physiological concentrations of NH_4Cl and *H. pylori*-derived $\text{NH}_3/\text{NH}_4^+$ alters their tight-junction behaviour and if so, to what extent the effects are manifest at the level of tight-junction protein expression. In previous work, ammonium treatment of Madin-Darby canine kidney (MDCK) cells was found to be associated with the expression of low-molecular-weight (LMW) occludin (Vastag *et al.*, 2005), and the expression levels of both claudins and occludin have been shown to influence tight-junction function in human colon carcinoma cell lines (Li *et al.*, 2004; Bojarski *et al.*, 2004). The aim of the present study was to assess the time-course of the effects of ammonium on Caco-2 cell tight junctions and to determine if the ammonium derived from *H. pylori* metabolism of urea modifies the expression of occludins. The rationale was that the *H. pylori*-mediated disruption of gastric epithelial tight junctions is linked to the formation of a LMW occludin that is dependent on bacterial urease activity and the production of ammonium. This proposed mode of action provides an alternative to the vacuolating cytotoxin VacA, or the translocation of the bacterial protein CagA, encoded by the *cag* pathogenicity island (*cag*-PAI), both of which have been shown to influence TER (Amieva *et al.*, 2003; Guillemin *et al.*, 2002; Papini *et al.*, 1998). In this work, we show that the reduction of TER by *H. pylori* has an ammonium-specific component that is independent of VacA and CagA. We also demonstrate that the level of ammonium produced by *H. pylori* is correlated with the expression of LMW occludin. These results suggest that *H. pylori*-derived ammonium exerts a rapid and specific change in the production of LMW occludin that coincides with the disruption of tight-junction function.

METHODS

Antibodies and chemicals. The following primary antibodies were used: rabbit anti-C-terminal occludin and mouse monoclonal anti-C-terminal occludin (Zymed Laboratories Inc., San Francisco), goat anti-N 19-terminal occludin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-ZO-1 (Zymed Laboratories) and mouse monoclonal anti-human cathepsin B (Oncogene Research Products Inc., San Diego). The secondary antibodies were from Dako, Jackson Laboratories Inc. (Bar Harbor, Maine, USA) or Molecular Probes. All chemicals were from either Sigma or Merck, except for non-fat freeze-dried milk (Vitalia GmbH, Sauerlach, Germany), lucifer yellow LY 476 Da (Molecular Probes) and TO-PRO3 (Molecular Probes).

Cell cultures. Cells of the human colon carcinoma epithelial cell line Caco-2 (ATCC clone HTB-37) were obtained from the laboratory of Professor Gstraunthaler (Institut für Physiologie Innsbruck, Austria) and cultured in Minimum Eagle's alpha Medium (Invitrogen) supplemented with heat-inactivated fetal calf serum (20%), L-glutamine (2 mM), non-essential amino acids (2 mM), penicillin and streptomycin (100 U ml^{-1} each), and amphotericin B (2.5 U ml^{-1}). Caco-2 cells between passages 4 and 6 only were grown on semipermeable transwell tissue-culture inserts of uncoated polycarbonate (8 μm pore size, 10 mm diameter, Nunc) or on collagen- and fibronectin-coated cover slips (12 mm, Marienfeld GmbH, Lauda-Koenigshofen, Germany). In addition to the Caco-2 cells mentioned above, Caco-2 cells of passage 85, MDCK cells of passages 27–30, and human lymphoid T cell line Jurkat (gift of Professor G. Hacker, Institute for Medical Microbiology, Technische Universität München) were grown in plastic Petri dishes (15 mm, Griener Bio-One) and flasks (75 cm^2 , Nunc). The confluence of epithelial cell monolayers was assessed by phase-contrast microscopy.

Bacterial strains and culture conditions. The *H. pylori* wild-type strain P12 and its isogenic mutants were grown on GC agar plates (Difco) supplemented with horse serum (8%), vancomycin (10 $\mu\text{g ml}^{-1}$), trimethoprim (5 $\mu\text{g ml}^{-1}$) and nystatin (1 $\mu\text{g ml}^{-1}$), and incubated for 24–48 h in a microaerobic atmosphere (85% N_2 , 10% CO_2 , 5% O_2). The P12 ΔureA mutant was constructed by allelic replacement mutagenesis with plasmid pBS1, containing a TnMax8 transposon insertion in the *ureA* gene (S. Odenbreit and others, unpublished results); P12 $\Delta\text{vacA}\Delta\text{cagA}$ has been described previously (Gebert *et al.*, 2003).

Preparation of cell extracts and culture supernatants. Confluent Caco-2 cell monolayers were infected by inoculation of bacterial suspensions into MEM medium supplemented with urea (2.5–15 mM) at a m.o.i. of 50. After 16–24 h incubation at 37 °C in humidified CO_2 (5%), the Caco-2-infected monolayers were washed twice in 5 ml ice-cold PBS and immediately frozen at –70 °C in lysis buffer [1% Triton X-100, 20 mM HEPES, 150 mM NaCl, 1.5 mM MgCl_2 , containing protease inhibitors leupeptin (2 $\mu\text{g ml}^{-1}$), sodium vanadate (2 mM), PMSF (4 mM), pepstatin (1 mM), aprotinin (10 $\mu\text{g ml}^{-1}$) and benzamidine (5 mM)]. The thawed lysates were sonicated (5 × 15 s) on ice. Protein from the pellet and supernatant fractions after centrifugation (13 000 g for 45 min) was measured by Bio-Rad Assay Dye Reagent and analysed on SDS-PAGE immunoblots. The medium from *H. pylori*/Caco-2 cell co-cultures was centrifuged (4000 g for 30 min), the pH was recorded, and the filtered (0.2 μm , Millipore) supernatants, referred to as co-culture supernatants (ccs), were kept frozen at –20 °C.

Ammonium determination. Ammonium concentrations in the ccs were measured on an automated clinical chemistry analyser (ARCHITECT c8000, Abbott Laboratories) using the ammonia assay (Roche Diagnostics), an enzymic method based on glutamate dehydrogenase and the NADPH-dependent conversion of 2-oxoglutarate to L-glutamate (Kirsten *et al.*, 1963).

Patient tissue sampling. Endoscopic biopsies from patients with stomach carcinoma ($n=9$) or patients without gastric carcinoma ($n=3$) were obtained from defined locations in the antral or corpus mucosa or the tumour site (Rieder *et al.*, 2001) in accordance with the Ethics Committee approval of Ludwig-Maximilians-Universität, München, project no. 240/01. Specimens were immediately frozen in liquid nitrogen and stored at –70 °C. Freshly thawed specimens (approx. 1 mm × 1 mm) were transferred on ice to 2 ml lysis buffer and homogenized four times with a Dounce teflon homogenizer. The Triton X-100-soluble extracts were analysed on SDS-PAGE

immunoblots. The *H. pylori* status of each patient was determined by assay of urease activity in gastric biopsies and by testing of patient sera for *H. pylori*-positive IgG and IgM antibodies.

SDS-PAGE and immunoblotting. SDS-PAGE was performed by the method of Laemmli (1970). Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (Amersham Biosciences) in a tank mini-blot apparatus (Scie-Plas Biochrom Ltd, Southam, UK) at constant current (45 mA for 16 h at 4 °C). Unreacted sites of the nitrocellulose membrane were blocked (2 h at room temperature) in non-fat milk (5 %) in PBS/Tween 20 (0.1 %), pH 7.4. The nitrocellulose membrane was then incubated with the anti-occludin or anti-ZO-1 antibodies at 1:2000 dilution for 3 h at room temperature and washed three times with PBS/Tween 20 (0.1 %), pH 7.4. The horseradish peroxidase (HRP)-conjugated secondary antibodies goat anti-mouse IgG or goat anti-rabbit IgG were diluted 1:12 000 in blocking buffer and incubated with nitrocellulose membranes (1 h at room temperature). After three washings, the nitrocellulose membranes were developed using enhanced chemiluminescent reagent (Pierce) on photographic film (Hyperfilm ECL, Amersham Biosciences). The images of the scanned nitrocellulose blots were processed with Adobe Photoshop version 6.0 software and the densitometry (arbitrary units, a.u.) of the immunoreactive occludin proteins was calculated by Image J software (NIH).

Occludin cDNA analysis. Occludin cDNA was amplified by PCR of reverse-transcribed RNA from Caco-2 cells using Invitrogen Superscript II. Oligonucleotides were obtained from Metabion (Martinsried, Germany). The following primer sequences were used: FOCLN218 (5'-CAAACCGAATCATTATGCAC-3'), FOCLN431 (5'-CTGGGACAGAGGCTATGGAA-3') and ROCLN1726 (5'-GCAT-CAGCCTTCTATGTTTTC-3'). PCR products were analysed on 1 % agarose gels.

Electrophysiological and paracellular flux measurements. TER was measured with a dual-voltage ohmmeter clamp (Vastag *et al.*, 2005); readings in ohms using Ag/AgCl electrodes were determined from the voltage response to 5 μ A cm⁻² current for 150 ms every 2 s. The TER results are expressed as the measured resistance in ohms multiplied by the area of the filter (1 cm²). All conditions were established in duplicate for each experiment. Inserts were used between days 16 and 22 after seeding, when the TER was greater than 500 Ω cm². Measurements of TER were recorded at the start of each experiment and at indicated time intervals, and are expressed in arbitrary units (a.u.) as the ratio of TER at each time point divided by TER at time 0. In order to provide the Caco-2 cells with sufficient stores of glutamate and growth factors, the medium was removed at 10–12 h intervals and fresh medium containing either NH₄Cl or ccs was added immediately following the measurement of TER. The lucifer yellow (LY) permeability assay of BD Biosciences (Chong *et al.*, 1997) was used to assess the permeability of the Caco-2 cells on the transwell filters. Monolayers were washed once with Hanks' balanced saline solution (HBSS), pH 7.4, and volumes of 50 μ l 0.4 M LY 476 Da in HBSS were placed in the apical compartment and 270 μ l of the HBSS transport buffer in the basal compartment. After 1 h incubation at 37 °C in 5 % CO₂, the LY fluorescence flux was determined at 485 nm excitation and 530 nm emission using a fluorescent plate reader (TECAN, Kirchheim, Germany). Permeability coefficients (P_{coeff}) were calculated according to the formula $P_{\text{coeff}} = V/(A \times C_i) \times C_f/t$, where V is the volume of the basal chamber, A the area of the membrane insert (0.804 cm²), C_i the initial concentration or fluorescence units in the apical compartment, C_f the final concentration or fluorescence units in the basal compartment, and t the assay time in seconds.

Immunofluorescence. Confluent Caco-2 cell monolayers, 10–14 days after seeding on collagen- or fibronectin-coated glass

cover slips, were exposed to NH₄Cl- or NH₃/NH₄⁺-containing *H. pylori*/Caco-2 ccs. At various time intervals, the cells were fixed in 4 % paraformaldehyde/PBS (15 min, 37 °C) and incubated (30 min, room temperature) in blocking buffer consisting of PBS containing 10 % goat serum or 10 % BSA, 0.1 % Triton X-100, 0.1 % Tween 20, pH 7.4. The primary mouse or rabbit antibodies, diluted 1:800 in goat serum blocking buffer, or the goat anti-N 19-terminal occludin diluted 1:800 in BSA blocking buffer, were added for 1.5 h at room temperature. After removal of antibodies, the cells were washed three times in PBS, 0.1 % Triton X-100, 0.1 % Tween 20, pH 7.4. Indirect detection of occludin protein was performed using the Cy3-coupled (Molecular Probes) or Alexa Fluor-coupled (Molecular Probes) goat secondary antibodies. After five washings, the cells were embedded in Mowiol 4-88 (Hoechst, Frankfurt). For visualization, an Olympus BX50F microscope equipped with an Olympus FluoView laser scanning system was used. Confocal images acquired at 0.75 nm sections were projected using Image J software (NIH) and processed by Adobe Photoshop version 6.0 software.

Data analysis. Data are presented as the mean $\pm$ SEM. The significance of difference between the means was evaluated by Student's *t* test for unpaired samples or by ANOVA; $P < 0.05$ was considered significant.

RESULTS

Ammonium salts and *H. pylori* culture supernatants disrupt the epithelial barrier function of Caco-2 cells

Within 14–21 days of seeding on transwell filters, Caco-2 cells formed monolayers with TER values in the range 680–1800 Ω cm². To determine the influence of *H. pylori*-generated ammonia on TER, co-culture supernatants (ccs) derived from the wild-type *H. pylori* strain P12 (wt-ccs) or isogenic mutant strains (Δ ureA-ccs, Δ vacA Δ cagA-ccs) were prepared and added to Caco-2 cell monolayers. For the control, 15 mM NH₄Cl was added. The TER decreased by 20–40 % after 1–4 h exposure to 15 mM NH₄Cl or upon incubation with wt-ccs diluted 1:3 in culture medium (Fig. 1a). The TER of Caco-2 cells treated with wt-ccs was 25–40 % and 60–70 % lower at 24 and 48 h, respectively (Fig. 1a). In contrast, no significant changes in the Caco-2 cell TER were found at 24 h and 48 h in a medium consisting of 1:3 diluted supernatant derived from control Caco-2 cultures or the Δ ureA-ccs, for which no ammonium was generated in the co-cultures. During the 24–48 h Caco-2 cell exposures to ammonium or ccs there was no cytolysis or cell detachment, as determined by histological examination using phase-contrast microscopy and by TER measurement.

We further examined the effect of 24 h ccs and ammonium treatments on epithelial barrier function by assaying paracellular transport by Caco-2 cells of the fluorescent substrate lucifer yellow (LY). The paracellular transport of Caco-2 cells after treatment with ammonium was 4–8-fold higher than in the control cultures. The P_{coeff} of LY in Caco-2 cells treated for 24 h was $0.44 \pm 0.12 \times 10^{-6}$ cm s⁻¹ with wt-ccs, $0.78 \pm 0.1 \times 10^{-6}$ cm s⁻¹ with 15 mM NH₄Cl, and $0.1 \pm 0.2 \times 10^{-6}$ cm s⁻¹ for control cultures. Thus, wt-ccs

Fig. 1. Effects of NH_4Cl and 1:3 diluted ccs on the TER of 21–23 day Caco-2 cell monolayers. (a) The TER values for each time point in the treatment groups were normalized to the TER values of controls with normal culture medium (dotted line), such that $\text{TER a.u.} = \text{TER a.u.}_{\text{treatment}} / \text{TER a.u.}_{\text{control}}$. TER values represent the mean and SD of three independent experiments; control culture TER range = $1000\text{--}1800 \Omega \text{ cm}^2$, mean = $1177 \Omega \text{ cm}^2$. Culture medium contained 15 mM NH_4Cl ($\blacktriangledown$), $\Delta ureA$ -ccs ($\triangle$), wt-ccs ($\bullet$) or $\Delta vacA \Delta cagA$ -ccs ($\blacksquare$). The difference between the TER a.u. of the treatment and the control groups is of statistical significance. *, $P < 0.01$ (ANOVA two way). (b) Caco-2 under treatment with 1:3 diluted $\Delta ureA$ -ccs ($0.2 \text{ mM NH}_3/\text{NH}_4^+$), $\Delta cagA \Delta vacA$ -ccs ($11 \text{ mM NH}_3/\text{NH}_4^+$), wt-ccs ($9 \text{ mM NH}_3/\text{NH}_4^+$), control medium ($1 \text{ mM NH}_3/\text{NH}_4^+$) or 15 mM NH_4Cl for 48 h (filled bars), 48 h + wash and 1.5 h replacement in control medium (cross-hatched bars), 48 h + wash and 24 h replacement in control medium (open bars). The difference between TER values at 0 h and at the 24 h wash period are of statistical significance **, $P < 0.05$ (Student's *t* test).

induces a level of paracellular transport intermediate between that of the control and NH_4Cl -treated cultures.

Ammonium-mediated decrease of TER is reversible

To determine if the effect of *H. pylori*-derived ammonium on tight-junction function is reversible, the 48 h Caco-2 cell cultures were washed and reinstated in normal control medium (Fig. 1b). The TER measurements of cultures that were exposed to the ammonium-containing $\Delta vacA \Delta cagA$ -ccs or wt-ccs showed a significant upward readjustment of their TER to a range of $0.7\text{--}0.8$ a.u. within 24 h. These results provide evidence that *H. pylori*-derived ammonium alters the tight-junction function of Caco-2 cells, but that the effects are reversible upon removal of ammonium.

H. pylori-derived ammonium induces the production of LMW occludin in Caco-2 cells

Previous work has shown that antibodies produced against pure forms of occludin bind to proteins in the 60–66 kDa area (Mankertz *et al.*, 2002; Bojarski *et al.*, 2004). However, an additional low-molecular-weight form of occludin (LMW occludin) can be induced in MDCK cells by treatment with NH_4Cl (Vastag *et al.*, 2005). Accordingly, the dose-dependent effects of NH_4Cl on LMW occludin production were assessed in Caco-2 cells after 24 h treatment with 0–50 mM NH_4Cl (Fig. 2). The appearance of LMW occludin of 42 kDa was most prominent after exposure to 15 mM NH_4Cl or 15 mM $(\text{NH}_4)_2\text{SO}_4$ (results not shown). The production of 60–66 kDa occludins (Fig. 2) or the tight-junction protein ZO-1 (results not shown) did not significantly change during treatment with ammonium salts.

To investigate whether or not cultures exposed to ammonium generated by *H. pylori* would also demonstrate increased production of the 42 kDa occludin, we infected

Fig. 2. Occludin production in Caco-2 cells exposed to NH_4Cl . SDS-PAGE immunoblots with rabbit anti-human occludin of Triton X-100 extracts of Caco-2 cells after 24 h exposure to 0–50 mM NH_4Cl . Each lane contains 40 μg of protein. The 42 kDa LMW occludin is indicated by an arrow.

monolayers of Caco-2 with either *H. pylori* P12 or the mutant strains P12 Δ ureA and P12 Δ vacA Δ cagA in the presence of increasing concentrations of urea. Occludin-specific immunoblots were then performed on the Triton X-100-soluble extracts and the insoluble membrane fractions (Fig. 3). Caco-2 cells infected with either *H. pylori* P12 or the double-mutant strain had 5–10-fold higher expression of the 42 kDa occludin than the background levels in cultures lacking urea (Fig. 3a). No significant amount of 42 kDa occludin was produced by infection of Caco-2 cells with the urease mutant. Since co-cultures undergoing urea metabolism for 16 h showed a pH 0.3–0.5 units higher than that of the control medium, we readjusted the pH of the control medium to 7.8–8.4 to determine if alkaline conditions could account for the presence of the 42 kDa occludin. No

significant amounts of 42 kDa occludin were found in normal Caco-2 cells cultured in alkaline medium (results not shown). The urea-dependent and NH₄Cl-dependent changes in occludin production were found only in the Triton X-100-soluble extract (Fig. 3b) and not in the insoluble membrane fraction (Fig. 3c).

In order to investigate whether the LMW occludin might also be produced *in vivo*, anti-C-terminal occludin immunoblots were made of detergent-soluble extracts of gastric tissue obtained from endoscopic biopsies. Full-length 66 kDa occludin was found in corpus and antrum gastric biopsies. LMW occludin at the position of the 42 kDa protein was detected in six of nine stomach carcinoma biopsies of patients that had tested positive for *H. pylori* urease activity. Further sampling of normal control individuals is required to determine the statistical significance of these findings with respect to *H. pylori* infection and stage of malignancy.

Because occludin is postulated to play a role in the trans-epithelial migration of activated lymphocytes, we investigated whether or not the exposure of Jurkat T cells to ammonium would modify their occludin expression. As described for peripheral blood lymphocytes (Alexander *et al.*, 1998), we found 60 kDa occludin in immunoblots of Jurkat T cells that were activated for 24 h with 30 ng ml⁻¹ phorbol ester (phorbol myristoyl acetate, PMA) and 125 ng ml⁻¹ of the calcium ionophore A23187, but no occludin expression in Jurkat T cells maintained in normal RPMI culture medium. LMW occludins were not detected in Jurkat T cells that were exposed to ammonium salts during their activation (results not shown). These results suggest that ammonium does not induce the formation of LMW occludins in T lymphocytes and that the effects of ammonium on occludin expression are specific for epithelial cells.

LMW occludin production induced by *H. pylori* ccs correlates with ammonium production

To determine if the ammonium generated in the *H. pylori*/Caco-2 co-cultures is sufficient for the formation of 42 kDa occludin, the ccs were assayed for their effects on occludin production in non-infected Caco-2 cells (Figs 4 and 5). The immunoblot intensities of 42 kDa occludin, but not the intensities of 60–66 kDa occludins, showed a significant correlation with the amount of ammonium production (Fig. 4). The ammonium levels in the diluted wt-ccs, 2.75–13 mM NH₄⁺, were in agreement with the amounts expected from the stoichiometric conversion of urea to ammonium, whereas the supernatants of Δ ureA-ccs had 0.125–0.75 mM NH₄⁺. The kinetics of ammonium-dependent 42 kDa occludin production showed a first-order exponential rate with $k=0.4303$ and $t_{1/2}=1.6$ h (Fig. 5, left panel). During the course of 8 h NH₄Cl exposure, the production of 42 kDa occludin increased approximately threefold compared to no major change of 60–66 kDa occludin production (Fig. 5, left panel).

Fig. 3. Occludin production in *H. pylori*-infected Caco-2 cells is dependent on urea metabolism. (a) Immunoblot densitometry. The bar graphs represent the mean and standard deviation of 42 kDa occludin in two immunoblot experiments of Caco-2 cell monolayers with 20 h *H. pylori* infections. Filled bars, P12 (wt); open bars, P12 Δ ureA; cross-hatched bars, P12 Δ vacA Δ cagA. (b) Triton X-100-soluble and (c) Triton X-100-insoluble occludin from a representative immunoblot, as described in (a). The position of the 42 kDa occludin is indicated by an arrow. The densitometry (a.u.) represents the immunoblot intensities of 42 kDa occludin protein.

Fig. 4. Production of 42 kDa occludin is correlated with the amount of urea-derived ammonium. Triton X-100-soluble occludin from Caco-2 cells after 24 h exposures to wt-ccs (filled symbols) or $\Delta ureA$ -ccs (open symbols). The densitometry plots of 42 kDa (●, ○) and 60–66 kDa occludin (■, □) are relative to the values in Caco-2 cells treated with supernatant of 1:8 diluted $\Delta ureA$ -ccs; 18300 ± 3067 a.u. 66 kDa and 505 ± 270 a.u. 42 kDa. The NH_4^+ concentrations are the means of two experiments corresponding to the 1:8, 1:4 and 1:2 diluted ccs.

Production of LMW occludin requires continuous exposure to ammonium

Further immunoblot experiments were performed to investigate whether or not the effect of ammonium on the 42 kDa occludin is reversible after the removal of ammonium by washing and the reinstatement of the Caco-2 cells in fresh normal medium (Fig. 5). Evaluation of the occludin immunoblot intensities after 8 h ammonium treatment and 16 h in recovery medium indicates that the production of 42 kDa occludin is abolished after removal of ammonium (Fig. 5), whereas no significant changes occur in the amount of 60–66 kDa occludins (Fig. 5, right panel). It is not clear from these results if the 42 kDa occludin is derived from the degradation of full-length occludin protein in the presence of ammonium or if it is generated by a process of ammonium-dependent *de novo* synthesis. Since detergent-soluble LMW occludins were not detected in the immunoprecipitates of ³⁵S-labelled proteins of Caco-2 cells, the effects of ammonium on occludin production could not be assessed using the available antibodies in pulse-chase experiments.

Evidence that the 42 kDa occludin is derived from N-terminal cleavage of full-length protein

To determine whether the LMW occludin is a product of ammonium-dependent post-translational events (i.e. protease cleavage) or transcription from alternative mRNA templates (as reported by Mankertz *et al.*, 2002), we performed N-terminal-specific immunoblots of protein extracts and RT-PCR of cDNA obtained from Caco-2 cells after 24 h NH_4Cl treatment (Fig. 6). The polyclonal antibody specific for occludin N-terminal peptide detected only the 66 kDa protein in extracts of cultures treated with NH_4Cl and control cultures, whereas immunoblots of the same extracts with the anti-C-terminal antibody revealed the 42 kDa occludin (Fig. 6a). In RT-PCR experiments, the use of 5' FOCLN218 and 3' oligonucleotide primer ROCLN1726 gave a single amplification product of 1530 bp that corresponds to the full-length occludin transcript. To favour amplification of shorter-length transcripts, the cDNA concentration was reduced by 10-fold in the presence of FOCLN218 and ROCLN1726 primers (Fig. 6b, lane 2). In addition, another 5' oligonucleotide

Fig. 5. Time-course of occludin production. Left panel, treatment of Caco-2 cells with 15 mM NH_4Cl ; right panel, determination of Triton X-100-soluble occludin after removal of *H. pylori* NH_3/NH_4^+ . The Caco-2 cells were treated with 1:3 diluted wt-ccs for 8 h (A), 1:3 diluted wt-ccs (9 mM NH_3/NH_4^+) for 8 h followed by washings and reinstatement for 16 h in fresh control medium (B), or Caco-2 control medium for 8 h (C). The densitometry values are expressed as mean and standard deviation of two independent experiments relative to the 42 kDa occludin at 0 h. ■, 60–66 kDa; ●, 42 kDa occludins.

Fig. 6. Effect of ammonium on N-terminal-specific occludin protein and gene expression. (a) Occludin-specific immunoblots using anti-C-terminal versus anti-N 19-terminal antibodies. Caco-2 cells were treated in two independent experiments for 24 h with NH₄Cl at the indicated concentrations. (b) RT-PCR of occludin transcripts. Amplification of Caco-2 cDNA using occludin-specific oligonucleotides FOCLN218 (5') and ROCLN1726 (3') with 3 μg (lanes 1 and 3) or 0.3 μg cDNA (lane 2). Caco-2 cells were left for 24 h in control medium (lane 1) or medium containing 15 mM NH₄Cl (lanes 2 and 3).

(FOCLN431), corresponding to amino acid position ⁸⁹Trp after the first transmembrane domain, was used with ROCLN1726 (results not shown). Both of these conditions gave only a single PCR product. The lack of evidence for an alternative mRNA transcript generated during ammonium treatment and the absence of 42 kDa occludin in N-terminal-specific antibody immunoblots suggest that the effect of ammonium on occludin expression is at the post-translational level and likely to involve N-terminal cleavage events.

Intracellular distribution of occludin during ammonium treatment

We next examined the intracellular distribution of occludin in Caco-2 cell monolayers that were exposed to control medium or to control medium that contained ammonium in the form of NH₄Cl or supernatants from *H. pylori* co-cultures. The labelling of occludin with anti-N-terminal occludin (Fig. 7a, b) and anti-C-terminal occludin (Fig. 7c, d) indicates that tight junctions are well established in control and ammonium-exposed cultures. The nuclear fluorescence of DNA-binding dye TO PRO-3 confirms that the monolayers are intact and uniformly fixed, with no indication that the ammonium and ccs had apoptotic effects in Caco-2 cells. Confocal immunofluorescence revealed the known pattern of defined occludin rings in the apical part of the cells (Fig. 7a, c) and less-defined fluorescence toward the basolateral border (Fig. 7b, d). The occludin in Caco-2 cells exposed to ammonium revealed diffuse and dot-like intracellular staining, in addition to the honeycomb rings. The diffuse intracellular dots were most pronounced in C-terminal occludin staining of Caco-2 cells exposed to NH₄Cl or ammonium derived from $\Delta vacA\Delta cagA$ -ccs.

The effect of ammonium on the intracellular distribution of occludin could be related to accumulation of

N-terminal-truncated protein in lysosomal compartments or to a non-specific redistribution of occludin away from the plasma membrane. To resolve this question, we tested whether or not cy3-labelled anti-C-terminal occludin co-localized with the lysosomal marker anti-cathepsin B labelled with Alexa Fluor 488 (Fig. 7c, d). Caco-2 cells with control ccs and $\Delta ureA$ -ccs showed cathepsin B staining in the form of sparse but bright speckles confined within the occludin tight junction in the apical sections (arrows, Fig. 7c, Control and $\Delta ureA$). In contrast, the NH₄Cl- and NH₃/NH₄⁺-containing ccs revealed a halo of low-intensity cathepsin B staining that was detected alongside the occludin tight junctions in apical and basolateral confocal sections. We cannot determine from the present results if the altered pattern of cathepsin and occludin distribution indicates leakage of cathepsin from lysosomes or alternatively co-localization of N-terminal truncated occludin that has accumulated in lysosomes.

DISCUSSION

We show that Caco-2 cells undergo major disturbance of their tight junctions, 20–40% loss of TER, during ammonium exposures of 1–8 h duration, and that they undergo partial recovery and readjustment of TER to initial values during 24–48 h NH₄Cl exposures. In previous work (Vastag *et al.*, 2005), TER measurements were made with solutions of NaCl and NH₄Cl of equivalent osmolarity, excluding the possibility that chloride ions cause the changes in tight junctions. A sustained diminution of TER was found upon continuous exposure to ammonium generated by either wt *H. pylori* or $\Delta vacA\Delta cagA$ mutant strains. This finding suggests that soluble factors other than VacA act together with ammonium to modulate epithelial tight junctions and to increase Caco-2 cell monolayer permeability. The restoration of TER after

Fig. 7. Effects of NH_4Cl and *H. pylori*-derived $\text{NH}_3/\text{NH}_4^+$ on occludin distribution. Caco-2 cells were treated for 24 h with control medium, medium containing 15 mM NH_4Cl , or ccs (1 : 3 diluted), with concentrations as indicated in Fig. 1. The confocal immunofluorescence was done using goat anti-N 19-terminal occludin and mouse anti-goat (FITC, green) with the nuclear dye (TO-PRO3, red) (a, b) or rabbit anti-C-terminal occludin with goat anti-rabbit IgG (Cy3, red) and mouse anti-cathepsin B with goat anti-mouse IgG (Alexa Fluor 488, green) (c, d). Apical (a, c) and basolateral (b, d) scanned sections are shown. Arrows indicate cathepsin B staining in apical sections. Bar, 10 μm .

removal of ammonium and replacement of the co-culture supernatants with normal culture medium is similar to previous findings (Terres *et al.*, 2003). In the latter work, polarized monolayers of T84 and Caco-2 cells showed laminin-dependent dome formation and decreased TER upon stimulation with *H. pylori* soluble extracts (HPE) followed by a recovery to normal phenotype after removal of HPE. We do not rule out the possibility that our preparations of filter-sterilized 16 h ccs contain soluble *H. pylori* factors that are the same as that of HPE. However, unlike the HPE which was obtained from the supernatant of bacterial suspensions, the ccs used in the present study are derived from established bacterial infections of epithelial cell monolayers, and their biological activity shows strict dependence on ammonium.

The rapid restoration of TER in polarized epithelial cells that have been treated with *H. pylori* $\text{NH}_3/\text{NH}_4^+$ and the abolishing of the 42 kDa occludin after removal of $\text{NH}_3/\text{NH}_4^+$ are consistent with the fact that occludins are not an essential structural component of tight junctions, but

instead play a role in the modulation of epithelial barrier function. Our findings support the prevailing view that the breakdown of the transepithelial barrier function of Caco-2 cells is reversible (Tavelin *et al.*, 2003; Nusrat *et al.*, 2000) and that epithelial cells have the capacity to undergo dynamic remodelling of their tight-junction protein apparatus in response to external stimuli, without suffering permanent cell damage (Bojarski *et al.*, 2004; Tavelin *et al.*, 2003; Ivanov *et al.*, 2004; Singh *et al.*, 2000). The production of LMW occludin during 1–4 h wt-ccs exposures indicates that occludin turnover is rapidly perturbed in the presence of *H. pylori*. It has been shown previously that *H. pylori* infection of AGS epithelial cells leads to an induction of occludin expression which is dependent on CagA (Guillemin *et al.*, 2002). Moreover, a disturbance of epithelial tight junctions in response to translocated CagA has been found in MDCK cells (Amieva *et al.*, 2003). These effects of *H. pylori* infection are only documented for full-length occludins. The study of Amieva *et al.* (2003) does not examine changes in occludin distribution in *H. pylori*-infected versus uninfected cells. Our detection of a 42 kDa

occludin in Caco-2 cells is in accordance with the fact that the redistribution of occludins (possibly due to formation of LMW occludin complexes) occurs in epithelial cells solely in the presence of ammonium and is independent of the *cag* pathogenicity island of *H. pylori*. Here we show no difference in occludin distribution between wild-type and $\Delta vacA\Delta cagA$ mutant strains, and suggest that ammonium produced by *H. pylori* urease activity constitutes an additional stress to epithelial tight-junction integrity that is independent of CagA or VacA. The action of inflammatory cytokines, known to disrupt epithelial barrier function by apoptosis-independent mechanisms (Bruewer *et al.*, 2003; Nusrat *et al.*, 2000), is not likely to explain the presence of LMW occludins in epithelial tissue in so far as we have found no inducible 42 kDa occludin production by Caco-2 under treatment with IFN γ or TNF- α (S. D. Lytton, unpublished observations).

Studies of occludin constructs bearing mutations or deletions of their N-terminal transmembrane regions provide evidence that the N-terminal and C-terminal cytosolic domains play a role in maintenance of the membrane-protein extracellular loop structure and in the organization and modulation of the tight-junction barrier (Nusrat *et al.*, 2000; Tavelin *et al.*, 2003). The accumulation of LMW occludin and its impact on the function of the full-length protein in intact cells should be resolved in the future by transfection of epithelial cells with N-terminal-truncated occludins. Such studies need to discriminate between the C-terminal caspase cleavage site of occludin (Bojarski *et al.*, 2004) and processing of occludin by proteases that show ammonium and/or pH sensitivity, as indicated by the results presented here.

Our data underline the importance of ammonium production for *H. pylori*-mediated modulation of the epithelial cell barrier and suggest that ammonium contributes to *H. pylori* pathogenicity. Because the effects of ccs on Caco-2 TER and the effects of ammonium on 42 kDa occludin formation are both reversible upon removal of ammonium, we might predict that truncated occludins and bacterial soluble factors have a synergistic action on the disturbance of epithelial tight-junction function. The presence of LMW occludins may not only suggest a link to the disturbance of epithelial cell tight junctions, but may also warrant an investigation of whether or not their presence is associated with other clinical consequences, such as gastric inflammation, alteration in gastrointestinal absorption or a specific autoimmune response to truncated membrane protein.

ACKNOWLEDGEMENTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft to F.X.B. and DFG HA2697/7-1 to R.H. We are very grateful for the help of Dr M. Schmolke in the measurement of ammonium, of Dr N. van Engel and Professor R. Hatz in providing endoscopic biopsy specimens from gastric carcinoma patients, and of Dr R. Blum with confocal microscopy.

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