Helicobacter pylori interferes with leukocyte migration via the outer membrane protein HopQ and via CagA translocation

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ABSTRACT

The human gastric pathogen *Helicobacter pylori* is a paradigm for chronic bacterial infections. Persistent colonization of the stomach mucosa is facilitated by several mechanisms of immune evasion and immune modulation, such as avoidance of Toll-like receptor recognition or skewing of effector T cell responses. Interactions of *H. pylori* with different immune cells have been described with respect to immune cell activation, cytokine release, or oxidative burst induction. We show here that *H. pylori* infection of human granulocytes, or of HL-60 cells differentiated to a granulocyte-like phenotype (dHL-60 cells) results in inhibition of cell migration under different conditions. Migration of dHL-60 cells in a three-dimensional collagen gel was found to be inhibited independently of the *cag* pathogenicity island, whereas migration inhibition in an under agarose assay was dependent on the *cag* pathogenicity island, on its effector protein CagA, and on the outer membrane protein HopQ. CagA translocation into leukocytes is accompanied by its tyrosine phosphorylation and by proteolytic processing into an N-terminal 100 kDa and a C-terminal 35 kDa fragment at a distinct cleavage site. By using complemented *H. pylori* strains producing either phosphorylation-resistant or cleavage-resistant CagA variants, we show that CagA tyrosine phosphorylation is required for migration inhibition, but CagA processing is not. Our results suggest that direct contact of *H. pylori* with immune cells subverts not only their activation characteristics, but also their migratory behaviour.

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Introduction

Colonization of the human stomach with *H. pylori* invariably results in a strong immune response, characterized by neutrophil infiltration of the gastric submucosa during the acute phase of infection, and a predominantly lymphocytic infiltration during chronic infection. Despite this marked inflammatory response and the production of *H. pylori*-specific antibodies, spontaneous elimination of the bacteria in the absence of antibiotic treatment is very rare. Several persistence strategies are discussed, including an evasion of recognition by the innate immune system, and a suppression of T cell-mediated immunity by several mechanisms (Salama et al., 2013). Remarkably, protective immunity against, or control of, *H. pylori* infection in animal models crucially depends on the T cell-mediated, rather than the humoral, immune response (Kusters et al., 2006). Although *H. pylori* is considered as an extracellular pathogen residing at the luminal surface of gastric epithelia,

the bacteria can also be observed in intracellular compartments (Dubois and Borén, 2007) or in the submucosa, for example as a consequence of impairment or destruction of gastric epithelial integrity after prolonged colonization (Necchi et al., 2007). In the latter case, bacterial cells might be in direct contact with immune cells. On the other hand, there is also histological evidence for the presence of transepithelial dendritic cells in infected human gastric tissue, and for contacts of these cells with *H. pylori* (Necchi et al., 2009). In *H. pylori*-infected mice, gastric dendritic cells with extensions across the epithelial layer could be directly detected by two-photon microscopy (Kao et al., 2010).

The fate of gastric dendritic cells after contact with *H. pylori* is not well-understood. Generally, gastric mucosal dendritic cells are more prevalent after *H. pylori* infection and display more activation markers (CD83, CD86, CCR7) (Hafsi et al., 2004; Hansson et al., 2006; Kranzer et al., 2004). In *H. pylori*-infected mice, dendritic cells have been shown to migrate to the paragastric lymph nodes (Algood et al., 2007), also indicating their activation in the gastric mucosa. However, activation by *H. pylori* might be incomplete or inefficient. For example, it has been shown that upregulation of the CCR7 receptor on monocyte-derived dendritic cells upon contact with *H.*

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pylori is less efficient than that induced by *E. coli* (Hansson et al., 2006). Nevertheless, *H. pylori*-activated DCs acquire the ability to migrate towards CCL19 (Hansson et al., 2006), consistent with their later arrival in gastric lymph nodes. Upon coincubation with naive CD4^{+} T cells, *H. pylori*-exposed DCs are able to induce IFN-γ, resulting in a polarization towards a Th1 or Th17 phenotype (Bimczok et al., 2010; Hafsi et al., 2004). However, the T cell priming capabilities of DCs may depend on the tissue environment; for instance, *H. pylori*-pulsed monocyte-derived DCs induce much less IFN-γ production in T cells when they are incubated with gastric or intestinal stroma-conditioned media prior to T cell interaction (Bimczok et al., 2011). Recent studies have also shown that *H. pylori* infections are characterized by a significant infiltration of regulatory T cells in the gastric submucosa, which counteract the proinflammatory activity of Th1 or Th17 cells (Hitzler et al., 2011; Lundgren et al., 2003; Oertli et al., 2012; Rad et al., 2006). T cell priming towards these regulatory effector functions depends on a tolerogenic reprogramming of DCs by the *H. pylori* virulence factors VacA, γ-glutamyltransferase, or CagA (Kaebisch et al., 2013; Oertli et al., 2013).

The gastric immune response to *H. pylori* infection and the development of gastritis thus involves chemotactic migration of several leukocyte types. Apart from dendritic cells migrating towards the lymph nodes, neutrophils are recruited to the gastric submucosa via chemotaxis towards CXCL1–3 (GROα, β and γ) and CXCL8 (IL-8) (Yamaoka et al., 1998), and via endothelial cell activation characterized by upregulation of E-selectin, VCAM-1 and ICAM-1 (Innocenti et al., 2002; Svensson et al., 2009). Moreover, the bacterial protein HP-NAP (*H. pylori* neutrophil-activating protein) has been shown to attract and to activate neutrophils (Evans et al., 1995; Polenghi et al., 2007; Satin et al., 2000). The activity of HP-NAP might also be involved in priming of T cells to a proinflammatory phenotype (Amedei et al., 2006). Infiltrating T lymphocytes have been reported to be recruited to the gastric mucosa via CCL20 binding to their CCR6 chemokine receptors (Cook et al., 2014; Wu et al., 2007), via L-selectin receptors (CD62L) binding to peripheral lymph node addressin (PNAd) (Kobayashi et al., 2004), and via $\alpha_4\beta_7$ integrins, which are known to bind to MAdCAM-1 adhesion molecules expressed by endothelial cells in the gastrointestinal tract (Michetti et al., 2000; Quiding-Järbrink et al., 2001).

While the influence of *H. pylori* virulence factors on activation of dendritic cells and on their acquisition of distinct T cell-priming capacities has been examined in several studies, a direct impact of virulence factors on leukocyte migration has not been determined so far. We show here that one of the major *H. pylori* pathogenicity factors, the *cag* pathogenicity island-encoded type IV secretion system with its effector protein CagA, has an inhibitory activity on leukocyte migration. The inhibitory activity of CagA depends on its tyrosine phosphorylation, but not on its processing into two fragments, which is a characteristic feature of CagA translocated into leukocytes. We can also show that the outer membrane protein HopQ is involved in migration inhibition, suggesting an unprecedented role of the HopQ–CagA axis in immune modulation.

Materials and methods

Bacterial and cell culture

H. pylori strains were grown on GC agar plates (Oxoid) supplemented with vitamin mix (1%), horse serum (8%), vancomycin (10 mg/l) and trimethoprim (5 mg/l) (serum plates), and incubated for 16 to 60 h in a microaerobic atmosphere (85% N_2, 10% CO_2, 5% O_2) at 37 °C. *E. coli* strains Top10 (Invitrogen) and DH5α (BRL) were grown on Luria–Bertani (LB) agar plates or in LB liquid medium supplemented with ampicillin (100 mg/l), chloramphenicol (30 mg/l), or kanamycin (40 mg/l), as appropriate. For the generation of isogenic mutants in *H. pylori* strain P12, plasmids were introduced by

natural transformation, as described (Haas et al., 1993), and transformants were selected on serum agar plates containing 6 mg/l chloramphenicol, 10 mg/l erythromycin, or 8 mg/l kanamycin, as appropriate.

AGS gastric epithelial cells and J774A.1 cells were cultivated under standard conditions as described previously (Odenbreit et al., 2000, 2001). HL-60 cells were cultivated in RPMI medium (Life Technologies) supplemented with 10% FCS and kept at a density of 1×10^5 to 2×10^6 cells/ml. Differentiation of HL-60 cells to a granulocyte-like phenotype (dHL-60 cells) was performed as described (Collins et al., 1978). Briefly, 10^7 HL-60 cells were harvested by centrifugation at $100 \times g$ for 5 min at room temperature, resuspended in 10 ml RPMI supplemented with 10% FCS and 1.3% dimethylsulfoxide, and cultivated for further 6 days without medium exchange. Differentiation was controlled by flow cytometry using CD11b and CD66acde markers (data not shown).

Isolation of human granulocytes

Human PMNs were isolated from blood donors by density gradient centrifugation. Briefly, 20 ml of blood were drawn into a syringe coated with heparin (10 U/ml blood) and diluted with PBS to a final volume of 50 ml. Subsequently, 25 ml of diluted blood were layered on top of 15 ml Ficoll (Biochrom) in a 50 ml tube. The Ficoll gradient was centrifuged at $400 \times g$ for 30 min at room temperature without brake. The clear supernatant including an opaque layer was removed and the remaining pellet was resuspended in 50 ml PBS. Cells were collected by centrifugation at $350 \times g$ for 10 min at 4 °C without brake, the supernatant was removed, and the pellet resuspended in erythrocyte lysis buffer (10 mM $KHCO_3$, pH 7.4; 155 mM NH_4Cl, 0.12 mM EDTA) and incubated for 30 min at 4 °C. PMNs were harvested by centrifugation and resuspended after an additional erythrocyte lysis step in RPMI medium with 10% FCS.

Plasmid constructions

For construction of the *cagA* 3′ deletion plasmid pCW1, a 600 bp *cagA* downstream region of strain P12 was amplified by PCR using primers WS390 (5′-CGGGATCCTA AAGGATTAAG GAATACC-3′) and WS391 (5′-ACCTGCGGCC GCTAAAGTGG AATTTCATGC G-3′) and cloned into the *Bam*HI and *Not*I sites of pBluescript II KS. Subsequently, an internal 500 bp *cagA* fragment (codons 613–781) amplified with primers WS452 (5′-GCGGTACCGT CGACGATCTT GAAAAATCTC TAAAGAAAC-3′) and WS359 (5′-ACCGCTCGAG GTTATCTTTT GATTGATGAT C-3′) was cloned together with a *Sal*I/*Bam*HI-restricted *rpsL-erm* cassette (Fischer et al., 2010) into the *Kpn*I and *Bam*HI sites of the resulting plasmid to obtain plasmid pCW1. The cognate *cagA* reconstitution plasmid pCW2 was generated by subcloning the *cagA* 3′ region (codons 613–1214) amplified with primers WS452 and WS425 (5′-ACCGCTGCAG GGTACCTTAA GATTTTTGGA AACCAC-3′) and restricted with *Sal*I and *Pst*I together with a *Bam*HI/*Sac*I-restricted *cagA* downstream fragment as described above and a *Pst*I/*Bam*HI-restricted terminatorless *cat* cassette (Fischer et al., 2001) into the *Sal*I and *Sac*I restriction sites of pUC18. Transformation of an *H. pylori* strain, which carries a *cagA* 3′ deletion introduced by plasmid pCW1, with plasmid pCW2 thus replaces the *rpsL-erm* cassette with a complete *cagA* 3′ region and introduces a downstream chloramphenicol resistance cassette for selection. For generation of a processing-resistant CagA variant, plasmid pCW2 was subjected to an inverse PCR with primers RW1 (5′-GCTGCGGCCG CTGCTGCTGG ACTCAAAAAC GAACCCATTT ATG-3′) and WS454 (5′-GAAATTTCCA AGTTTGCAT TC-3′), and the resulting PCR product was religated after *Dpn*I digestion of template DNA to obtain pRW2, in which the codons for asparagine residues 880 to 885 are thus replaced with six alanine codons. A phosphorylation-resistant CagA