Acute inflammatory response induced by *Helicobacter pylori* in the rat air pouch

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Abstract

Infection by *Helicobacter pylori* elicits persistent neutrophil infiltration in the gastric mucosa and stimulates the release of substances that may contribute to the establishment of gastritis. In this study, we used the rat air pouch model to evaluate the acute inflammatory response to *H. pylori*, in vivo. A pronounced neutrophil infiltration was observed 6 h and 12 h after the injection of *H. pylori* into the air pouch. Strains with different genotypes were able to induce cellular influx. This response was dependent upon the amount of bacteria injected and still occurred when heat-killed bacteria were employed. An increase in prostaglandin E2 levels was observed, indicating that *H. pylori* induced cyclooxygenase 2 in this model. The production of interleukin-1β and tumor necrosis factor-α by leukocytes was also enhanced, suggesting that this model may be useful for studying the direct activation of neutrophils by *H. pylori* in vivo.

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Keywords: Air pouch; *Helicobacter pylori*; Inflammation; Interleukin-1β; Neutrophil; Tumor necrosis factor-α

1. Introduction

*Helicobacter pylori* is one of the most common human gastrointestinal pathogens, infecting 50% of the human population [1]. Infection by *H. pylori* induces acute gastritis, which later develops into chronic gastritis. Both forms of gastritis are characterized by a considerable neutrophil infiltration, which can contribute to gastritis induction by releasing pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, IL-8 and tumor necrosis factor-α (TNF-α), as well as reactive oxygen species [2–4]. *H. pylori* or its products may recruit and activate neutrophils by directly stimulating these inflammatory cells or by stimulating the release of epithelial chemokines [5].

Many virulence factors contribute to the pathogenesis of *H. pylori*, but it is still unclear which of these modulate, directly or indirectly, neutrophil migration during *H. pylori* infection. Vacuolating toxin (VacA) induces the formation of large cytoplasmic vacuoles in cultured cells and increases the permeability of the epithelial barrier, probably by acting on tight junctions to reduce transepithelial resistance and, consequently, potentiating neutrophil migration into the gastric mucosa [6,7]. Several events occur after cell contact with bacteria possessing the *cag* pathogenicity island, for example, IL-8 may be induced by gastric epithelial cells and IL-8, in turn, is a potent neutrophil chemo-attractant [8].

Protein components in *H. pylori* extracts can attract and activate neutrophils and other inflammatory cells, thereby stimulating the production of TNF-α and IL-1β [9]. A 150-kDa protein in aqueous extracts of *H. pylori* promoted neutrophil adhesion to endothelial cells and chemotaxis in vitro, suggesting that this molecule could have a role in the accumulation of these cells at the site of infection [10,11]. This protein, designated HP-NAP (*H. pylori* neutrophil-activating protein), stimulates the production of reactive oxygen intermediates and the expression of...
cyclooxygenase 2 (COX-2) in neutrophils [11,12]. H. pylori lipopolysaccharide (LPS) possesses little biological activity compared to LPS from other bacteria [13]. This LPS, however, can stimulate the secretion of IL-8 by gastric epithelial cells and mononuclear cells, in vitro [14]. The priming of neutrophils by pre-incubation with LPS has also been described [13,15]. Reports on the ability of H. pylori to induce inflammation have been restricted almost entirely to the analysis of biopsies from patients. Only a few animal models have been used, although several aspects of the interaction between leukocytes and H. pylori have been evaluated in experiments in vitro [16]. The rat air pouch model of inflammation has been used to investigate the inflammatory response to a variety of agents, including zymosan, carrageenan and bacteria [17–20]. This model allows a quantitative assessment of the progress of inflammation and measurement of the formation of chemical mediators such as cytokines. In this study, the air pouch model was used to characterize the acute inflammatory response induced by H. pylori. The kinetics of leukocyte recruitment and the levels of prostaglandin E2 (PGE2) and cytokines formed were evaluated.

2. Materials and methods

2.1. Culture conditions and characterization of H. pylori

H. pylori isolates obtained from patients with non-ulcer dyspepsia and with peptic ulcer disease were included in the study (Table 1). Isolates of H. pylori were inoculated into selective medium [brain heart infusion (BHI) agar (Merck, Germany), 10% sheep blood, 10 mg l⁻¹ vancomycin, 20 mg l⁻¹ nalidixic acid, 2 mg l⁻¹ amphotericin B and 40 mg l⁻¹ 2,3,5-triphenyltetrazolium chloride (Sigma, Germany)], followed by incubation for 3–7 days at 37°C under microaerophilic conditions (8–10% CO₂, 5–6% O₂, 80–85% N₂, at 98% humidity). The identity of the colonies was confirmed by Gram staining and oxidase, catalase and urease production. The strains were inoculated on a plate, transferred to liquid medium [BHI agar, 10% fetal calf serum (Cultilab, Brazil), 10 mg l⁻¹ amphotericin B] and incubated for 3–5 days at 37°C under microaerophilic conditions as above, with continuous shaking at 50 rpm. Genomic DNA was extracted with DNAzol® reagent (Gibco BRL, Cincinnati, OH, USA). Polymerase chain reactions (PCR) were performed in a total volume of 50 µl containing 50 pmol primer, 100 ng genomic DNA, 1.0 mmol l⁻¹ of each of the four dNTPs (Invitrogen® Life Technologies, Alameda, CA, USA) and 2.5 U Taq DNA polymerase (Invitrogen® Life Technologies). The reaction mixtures were cycled in an automated GeneAmp® PCR System 9700 thermal cycler (PE Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 95°C for 1 min, with annealing temperature ranging from 45 to 60°C for 1 min and 72°C for 1 min. The final cycle included a 7-min step to ensure full extension of the PCR products. The presence of H. pylori was confirmed by PCR of the 16S rRNA [21] and glmM [22] genes. The cagA, cagE, vacA (m and s regions) and HP-NAP genes were analyzed (Table 1) using previously described primers [23–26].

2.2. Animals

Male Wistar rats (180–200 g) free of specific pathogens were obtained from CEMIB (State University of Campinas, Campinas, SP, Brazil). The experiments were performed in accordance with the principles outlined by the Brazilian College for Animal Experimentation (COBEA).

2.3. Air pouch model

Air pouches were produced as described elsewhere [27]. Briefly, 20 ml of sterile air was injected subcutaneously in the dorsal skin of rats. Three days later, another 10 ml of air was injected at the same site. On the sixth day after the first injection, the pouch received 2 ml of an H. pylori suspension. After the injection of H. pylori, the rats were anesthetized with halothane and the pouch was carefully opened by a small incision. The exudate was collected and transferred to a sterile tube and the exudate volume was measured gravimetrically. An aliquot of the exudate was used to determine the leukocyte number in an automatic counter (Celm 550, Brazil). The profile of infiltrated cells was determined in exudate smears stained with May–Grunwald–Giemsa. Samples of exudate were also frozen and stored at −20°C for subsequent quantification of PGE₂, TNF-α and IL-1β using commercially available ELISA kits (Amersham Pharmacia Biotech, Amersham, UK). A sample of blood was drawn from the inferior vena cava for determination of leukocyte numbers and plasma cytokine levels.

To determine the occurrence and the kinetics of the inflammatory response, two H. pylori strains (109, 551) were initially used to inoculate BHI broth and both strains were co-injected in the same animal. Thereafter, individual suspensions of H. pylori collected from solid plate cultures in phosphate-buffered saline (PBS) were used. In another experiment, bacteria were collected in PBS and heated to

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Strains used and genotypic status</th>
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<tr>
<td>Strain</td>
<td>Origin</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>109</td>
<td>NUD</td>
</tr>
<tr>
<td>219</td>
<td>PUD</td>
</tr>
<tr>
<td>320</td>
<td>PUD</td>
</tr>
<tr>
<td>551</td>
<td>PUD</td>
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NUD, non-ulcer dyspepsia; PUD, peptic ulcer disease.
100°C for 5 min. Control rats received only BHI broth or PBS.

2.4. Statistical analysis

All data were expressed as the mean ± S.E.M. Comparisons among groups were performed using one-way analysis of variance followed by the Student t-test or Dunnett multiple comparisons test, when convenient. An associated probability (P value) of less than 5% was considered significant.

3. Results

3.1. Inflammatory response at different times after the injection of H. pylori cultured in BHI broth

The volume of exudate recovered from air pouches at different times after the injection of H. pylori did not differ from that of the control group (data not shown). The co-injection of H. pylori (strains 109 and 551) into air pouches resulted in a significant (P < 0.01) accumulation of leukocytes after 6 h and 12 h compared to that observed with BHI broth alone (Fig. 1). The leukocyte number returned to basal levels after 24 h. After 1, 6 and 12 h, there was a significant increase in the number of neutrophils, when compared to the control group (Table 2), although there was no change in the number of peripheral blood leukocytes at these same times (data not shown). When the responses in PBS and liquid medium control groups were compared, a high level of leukocyte accumulation was observed in the liquid medium group. Thus, suspensions of H. pylori collected from solid plate cultures collected in PBS were used in further experiments, due to the fact that the liquid culture medium (BHI broth) was able to induce a significant cellular response when used as a negative control (Fig. 1).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>BHI broth (control)</th>
<th>H. pylori suspension</th>
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<tbody>
<tr>
<td></td>
<td>NE (×10⁶)</td>
<td>EO (×10⁶)</td>
</tr>
<tr>
<td>1</td>
<td>0.42 ± 0.10</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>6</td>
<td>26.65 ± 2.88</td>
<td>0.54 ± 0.29</td>
</tr>
<tr>
<td>12</td>
<td>6.57 ± 1.85</td>
<td>0.40 ± 0.13</td>
</tr>
<tr>
<td>24</td>
<td>5.08 ± 2.24</td>
<td>0.31 ± 0.18</td>
</tr>
</tbody>
</table>

The values are the means ± S.E.M. of five rats. **P < 0.01 compared to the respective control groups (liquid medium).
The injection of *H. pylori* suspension (3–18 × 10⁸ bacteria; strain 551), obtained from solid medium in PBS, increased the formation of exudate, which increased with the number of bacteria (Fig. 2A). An increased migration of inflammatory cells into the pouches was also observed after the injection of *H. pylori* in PBS. This response also varied with the number of bacteria used (Fig. 2B). The killed bacteria (3 × 10⁸ bacteria, strain 551) also induced cellular influx and exudate production (Fig. 2A,B). No statistical difference was seen when the experiments performed with live and killed bacteria were compared. Neutrophils were the predominant inflammatory cells recruited to air pouches in response to 3 × 10⁸ *H. pylori* per pouch. The same result was observed when 3 × 10⁸ killed bacteria were injected (Table 3). Higher numbers of microorganisms caused significant migration of eosinophils and mononuclear cells, in addition to neutrophils (Table 3). A predominant neutrophil recruitment was seen, compared to PBS, when different strains of *H. pylori* were injected at the same concentrations (Fig. 3). No statistical differences were observed when the experiments were performed with different strains.

### 3.2. Leukocyte migration 6 h after the injection of *H. pylori* suspension in PBS

The injection of *H. pylori* suspension (3–18 × 10⁸ bacteria; strain 551), obtained from solid medium in PBS, increased the formation of exudate, which increased with the number of bacteria (Fig. 2A). An increased migration of inflammatory cells into the pouches was also observed after the injection of *H. pylori* in PBS. This response also varied with the number of bacteria used (Fig. 2B). The killed bacteria (3 × 10⁸ bacteria, strain 551) also induced cellular influx and exudate production (Fig. 2A,B). No statistical difference was seen when the experiments performed with live and killed bacteria were compared. Neutrophils were the predominant inflammatory cells recruited to air pouches in response to 3 × 10⁸ *H. pylori* per pouch. The same result was observed when 3 × 10⁸ killed bacteria were injected (Table 3). Higher numbers of microorganisms caused significant migration of eosinophils and mononuclear cells, in addition to neutrophils (Table 3). A predominant neutrophil recruitment was seen, compared to PBS, when different strains of *H. pylori* were injected at the same concentrations (Fig. 3). No statistical differences were observed when the experiments were performed with different strains.

### 3.3. Exudate levels of PGE₂, IL-1β and TNF-α

The results from exudate samples, shown in Table 4, were obtained from groups injected with strains 551, 109 and 320 (3 × 10⁸ bacteria per pouch; n = 2–3 for each group). A significant increase in the exudate content of PGE₂ was observed 6 h after the injection of *H. pylori* when compared to the PBS group (Table 4). The levels of IL-1β and TNF-α were also greatly increased (Table 4). When individual results of PGE₂, IL-1β and TNF-α production from these strains were compared, no statistical differences were observed. These cytokines were not detected in plasma from control and *H. pylori*-injected rats (data not shown).

### 4. Discussion

The injection of *H. pylori* into rat air pouches evoked a pronounced infiltration of neutrophils after 6–12 h. No cellular influx was observed after 24 h, indicating that this was an acute inflammatory response. The cellular response was dependent upon the amount of bacteria injected and was observed with different strains, independently of cagA and vacA genotypes. A similar leukocyte activation induced by *H. pylori* has been reported in vitro, where *H. pylori* strains cagA+ and cagA− induced neutrophil chemotaxis in Boyden chambers when there was no contact with or interference from epithelial cells [28]. Up-regulation of the adherence molecule CD11b and an oxidative burst were observed in neutrophils following exposure to a sonicate from several *H. pylori* strains. Strains without the cagA and pic B genes showed the same ability to upregulate CD11b as strains positive for these genes [29]. In these cases, the virulence factor cagA, which depends on epithelial contact for its activity, apparently did not participate in leukocyte migration.

Neutrophil influx was also observed when killed bacteria were injected into the air pouches. The heat treatment used efficiently killed bacteria, demonstrated by the absence of growth on BHI agar plates (data not shown). A previous report has demonstrated the chemotactic activity of crude sonicates from *H. pylori* to be proteinase-sensitive but stable to such heat treatment [30]. Thus, it is possible that small protein fragments retaining biological activity may remain following the heat treatment. Another possibility is that LPS may be responsible for the cellular response observed after heat treatment. *H. pylori* LPS has received limited attention compared with other surface antigens. Nevertheless, this molecule seems to have similar properties to the LPS of other Gram-negative bacteria [13,31]. Future experiments with isogenic mutants (cagA, cagE, vacA and HP-NAP gene) or purified factors (LPS...
and HP-NAP) will be required to generate more evidence about which factors are involved in this phenomenon.

COX, also known as prostaglandin H synthase, catalyzes a fundamental reaction in the metabolism of arachidonic acid to PGs and thromboxanes. This enzyme occurs as constitutively expressed COX-1 and inducible COX-2 [32]. In neutrophils from normal subjects, arachidonic acid is metabolized predominantly to leukotrienes by constitutively expressed 5-lipoxygenase [33]. COX-2 is inducible in LPS or pro-inflammatory cytokines. The expression of COX-2 by neutrophils results in the synthesis and secretion of PGE2 [34]. Up-regulation of COX-2 mRNA and an increase in PGE2 synthesis by neutrophils were observed after stimulation of these cells with live H. pylori or an aqueous extract of this bacterium [12]. Gastric biopsies from H. pylori-infected patients with active gastritis express COX-2 and produce significant amounts of PGE2 when compared to normal biopsies [35]. An increase in PGE2 synthesis was detected in rat air pouches treated with H. pylori, suggesting that COX-2 may be induced in this model.

Cytokines released by neutrophils, including IL-1α and β, IL-12, TNF-α, transforming growth factor-β and others [36], exert pro-inflammatory and immunoregulatory actions. H. pylori components can induce the migration of neutrophils to the gastric mucosa and, following extravasation, neutrophils themselves may be a source of cytokines, thereby further amplifying the inflammatory response [37]. We observed a pronounced increase in the levels of IL-1β and TNF-α in air pouches treated with H. pylori, showing that neutrophils produced pro-inflammatory cytokines in response to H. pylori. These cytokines may play a role in the pathogenesis of the acute inflammation induced by this microorganism. In addition, the production of other cytokines, such as IL-8, in H. pylori rat air pouch should be evaluated.

Our results suggest that the rat air pouch model of inflammation induced by H. pylori may be useful for evaluating the responses of inflammatory cells to bacteria and the drugs that act upon these cells to attenuate these phenomena, without the disadvantages presented by in vitro models, such as the activation of leukocytes due to separation procedures or by exposure to artificial environments.

References


Fig. 3. Neutrophil counts in inflammatory exudates obtained 6 h after the injection of different H. pylori strains (3×10⁶ bacteria per pouch). The columns represent the means±S.E.M. of five rats. **P < 0.01 and ***P < 0.001 compared to the control group.


