Specific Neutrophil Hyporesponsiveness in Chronic *Helicobacter pylori* Infection

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The release from mucosal neutrophils of reactive oxygen species (ROS) is believed to be an important part of the pathogenesis of *Helicobacter pylori*–associated gastritis and duodenal ulcer. In this study, bacterial sonicates prepared from 1 reference and 29 patient strains were used to stimulate ROS release from peripheral blood neutrophils. In 29 *H. pylori*–positive patients, the neutrophil response toward the homologous strain was absent. In contrast, a significant response was observed toward the reference strain. In addition, on stimulation of neutrophils from 29 patients without *H. pylori* infection, ROS release was significant. The stimulatory effect on neutrophils by FMLP was comparable in the 2 groups. It is concluded that a specific neutrophil hyporesponsiveness in ROS release toward the homologous *H. pylori* strain exists. This feature has, to our knowledge, not been described previously for neutrophil responses to any human pathogen.

The causative role of *Helicobacter pylori* in duodenal ulcer disease and chronic gastritis is established [1], but the majority of subjects with *H. pylori* infection remain asymptomatic. The determinants for the clinical outcome of the infection and the pathogenic mechanisms have not been elucidated. Differences in bacterial virulence factors [2] and in host immune responses [3] have been suggested as crucial factors in eliciting clinical *H. pylori* infection. Histopathology of the infected gastric mucosa shows infiltration with neutrophils and mononuclear cells and tissue damage [4]. A potential role for reactive oxygen species (ROS) released from phagocytes in the pathogenesis of chronic inflammatory disorders has been suggested [5]. A similar role for ROS in duodenal ulcer disease has been implicated by the observation that increased mucosal concentrations of ROS correlate with a high density of *H. pylori* in the mucosa [6]. We have previously shown that *H. pylori* induce phagocyte chemotaxis, oxidative burst response, and neutrophil degranulation with myeloperoxidase release from azurophilic granules. This is mainly due to a protein of 25–35 kDa distinct from N-formylated oligopeptides [7–10]. *H. pylori* lipopolysaccharide has only weak stimulatory activity on neutrophil ROS release [11]. Neutrophil activation has also been reported by others [12, 13].

Even though the majority of the bacteria are on the mucosal epithelial surface, they may occasionally disrupt the tight junctions and penetrate the epithelial cells; bacteria have also been observed in lamina propria [14, 15]. The bacteria give rise to a humoral immune response characterized by the production of systemic IgG and secretory IgA antibodies [2, 16]. Despite these immunologic defense mechanisms, the infection remains chronic. To further elucidate the role of neutrophil ROS release in *H. pylori*–related diseases, we investigated the interaction of *H. pylori* sonicates with neutrophils isolated from *H. pylori*–positive patients with gastroduodenal abnormalities and from *H. pylori*–negative patients without gastroduodenal abnormalities. The neutrophil response to the patient’s own (homologous) strain was also compared with the neutrophil response to 1 selected reference (heterologous) strain.

**Materials and Methods**

**Patients and blood samples.** The study population consisted of 58 consecutive patients (31 men, 27 women; median age, 54 years [range, 21–84]) admitted for upper gastrointestinal endoscopy due to dyspeptic symptoms. The patients were not treated with antibiotics prior to the study. At least 5 days before gastroduodenoscopy, the treatment with H2 receptor antagonists or proton-pump inhibitor (PPI) was discontinued. During gastroscopy, biopsies were taken from both gastric antrum and corpus according to the Sydney classification of gastritis [17]. Venous blood samples (30 mL) were drawn from all patients for neutrophil functional assay a median of 4 weeks (range, 4–20) after gastroduodenoscopy.

**Histology.** Four biopsies were stored in formaldehyde prior to histologic examination. Hematoxylin-eosin–stained sections were evaluated morphologically. The presence of *Helicobacter*-like organisms (HLO) and neutrophil infiltration were recorded.

**Culture of *H. pylori.*** Four biopsies from each patient were transported in enriched serum broth and spread on 7% lysed horse blood agar plates within 3 h. The plates were incubated microaerobically (5% O2, 10% CO2, 85% N2) at 36°C for up to 7 days.
Measurement of *H. pylori* antibodies. IgG antibodies to low-molecular-weight antigens were assessed by ELISA as previously described [16]. In adult dyspeptic patients, this assay has a sensitivity of 95% and a specificity of 73% [16].

Characterization of the selected reference strain. As the reference strain in this study, we chose CH-20249, which had been used in earlier studies [7–11, 16, 18–21]. The strain was isolated from an adult male patient with a peptic ulcer and has the protein profile characteristic for *H. pylori*; it is CagA-positive and is representative of 8 other clinical isolates [18]. We used this strain for routine antigen preparation for ELISA [16, 18, 19]. It has a concentration-dependent chemotactic activity toward human phagocytes [7] and can prime the subsequent chemiluminescence of phagocytes when stimulated with the oligopeptide N-FMLP. In addition, strain CH-20249 has dose-dependent stimulatory activity on phagocyte chemiluminescence [8]. The activity is probably due to a protein of ~25–35 kDa [7, 8] and is distinct from FMLP [9]. The strain has priming activity on neutrophil degranulation [10] caused by protein(s), but our findings suggest that nonprotein molecules also have part of the activity. Lipopolysaccharide from the reference strain had no direct stimulatory activity on neutrophil chemiluminescence, but preincubation in lipopolysaccharide resulted in a significant priming of the subsequent stimulation [11]. Bactericidal assays done by testing the effect of neutrophils and monocytes on the strain showed that the phagocytes were ineffective in killing *H. pylori* [20]. Western blot assays show the strain to have cross-reactive common antigens with *Pseudomonas aeruginosa* [21].

Preparation of *H. pylori* sonicates. Subcultures of isolates from patients (4–6 passages) and the selected reference strain (numerous passages) were grown on 7% lysed horse blood agar plates under microaerobic conditions. After 48–72 h of incubation at 36°C, the noncontaminated plates were harvested in sterile distilled water. The bacteria were washed once in distilled water and resuspended to a concentration of 0.5 g/mL (~10^9/mL). The bacteria were disintegrated by sonication on ice five times for 45 s at 20,000 Hz (Rapidis 350; 19-mm probe, 9.5-mm tip; Ultrasoundics, UK). The cell lysate was centrifuged at 14,000 g for 1 h at 4°C. The antigen concentration was measured by refractometry using human immunoglobulin as a standard. Sonicates were stored in small aliquots at −20°C.

The reference strain sonicate was produced as a single batch, which was used throughout the study. Sonicates from the clinical isolates were prepared as needed.

Isolation of neutrophils. Peripheral blood cells were separated by dextran sedimentation and subsequently by density-gradient centrifugation on metrizoate/poly sacrose (Lymphoprep; Nyegaard, Oslo). After separation from mononuclear cells, the remaining erythrocytes were removed by hypotonic lysis; neutrophils were adjusted to 5 × 10^9/mL in Krebs Ringer’s solution with 5 mmol/L glucose. Neutrophils were >98% viable and pure.

Reagents for neutrophil chemiluminescence. N-FMLP, zymosan A, and luminol (5-amino-2,3-dihydro-1,4- phtalalazinedione) were obtained from Sigma (St. Louis). The synthetic oligopeptide FMLP is the major peptide neutrophil chemotactic factor in *Escherichia coli* [22].

Neutrophil chemiluminescence. The production of ROS from neutrophils was measured in a chemiluminescence assay. The assay was done in duplicate in a luminometer (model 1251; LKB-Wallac, Bromma, Sweden) in cuvettes containing 5 × 10^5 neutrophils and luminol at a final concentration of 7 × 10^{-5} mol/L. The cells were stimulated with bacterial sonicate from the homologous strain at 1000 μg/mL, the reference strain at 1000 μg/mL, serum- opsonized zymosan A (OZ) at 2 mg/mL, or FMLP at 2 × 10^{-6} mol/L. Sequential 10-s counts were made on each cuvette over 90 min. We measured the peak millivolts and the time it took to reach the peak.

Assessment of CagA by SDS-PAGE. To analyze whether the 29 strains were CagA-positive, SDS-PAGE was done in a 10% gel. The assay was done as previously described [18]. The relative molecular weight was determined by comparison of mobility of test proteins to that of reference proteins (high-molecular-weight kit; Pharmacia Fine Chemicals, Uppsala, Sweden). The gels were Coomassie brilliant blue–stained. CagA was recorded on a base of molecular mass being ~120 kDa.

Statistical analysis. Mann-Whitney *U* test was used to compare groups of patients; the Wilcoxon sign rank test was used to compare neutrophil responses to different cytotoxins. The one-sample *t* test was used to compare ROS release responses in neutrophils as results of stimulus from the homologous strain to that resulting from stimulus due to heterologous strains.

Results

Histology, culture, and antibody response. Twenty-nine patients were *H. pylori* culture–positive, and HLO were seen in sections from all patients. Examination of morphology revealed active chronic gastritis in all 29 patients; 8 had intestinal metaplasia, and anti-*H. pylori* IgG antibody levels were increased in 27 of the culture- and histology-positive patients. During endoscopy, duodenal ulcers were found in 8 patients, gastric ulcers in 8, esophagitis in 3, and gastritis type B in 6. Four patients had no gastroduodenal abnormalities.

The results of histology and antibody detection in 29 culture-negative patients are shown in table 1. One of the *H. pylori*–negative patients had intestinal metaplasia and was seronegative.

To analyze the influence of histologic and serologic status on the chemiluminescence responses, the data were divided into the following 3 patient groups: *H. pylori*–positive (*n = 29*), culture-negative but seropositive (*n = 8*; in 1 patient, HLO was seen by histology), and histology-, culture-, and seronegative (*n = 21*; figure 1).

Neutrophil ROS release stimulated by the heterologous strain. Neutrophils isolated from 58 patients were stimulated with FMLP, OZ, and *H. pylori* sonicate from the reference strain. By comparing the peak chemiluminescence values of the 29 patients with *H. pylori* infection with those of 29 patients without *H. pylori* infection (1 excluded due to HLO in the histologic sections), we found that ROS were generated in comparable amounts: FMLP (*P = .8*), OZ (*P = .7*), and the reference strain (*P = .7*). No significant differences in the neutrophil responses to FMLP, OZ, or the reference strain were observed between the group of patients without gastroduodenal
Table 1. Histology and antibody responses in 29 H. pylori culture-negative patients.

<table>
<thead>
<tr>
<th>Gastroscopic diagnosis</th>
<th>Ulcer</th>
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<tbody>
<tr>
<td></td>
<td>Duodenal (n=0)</td>
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<tr>
<td>Chronic inflammation</td>
<td>0</td>
</tr>
<tr>
<td>Active chronic inflammation with HLO</td>
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<tr>
<td>No inflammation</td>
<td>0</td>
</tr>
<tr>
<td>Unspecific inflammation</td>
<td>0</td>
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<tr>
<td>Anti-H. pylori IgG antibodies</td>
<td>0</td>
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</tbody>
</table>

NOTE: HLO, Helicobacter-like organisms.

Abnormalities (n = 22), duodenal ulcer (n = 8), gastric ulcer (n = 12), gastritis type B (n = 10), or esophagitis (n = 7).

No significant difference in neutrophil reactivity to FMLP or the reference strain could be demonstrated in H. pylori-positive patients compared with that in patients with or without increased IgG antibody levels to H. pylori (figure 1).

Specificity of neutrophil ROS release stimulated by the homologous H. pylori strains. It was possible to compare the neutrophil response to FMLP, sonicate from the reference strain, and sonicate from the homologous strain in the 29 H. pylori-positive patients. Neutrophil response to the homologous strain was very weak or absent (median, 0.0 mV; range,
Figure 2. Neutrophil chemiluminescence of 29 H. pylori-positive patients. Neutrophils were stimulated with H. pylori sonicate (1000 μg/mL) of patient’s own strain, reference strain, and FMLP (2 × 10⁻⁶ M). Mean and 95% percentile are indicated. Values are expressed as peaks in mV. P values (Mann-Whitney U test) compare responses when stimulating with patient’s own strain or reference strain.

0.0–3.4) in all 29 patients, despite the fact that the neutrophils could respond to FMLP (median, 8.5 mV; range, 0.1–93.3) and the sonicate from the reference strain (median, 31.1 mV; range, 0.2–266.3) (figure 2). In all patients, the neutrophil response to the homologous strain was lower than that to the reference strain (P < .001). The neutrophil response to the homologous strain was not significantly different between the groups of endoscopy patients.

Cross-stimulation of neutrophils from different patients with different H. pylori strains. Neutrophil responses to stimulation by sonicates prepared from strains originating from unrelated patients were tested. It was shown that sonicates of 5 different clinical isolates that did not stimulate the homologous neutrophils contained significant stimulatory activity toward the neutrophils from 12 unrelated patients and 3 healthy neutrophil donors (table 2).

Relationship between endoscopic diagnosis and neutrophil ROS. There were no statistical differences within the groups of patients diagnosed by endoscopy in the responses obtained by stimulating with the reference strain, the homologous strain, or FMLP (data not shown).

Effect of treatment on neutrophil ROS. Neutrophil functional assays were done a median of 4 weeks (range, 4–20) after initial gastroduodenoscopies of the 58 patients. In the period from endoscopy to blood sampling, 7 of the H. pylori-positive patients received triple antibiotic therapy (amoxicillin, metronidazole, and PPI) for eradication of H. pylori; none of the remaining 51 patients received any antibiotic. One month after gastroscopy and start of eradication treatment, there were no significant differences in the neutrophil ROS stimulated by the homologous strain, the reference strain, or FMLP in antibiotic-treated and -untreated patients (figure 3).

Time course of the chemiluminescence response and neutrophil priming. To evaluate the nature of the neutrophil ROS beyond peak values, the times to peak values were determined (figure 4.) The time to peak chemiluminescence did not differ within the 3 different groups by culture, histology, or serology. Compared with the time course in the reference strain, the time courses of strains ii and iii (table 2) did not differ significantly (strain ii, P = .5; strain iii, P = .8). In none of the patients was the baseline response increased, that is, the neutrophils were not primed due to the H. pylori infection.

Neutrophil ROS versus mucosal neutrophil load and intestinal metaplasia. To evaluate our data with regard to stage of infection, we chose to define the occurrence of intestinal metaplasia as an indicator of the most severe stage of the disease. Intestinal metaplasia was found in histologic sections of biopsies from 9 patients, of whom 8 were H. pylori-positive. The data were evaluated in terms of peak chemiluminescence and time to peak (data not shown). There were no significant
Table 2. Stimulation of heterologous neutrophil chemiluminescence (reactive O₂ species release) by 5 *H. pylori* strains.

<table>
<thead>
<tr>
<th>Neutrophils</th>
<th>Reference</th>
<th>i</th>
<th>ii</th>
<th>iii</th>
<th>iii</th>
<th>iii</th>
<th>FMLP</th>
</tr>
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<tr>
<td>Homologous</td>
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<td>0.0</td>
<td>1.7</td>
<td>0.0</td>
<td></td>
<td></td>
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<tr>
<td>Heterologous</td>
<td></td>
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<td>25.3</td>
</tr>
<tr>
<td>1</td>
<td>81.0</td>
<td>1.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>25.3</td>
</tr>
<tr>
<td>2</td>
<td>111.0</td>
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<td>72.0</td>
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<td>—</td>
<td>—</td>
<td>8.5</td>
</tr>
<tr>
<td>3</td>
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<td>4.5</td>
<td>32.2</td>
<td>14.9</td>
<td>11.2</td>
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<td>39.5</td>
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<tr>
<td>6</td>
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<td>1.7</td>
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<td>—</td>
<td>25.0</td>
<td>7.0</td>
<td>—</td>
<td>—</td>
<td>44.0</td>
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<tr>
<td>8</td>
<td>244.1</td>
<td>—</td>
<td>69.2</td>
<td>50.0</td>
<td>—</td>
<td>—</td>
<td>14.9</td>
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<tr>
<td>9</td>
<td>1.1</td>
<td>—</td>
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<td>0.0</td>
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<td>—</td>
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<tr>
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<td>13.9</td>
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<td>3.4</td>
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<tr>
<td>12</td>
<td>11.8</td>
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<td>8.3</td>
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<tr>
<td>13</td>
<td>49.2</td>
<td>12.7</td>
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<td>2.4</td>
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<tr>
<td>15</td>
<td>35.5</td>
<td>3.5</td>
<td>26.5</td>
<td>2.2</td>
<td>11.4</td>
<td>12.8</td>
<td>6.3</td>
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<tr>
<td>Mean ± SE</td>
<td>51.2 ± 16.9</td>
<td>3.3 ± 1.5</td>
<td>25.7 ± 6.3</td>
<td>12.1 ± 4.3</td>
<td>12.9 ± 4.6</td>
<td>13.5 ± 7.0</td>
<td>11.0 ± 3.1</td>
</tr>
<tr>
<td><em>P</em></td>
<td>.07</td>
<td>.001</td>
<td>.015</td>
<td>.04</td>
<td>.04</td>
<td>.1</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. Heterologous neutrophils (5 × 10⁵/mL) from 12 dyspeptic patients (1–12) and 3 healthy neutrophil donors (13–15) were stimulated with reference strain, *H. pylori* (1000 μg/mL) isolated from 5 patients included in study, and FMLP (2 × 10⁻⁶ M). *P* (1 sample *t* test) comparing response when homologous and heterologous neutrophils were stimulated with same strain of *H. pylori*. Values are peak values in mV.

Figure 3. Influence of eradication therapy on neutrophil chemiluminescence of 58 patients. Seven patients received triple therapy for eradication. Neutrophils were stimulated with *H. pylori* sonicate (1000 μg/mL) of patient’s own strain, reference strain, and FMLP (2 × 10⁻⁶ M). Mean and 95% percentile are indicated. Values are expressed as peaks in mV. *P* values (Mann-Whitney *U* test) compare responses when stimulating with either patient’s own strain or reference strain.
Figure 4. Time course of neutrophil chemiluminescence of 58 patients. Neutrophils were stimulated with *H. pylori* sonicate (1000 μg/mL) of reference strain and FMLP (2 × 10^{-6} M). Twenty-nine patients were *H. pylori*-positive, 21 were *H. pylori*-negative by culture, histologic, and serologic examination; 8 were culture-negative but seropositive. Mean and 95% percentile are indicated. Values are expressed in time to peaks in minutes.

**Discussion**

*H. pylori* infection is often chronic, and it is associated with the accumulation of neutrophils and other inflammatory cells in the gastric mucosa [4, 6, 14]. It has been proposed that mucosal ROS production is of pathogenic importance [6], but it is unclear if differences in host immune responses to *H. pylori* could explain the diversity of clinical presentations of this infection, especially when most subjects remain asymptomatic.

In this study, severe hyporesponsiveness of peripheral neutrophil ROS release was demonstrated when stimulation was by the homologous strain of *H. pylori* (figures 2–5, table 2). The response was not due to defective neutrophil function, since the responses to a reference strain and a standard oligopeptide (FMLP) was not impaired. Neither was the low response due to defective stimulatory capacity of the isolated strains of *H. pylori*, since control experiments confirmed that
several isolates could stimulate neutrophils from heterologous donors (table 2). It appears that a specificity of the impaired response exists. Analysis of the data obtained, including antibody levels against *H. pylori*, histologic and gastroscopic diagnosis, treatment, or CagA positivity of the strains examined, does not explain this phenomenon. The low neutrophil responsiveness to stimulation with the homologous strain of *H. pylori* could be a mechanism for the infection to suppress the immune defense, thereby allowing the infection to become chronic.

The impaired responsiveness of homologous neutrophils is in contrast to the results of Davies et al. [6], who found a dose-response relationship between *H. pylori* and neutrophil load in the gastric mucosa and ROS production of the mucosal biopsies. They therefore suggested that ROS production from neutrophils stimulated with *H. pylori* was an important mechanism in the pathogenesis of peptic ulcer disease. The present assay is done on peripheral neutrophils under serum-free conditions, and any stimulation of neutrophils by agents other than *H. pylori* is negligible. This might explain the difference between our results and those of Davies et al. However, we can not exclude the possibility that immunoglobulins bound to Fc receptors on the neutrophil surface play a role. Another explanation could be that neutrophil homing is specific and that all the neutrophils capable of activation by *H. pylori* are accumulated at the site of infection.

The exact mechanisms of action in *H. pylori* stimulation of neutrophil oxidative burst are unknown, and a receptor has not been described. Consequently, it is unclear if the membrane interaction takes form as lectin-mediated binding or specific receptor binding. For some defined molecules capable of neutrophil activation (e.g., FMLP and complement factor C5a), a receptor-specific phenomenon of “desensitization” has been reported [23]. In the study reported here, we found no priming of the neutrophil response to the bacteria. Previous data indicate that the active molecule(s) in *H. pylori* sonicate have no relation to FMLP [9, 10], and the FMLP response in this study was

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**Figure 5.** Neutrophil chemiluminescence of 58 patients analyzed according to neutrophil infiltration into gastric mucosa. Burden of infiltration is graded 1–3. Neutrophils were stimulated with *H. pylori* sonicate (1000 μg/mL) of patient’s own strain, reference strain, and FMLP (2 × 10⁻⁶ M). Mean and 95% percentile are indicated. Values are expressed as peaks in mV.
similar in the *H. pylori*—positive and *H. pylori*—negative groups (figure 1). It is possible that neutrophils from patients with chronic *H. pylori* infection are desensitized to circulating components from the homologous strain and thus unable to respond to further stimulation ex vivo. This hypothesis requires the existence of wide strain diversity in neutrophil-activating antigens, which has not been reported yet. The neutrophil activation is, until now, believed to be unspecific in contrast to T lymphocyte responses to defined antigens, where tolerance is well recognized.

Humoral factors such as immunoglobulins could mediate the specificity in the interaction between neutrophils and *H. pylori*. In this study, no differences between the groups of antibiotic-treated and -untreated patient groups could be demonstrated. This does not exclude the possibility that immunoglobulins are implicated in the nature of our findings. The immunoglobulin levels decline slowly after eradication of *H. pylori* [24], and there are still circulating antibodies 6 months after successful eradication. The patients were examined too closely to the onset of *H. pylori* eradication. As a result, a significant fall in circulating antibodies could not be expected. For elucidating the mechanisms of the neutrophil ROS release, studies on neutrophil function of long-term *H. pylori*—eradicated patients are in progress.

Our work, to our knowledge, is the first report of specificity of neutrophil activation to a human bacterial infection. It will be interesting to see if neutrophil stimulation with the homologous strain in other chronic infections shows similar trends.

Acknowledgment

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References