# IMMUNOLOGY AND MICROBIOLOGY

# New Data on the Effect of *Helicobacter Pylori* on Oxygen Metabolism in Human Neutrophils

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A new phenomenon is discovered: *Helicobacter pylori* suppressed production of superoxide radical by neutrophils, while intracellular production of oxygen radicals is considerably activated. This phenomenon seems to play an important role in *Helicobacter* modulation of the inflammatory process in the stomach and persistence of the bacterium in the inflammatory focus.

**Key Words:** *Helicobacter pylori; neutrophils; active oxygen radicals; luminol; lucigenin*

In humans, infection with *H. pylori* leads to development of helicobacteriasis, a specific infectious process presenting as a syndrome of several nosological entities: chronic gastritis [3,9], peptic ulcer [10], MALT lymphoma, and distal gastric adenocarcinoma [6]. According to modern concepts, the pathophysiology of helicobacteriasis is associated with *H. pylori* interaction with epithelial cells of the stomach followed by the production of chemokines and cytokines stimulating the migration and activation of neutrophils in the involved zone [13]. Direct release of reactive oxygen radicals (OR) by phagocytes and enzymes into the mucosa induces inflammation causing damage to gastric cells and tissues [14]. It is important, that *H. pylori* also produces some substances activating neutrophils, maintaining the persistence of its own population and inflammatory process in a local zone [11].

However, the mechanism protecting *H. pylori* from the effects of active bactericidal substances produced by neutrophils is poorly understood. We studied production of OR during *H. pylori* phagocytosis by neutrophils, an important components of protection from pathogenic microorganisms.

## MATERIALS AND METHODS

Broth and agar cultures of *H. pylori* reference strains NCTC 11639 and NCTC 11637 were cultured routinely [4] in Columbia Agar (bioMerieux) with 7% (v/v) normal lyzed equine blood and Brucella Broth (BBL/Becton Dickinson) with 10% (v/v) normal equine serum, respectively. The cultures were incubated for 24-72 h at 37°C in microaerobic atmosphere of fixed composition (v/v): 5% oxygen, 10% carbon monoxide, and 85% nitrogen. Broth cultures were stirred on an S-3 rotating platform (ELMI) during incubation.

Venous blood from 8 healthy volunteers was put into tubes with 20 μl/ml 7.5% EDTA (Sigma) and differential blood count was determined on a hemocytometer Xcell-18 (Danam).

Chemiluminescent (CL) analysis of phagocytes in the whole blood was carried out as described previously [15]. To this end, 300 μl luminol ($5.6\times10^4$ M; Aldrich Chem. Co) or lucigenin ($4.9\times10^4$ M; Sigma) in

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Hanks solution (pH 7.2) and 50 µl opsonized zymosan (treated by pooled donor sera) or 50 µl *H. pylori* suspension were put in succession into plastic tubes (Clinicon). Optical density of *H. pylori* suspension was brought to the standard optical density of zymosan suspension on a Screen master Plus biochemical analyzer at λ=340 nm. Then, 10 µl whole blood was put into tubes and CL reaction was analyzed on an LKB-1251 luminometer (Wallak). Chemiluminescence intensity was expressed in mV integral response ($CL_{int}$) over 10 or 20 min per 100 granulocytes. For some experiments α coefficient (ratio of neutrophil CL during *H. pylori* phagocytosis in a medium with luminol to CL in medium with lucigenin) was determined.

The results were processed statistically using Student's *t* test.

## RESULTS

Two luminophores (luminol and lucigenin) were used for the analysis of induction of active OR generation during neutrophil interaction with *H. pylori*. Fluorescence of luminol reflects mainly the internal activation events of oxygen metabolism in neutrophils during phagocytosis involving myeloperoxidase, while fluorescence of lucigenin reflects production of $O_2^{\bullet-}$ superoxide radical by cells [5]. Based on previous findings [2], we considered that $O_2^{\bullet-}$ production by phagocytes and, presumably, its activity were realized mainly in the extracellular space.

The first series of experiments was carried out on 2-day agar culture of *H. pylori* NCTC 11639. Phagocytosis of *H. pylori* by whole blood neutrophils in the medium with luminol was associated with increased (4-fold on average) intracellular production of active OR in comparison with the standard concentration of zymosan used for clinical studies (optical densities of their suspensions were equalized). By contrast, CL during neutrophil phagocytosis of *H. pylori* in the medium with lucigenin reflecting mainly extracellular production of $O_2^{\bullet-}$ by phagocytes was markedly reduced (Table 1, donors 1-3). This regularity was demonstrated in experiments with suspension of purified blood leukocytes (66% neutrophils) obtained after erythrocyte lysis (Table 1, donor 4).

The second series of experiments was carried out on *H. pylori* broth culture (bacterial count was equal to that of agar culture suspension by optical density). The use of this culture revealed another regularity: CL in the presence of lucigenin reproduced the phenomenon described above (marked suppression of $O_2^{\bullet-}$ production), while phagocytosis of bacteria in the medium with luminol was not associated with intensive CL, as in experiments with *H. pylori* agar culture (Fig. 1). This fact suggests that activation of neutrophil CL response in the medium with luminol during phagocytosis of *H. pylori* agar culture was presumably due to specific features of *H. pylori* broth culture, not intrinsic of agar culture.

Analysis of the production of active oxygen forms by neutrophils during phagocytosis of 24, 48, and 72-h broth cultures of *H. pylori* NCTC 11639 showed the highest coefficient α for 24-h bacterial culture: 7.03±0.09 *vs.* 4.50±0.20 (48 h) and 4.00±0.11 (72 h).

Experiments with the blood from 4 donors with initially different CL activities of granulocytes gave interesting data. The higher was activity, the more marked was suppression of $O_2^{\bullet-}$ generation by *H. pylori* NCTC 11637 culture (Table 2). This fact indicates that *H. pylori* can modulate neutrophil activity.

Comparison of broth cultures of two strains of *H. pylori* showed higher inhibitory activity of strain 11639, which suppressed $O_2^{\bullet-}$ generation stronger (Fig. 1).

It is noteworthy that 24-h storage of broth and agar cultures of *H. pylori* at 4°C in normal atmosphere (at least 90% cells in the bacterial population die under these conditions) virtually completely abolished the detected phenomenon (coefficient α was equal to 1), this suggests that the phenomenon was determined by viable bacterial cells.

**TABLE 1.** Production of OR by Blood Neutrophils during Phagocytosis of Zymosan and *H. pylori* NCTC 11639 (Agar Culture; $CL_{int}$ for 20 min; *M*±*m*)

| Donors | Zymosan | | *H. pylori* | |
|---|---|---|---|---|
| | luminol | lucigenin | luminol | lucigenin |
| Whole blood | | | | |
| donor 1 | 13.90±0.39 | 6.20±0.35 | 73.10±0.78** | 2.70±0.06* |
| donor 2 | 16.0±0.6 | 4.0±0.2 | 50.6±0.2** | 1.680±0.044* |
| donor 3 | 12.8±1.0 | 2.6±0.5 | 593.0±2.3** | 1.90±0.15* |
| Purified leukocytes | | | | |
| donor 4 | 51.40±0.68 | 1.60±0.03 | 151.8±3.5** | 0.49±0.01* |

**Note.** *$p<0.01$, **$p<0.001$ compared to zymosan.

**TABLE 2.** Effect of *H. pylori* NCTC 11637 (Broth Culture) on OR Production by Neutrophils from Patients with Different Cell Activities ($CL_{int}$ for 20 min; *M*±*m*)

| Donors | | *H. pylori* | | α |
|---|---|---|---|---|
| | | luminol | lucigenin | |
| Whole blood | donor 5 | 52.0±3.5 | 8.25±0.50 | 6.30±0.49 |
| | donor 6 | 12.7±0.6 | 3.3±0.1 | 3.80±0.06 |
| | donor 7 | 128.0±3.0 | 6.9±0.3 | 18.6±1.1 |
| Purified leukocytes | donor 8 | 29.6±1.5 | 3.60±0.17 | 8.30±0.75 |

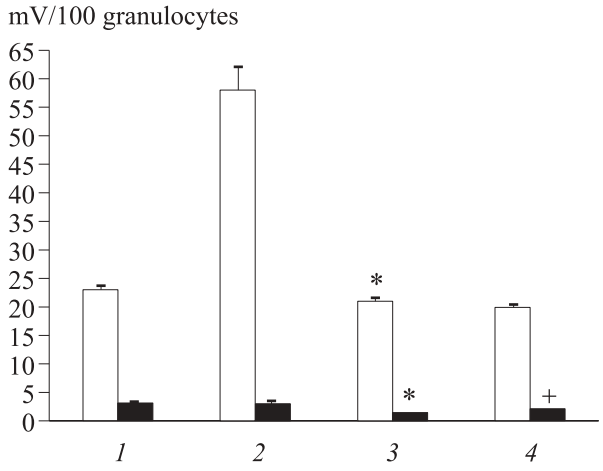


**Fig. 1.** Generation of active oxygen radicals by donor blood granulocytes during phagocytosis of zymosan (*1*), agar (*2*) and broth (*3*) cultures of *H. pylori* NCTC 11639 and broth culture of *H. pylori* NCTC 11637 (*4*). Light bars: medium with luminol; dark bars: with lucigenin. $p<0.01$ compared to: *zymosan, +*H. pylori* NCTC 11639.

Hence, the study showed that *H. pylori* agar (but not broth) culture activated oxygen metabolism of blood granulocytes. A possible explanation of the striking differences in oxygen metabolism of phagocytes caused by phagocytosis of *H. pylori* cultured under different conditions is phagocytosis initiation through different receptors. Since absorption of zymosan opsonized by pooled donor sera is mediated mainly through CR3 complement receptors (CD11b), the same mechanism can be involved in phagocytosis of *H. pylori* broth cultures, because they were cultured in medium with normal equine serum which no doubt opsonized bacterial cells by complement components.

Agar cultures of *H. pylori* were not opsonized, and hence, phagocytosis of bacterial cells by neutrophils could involve other cell receptors and mechanisms. It can be hypothesized that neutrophil Toll-like 1, Toll-like 4, and Toll-like 5 receptors participate in this event [7,8], as well as atypical protein kinase C zeta [1], which can lead to appreciable intensification of CL response and formation of megasomes in neutrophils, in which *H. pylori* cells survived.

On the other hand, *H. pylori* markedly suppressed generation of $O_2^{\bullet-}$ irrespective of the method of culturing, most likely at the expense of SOD [12].

The significance of the described phenomenon is great, as *H. pylori*-induced suppression of highly active $O_2^{\bullet-}$ released by phagocytes into the microenvironment during contact with the bacteria provides one of the mechanisms maintaining their survival and long persistence in the focus of inflammation.

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