In Vitro Treatment with Killed Helicobacter pylori Downregulates the Production of RANTES by Peripheral Blood Mononuclear Cells

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Summary

The mechanisms by which Helicobacter pylori colonizes and persists within the gastric mucosa are poorly understood. The gastric immune response observed in vivo during H. pylori infection, is characterized by a polarization of Th1 cell type that seems to be responsible for gastric pathology. The purpose of this study was to test the direct effect of H. pylori cagA/vacA (live and or gentamicin-killed) on human peripheral blood mononuclear cells (PBMCs) in order to evaluate the production of regulated activation normal T cell expressed and secreted (RANTES) in vitro. We also evaluated the possible relationship between RANTES release and the presence of IL-12 and IFN-γ in supernatants of the same cells. In the present study, we showed for the first time that the low amount of RANTES in supernatants of PBMC incubated with killed H. pylori is linked, at least in part, to the inhibition of IL-12 and IFN-γ release.

Key words: RANTES, IFN-γ, IL-12, Helicobacter pylori, PBMC.

INTRODUCTION

Helicobacter pylori is the most common bacterial pathogen involved in human gastrointestinal pathology. Gastric colonization by H. pylori, a minimally invasive Gram-negative bacterium, is the major cause of chronic active gastritis and is often associated with both duodenal and gastric ulceration, as well as gastric carcinoma and mucosa-associated lymphoid tissue lymphoma 1. The gastric immune response observed in vivo, during H. pylori infection, is characterized by a polarization of Th1 cell type that seems to be responsible for gastric pathology. 2-4 The local inflammation induced by H. pylori is characterized by infiltration of neutrophils, lymphocytes, plasma cells and monocytes in the gastric mucosa and by the local production of cytokines and chemokines 5,6. Among these, RANTES (regulated activation normal T cell expressed and secreted) seems to play an important role in recruiting and activating neutrophils in the gastric mucosa. RANTES is a CC chemokine produced by epithelial cells, CD8+ T cells, fibroblasts and platelets that mediates the trafficking and homing of classical lymphoid cells such as T cells and monocytes and acts on a range of other cells, including basophils, eosinophils, natural killer cells, dendritic cells, and mast cells 7,8.

In the present study we analyzed the production of RANTES from peripheral blood mononuclear cells (PBMCs) treated in vitro with killed and/or live H. pylori. Furthermore, we evaluated the possible relationship between RANTES release and the presence of IL-12 and IFN-γ in supernatants of the same cells.

In previous studies, in relation to the Th1/Th2 paradigm, we have demonstrated that infection of PBMCs with H. pylori induces high levels of inflammatory mediators such as IL-12, IL-18, IFN-γ, TNF-α and MCP-1. Furthermore, we have shown that
the treatment of PBMCs with killed *H. pylori* subverted the environment of cytokine patterns responsible for the inflammatory process inducing IL-4 and IL-10.  

In the present study, we demonstrate that stimulation of PBMCs with live *H. pylori* induces a higher amount of RANTES compared to that induced with killed *H. pylori* treatment as well as killed + live *H. pylori*.  

In order to confirm if RANTES production was supported by a Th1 response, we neutralized IFN-γ and IL-12 by adding monoclonal antibodies (mAbs) to PBMCs stimulated with live *H. pylori* as well as with killed + live *H. pylori*. In addition, in another series of experiments we added human recombinant IFN-γ (hrIFN-γ) and human recombinant IL-12 (hrIL-12) to PBMCs treated with killed + live *H. pylori* as well as with killed + live *H. pylori*.

**MATERIALS AND METHODS**

**Isolation of PBMCs**

PBMCs were obtained from healthy, *H. pylori*-seronegative donors, after centrifugation of heparinized venous blood over Ficoll-Hypaque gradient (Pharmacia, Milan, Italy). PBMCs were then washed twice in RPMI 1640 medium and cultured in 24-well plates (Corning, Bibby srl, Milan, Italy) at a concentration of 2x10^6 cells/mL per well in RPMI 1640 medium. PBMCs were cultured at 37°C in 5% CO2 atmosphere in RPMI 1640 (Biochrom KG Seromed, Milan) supplemented with 50 mM 2-mercaptoethanol, 1 mM pyruvate, 1 mM non-essential amino acids, 1 mM HEPES and 5% fetal calf serum (FCS) (Biochrom KG Seromed, Milan, Italy).

**Limitus test**

Culture media and reagents tested for the presence of endotoxin by Limulus test (Sigma, Milan, Italy) were found to contain <10 pg of endotoxin per mL.

**Chemokine and cytokine evaluations**

Supernatants from PBMCs in different experimental conditions were collected and analyzed for the presence of RANTES by an immunoenzymatic method: human RANTES Quantikine immunoassay (R&D Systems, Milan, Italy). The limit of detection ranged from 1.74 - 6.63 pg/mL.

Monoclonal anti-human IL-12 and anti-human IFN-γ antibodies were obtained from R&D Systems; Milan, Italy. ND50 of anti-human IL-12 antibody was determined to be approximately 0.3 - 0.9 pg/mL in the presence of 1.0 ng/mL of rhl IL-12; ND50 of anti-human IFN-γ antibody was determined to be approximately 0.2 - 0.6 pg/mL in the presence of 5 ng/mL of rhl IFN-γ using the HeLa cell line. Both antibodies were used at 1 ng/mL.

Human recombinant IFN-γ (specific activity: 1 x 10^7 U/mg protein) was obtained from R&D Systems (Milan, Italy) and used at a concentration of 50 U/mL.

Human recombinant IL-12 (ED50 = 0.05 - 0.2 ng/mL) was obtained from R&D Systems (Milan, Italy) and used at a concentration of 1 ng/mL.

**Bacterial strain**

*H. pylori* isolated from the antral mucosa of a patient and associated duodenal ulcer was plated on Skirrow's agar and incubated at 37°C in a microaerophilic environment for 5 days, harvested and diluted in sterile phosphate-buffered saline (PBS) (pH 7.2). The *H. pylori* strain was cytotoxin-associated antigen (CagA) positive and vacuolating toxin (VacA) s1/m1 genotype.

The concentration of bacteria was estimated by measuring the absorbance of the suspension and comparing the value to a standard curve. The standard curve was generated by measuring the absorbance of an array of serially diluted samples before quantifying the number of viable bacteria in each sample by a colony assay. After centrifugation at 2,500 x g for 15 min, bacteria were resuspended in PBS to a range of concentrations from 10^3 to 10^9 CFU/mL. The motility of the organisms was confirmed by phase-contrast microscopy prior to use. For the experiments with killed bacteria, *H. pylori* was treated with gentamicin (4 mg/mL; Seromed, Milan) for 45 min at 4°C, washed, and diluted in PBS to the same concentrations as the live bacteria.

The combined treatment was performed by adding to PBMCs a suspension of gentamicin-killed bacteria (1.2 x 10^9 killed CFU/mL) for 20 h and after this period adding 1.2 x 10^9 CFU/mL of live *H. pylori* for a further 24 h. After 44 hours supernatants were harvested, centrifuged and stored at -80°C until cytokine assays.

**Cytotoxicity**

To determine the effect of different incubation times on cell viability, a colorimetric assay was used as described by Moreira et al. 13 The assay is based on the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Sigma, Milan, Italy, a pale yellow substrate that is cleaved by active mitochondria to produce a dark blue formazan product. Briefly, PBMCs were seeded in 96-microwell plates at 2x10^4 per well, then treated with live or killed *H. pylori* for 24 h or killed + live *H. pylori* for 44 h. MTT diluted in solute solution was added to the cells and incubated for 4 h, then acid propion 2 at 0.04 M HCl in propion 2 50 (Sigma, Milan, Italy) was used to solubilize the formed crystals. The plates were read with a microELISA reader using a wavelength of 570 nm. Cytotoxicity percentage was calculated as follows: 1 (experiment OD - lysis control OD) / (cell control OD - lysis control OD) x 100.
Statistical evaluation

Results are expressed as the means of five experiments ± standard deviation (SD). Data were analyzed by one way analysis of variance (ANOVA) and the Student-Newman-Keuls test. Differences were considered statistically significant for p value of <0.05.

RESULTS

In all the experiments, untreated PBMCs did not produce RANTES. The effects of different concentrations (10^3, 10^4, 10^5 CFU/ml per well) of killed or live H. pylori at 24 hours after treatment on RANTES release by PBMCs were analyzed. In particular, the treatment with different concentrations of live H. pylori (10^3, 10^4, 10^5 CFU/ml) induced a dose-dependent production of RANTES (658±78 pg/ml; 963±64 pg/ml; 1680±252 pg/ml; p<0.05 respectively). Moreover, the treatment of PBMCs with killed H. pylori at the same concentrations induced a significant difference in RANTES production (23±4 pg/ml; 62±12 pg/ml; 151±17 pg/ml respectively; p<0.05). In light of these results, all the experiments were performed using the concentration of 10^5 CFU/ml per well of both live and killed H. pylori.

A second series of experiments was carried out to determine how treatment with killed H. pylori and/or live H. pylori may differentially influence the in vitro kinetics of RANTES release by PBMCs. It was found that live H. pylori treatment triggers PBMCs to release higher amounts of RANTES when compared to those induced by killed H. pylori. The amounts of RANTES released at 12, 24 and 48 hours were respectively 780±82 pg/ml vs 98±25 pg/ml (p<0.05). 1680±111 pg/ml vs 151±17 pg/ml (p<0.05); 1120±93 pg/ml vs 104±28 pg/ml (p<0.05). Since both live and killed H. pylori induced the highest RANTES production at 24 h post treatment, only data referring to this incubation time are shown in Figures 1 and 2. The combined treatment (killed + live H. pylori) induced a marked decrease in RANTES production compared to live H. pylori (319±31 pg/ml vs 1680±111 pg/ml respectively; p<0.05) (Figure 1).

In addition, the amounts of RANTES after the combined treatment (killed + live H. pylori) were similar to those obtained with killed H. pylori treatment (Figure 2).

In order to verify whether these results were influenced by the incubation time and additional treatment, the cell viability was analyzed by the MTT test. It was found that the different incubation times (24 and 44 h) as well as the treatment with killed and live H. pylori did not significantly influence cell viability (data not shown).

Moreover, to verify a possible correlation between RANTES and IL-12 as well as IFNγ monoclonal anti-IFNγ and/or anti-IL-12 antibodies were added to PBMCs treated with live or killed + live H. pylori. As shown in Figure 1, the addition of monoclonal anti-IL-12 and anti-IFNγ antibodies to PBMCs treated with live H. pylori determined a down-regulation of RANTES production. In particular the addition of mAb-IL-12 determined a higher RANTES decrease compared to the addition of mAb-IFNγ (761±82 vs 1298±94 p<0.05). RANTES decrease was more evident when both monoclonal antibodies were added together to PBMCs treated with live H. pylori (391±25 vs 1680±111 p<0.005). These results demonstrate that the strong increase in RANTES expression in PBMCs treated with live H. pylori was, at least in part, dependent on the presence in supernatants of IL-12 and, to a lesser extent, on IFNγ.

![Figure 1 - Production of RANTES by PBMCs at 24 hours post treatment with live or killed + live H. pylori in presence or not of mAb vs IL-12 and or IFNγ.](image-url)
extent of IFN-γ. On the contrary, the addition of monoclonal anti-IL-12 and/or anti-IFN-γ antibodies to PBMCs treated with killed + live H. pylori did not determine any significant effect.

These data are in agreement with our previous studies that demonstrated that PBMCs in the presence of a combined treatment (killed + live) produced lower levels of inflammatory cytokines when compared to those treated with live H. pylori. In order to confirm this datum, we added, as shown in Figure 2, human recombinant-IL-12 or human recombinant-IFN-γ and both together to PBMCs treated with killed and killed + live H. pylori. The results demonstrate that the addition of hr-IL-12 triggers PBMCs, treated with killed or killed + live H. pylori, to release a higher amount of RANTES compared with that obtained with the addition of IFN-γ (670±36 pg/mL vs 380±28 pg/mL and 785±49 pg/mL vs 450±24 pg/mL respectively p<0.05). Furthermore, the addition of both human recombinant cytokines determined a synergistic effect in up-regulating the RANTES release (p<0.05).

These findings confirm that RANTES production is supported by IL-12 and, to a lesser extent, by IFN-γ.

**DISCUSSION**

H. pylori is the major causative agent of chronic superficial gastritis and plays a central role in the etiology of peptic ulcer disease. Evidence suggests that H. pylori infection pre-exists in gastric carcinoma and precancerous lesion and is a risk factor for the development of gastric carcinoma.

The recruitment and migration of immune cells toward inflammatory sites in a target tissue is a multi-step process that depends on the nature and state of activation of the inflammatory cells and is coordinated by receptors recognizing a mosaic of chemoattractant and pro-inflammatory cytokines. Innate immunity against pathogens is in part orchestrated by the ordered release of different chemokines that function as chemoattractants and activators of various immune cells, a property that enables immune cells to serve as the first line of cell-mediated host defense against infections.

In the present study, we showed for the first time that the low amount of RANTES in supernatants of PBMCs incubated with killed H. pylori is linked, at least in part, to the inhibition of IL-12 and IFN-γ release. Furthermore, treatment with killed + live H. pylori is also able to inhibit the induction of RANTES. The inflammatory response to H. pylori infection is a complicated product of cytokine networks. Our results confirm that the immunological disorders determined by H. pylori infection could be partially restored by treatment with killed H. pylori.

In conclusion, treatment with killed H. pylori may reduce some inflammatory responses caused by H. pylori and the associated risk of gastric cancer development. This approach could be a strategy for therapeutic manipulation directed towards equilibrating the inflammatory process determined by H. pylori infection.

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