MODULATION OF GRO-α AND TNF-α PRODUCTION BY PERIPHERAL BLOOD MONONUCLEAR CELLS TREATED WITH KILLED HELICOBACTER PYLORI

G. STASSI, A. CASCIO[1], C. IARIA[1], D. GAZZARA, G.B. COSTA, D. IANNELLO and A. ARENA

Unit of Microbiology, Department of Surgical Science, Policlinico Universitario; [1]Section of Infectious Diseases, Department of Human Pathology, Policlinico Universitario, Messina, Italy

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GRO-alpha seems to play an important role in recruiting and activating neutrophils during *Helicobacter pylori* infection. In the present study, we examined how treatment with killed *H. pylori* or/and live *H. pylori* may differentially influence the *in vitro* GRO-alpha and TNF-alpha release by peripheral blood mononuclear cells (PBMC). The amounts of TNF-alpha and GRO-alpha produced by PBMC after stimulation with live *H. pylori* were higher than those produced after stimulation with a combination of killed and live *H. pylori* and the latter were higher than those produced after stimulation with killed *H. pylori*. In conclusion, the treatment of peripheral blood mononuclear cells with killed *H. pylori* down-regulates the production of GRO-alpha. Taken together, our data demonstrate that treatment with killed *H. pylori* could represent an innovative approach during gastric infection supported by *H. pylori*.

H. 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 (1-3). 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 (4-5). Among these, GRO-α, a CXC chemokine, seems to play an important role in recruiting and activating neutrophils in the gastric mucosa. In addition, TNF- α has been demonstrated to be important for the up-regulation of GRO-α production and that at high concentrations, it can by itself injure the gastric mucosa and may be responsible for severe pathology (6-7). GRO-α possesses potent neutrophil-stimulating activity by inducing chemotaxis, shape change, a rise in intracellular free calcium levels, exocytosis, and the respiratory burst in these cells (8). GRO-α was initially isolated and characterized by its growth stimulatory activity on malignant melanoma cells (9). Moreover, this peptide can regulate endothelial cell proliferation, stimulating angiogenesis (10).

In a previous study, carried out in an *in vitro* model (11), we demonstrated that the immunological disorders determined by *H. pylori* infection could be related to a shift from a Th-2 to a Th-1 type cytokine profile. In light of these results, in the present study we analyze the direct effect of *H. pylori* (live or

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Mailing address: Prof. Giovanna Stassi,
Unit of Microbiology,
Policlinico Universitario G. Martino,
Via C.Valeria 1,
98125 Messina, Italy
Tel: ++39 0902213305 Fax: ++39 0902213302
e-mail: gstassi@unime.it

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gentamicin-killed) on human PBMC in order to evaluate the *in vitro* production of TNF-α and of GRO-α which seems to represent a key factor in the clinical outcome of inflammatory diseases and malignancies including gastric carcinoma.

MATERIALS AND METHODS

PBMC were obtained from healthy, *H. pylori*-seronegative donors, after centrifugation of heparinized venous blood over Ficoll-Hypaque gradient (Pharmacia, Milan, Italy) (12). PBMC were then washed twice in RPMI 1640 medium and cultured in 24 well plates (Corning, Bibby srl, Milan, Italy) at a concentration of $2x10^6$ (colony-forming unit) CFU/mL per well in RPMI 1640 medium. PBMC were cultured at 37°C in 5% CO_2 atmosphere, in RPMI 1640 (Biochrom KG Seromed, Milan, Italy) supplemented with 50 mM 2-mercaptoethanol, 1 mM pyruvate, 1 mM non-essential aminoacids, 1 mM HEPES and 5% fetal calf serum (FCS) (Biochrom KG Seromed, Milan, Italy). Culture media and reagents tested for the presence of endotoxin by E-Toxate kit (Sigma, Milan, Italy) were found to contain <10 pg of endotoxin per mL.

Supernatants from PBMC in different experimental conditions were collected and analysed for the presence of TNF-α and GRO-α by an immunoenzymatic method: human TNF-α Quantikine immunoassay and human GRO-α Quantikine immunoassay, (all from R&D Systems, Milan, Italy); the limit of detection was respectively 4.4 pg/mL for TNF-α and 10 pg/mL for GRO-α. Monoclonal anti-human TNF-α antiboby (ND_{50} for this lot of anti-human TNF-α antibody was 0.015-0.06 μg /mL in the presence of 0.25 ng/mL of human TNF-α) (R&D Systems, Milan, Italy). Monoclonal anti-human GRO-α antibody (ND_{50} for this lot of anti-human GRO-α antibody was 1 μg /mL in the presence of 6 ng/mL of rhGRO-α) (R&D Systems, Milan, Italy).

H. pylori isolated from the antral mucosa 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 concentration of bacteria was estimated by measuring the adsorbance of the suspension and comparing the value to a standard curve. The standard curve was generated by measuring the adsorbance 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 re-suspended in PBS to a range of concentrations from 10^5 to 10^9 CFU/mL, diluted in PBS. 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, Italy) for 45 min at 4°C, washed, and diluted in PBS to the same concentrations as the live bacteria (13).

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

To determine the effect of different incubation times on cell viability a colorimetric assay was used as described by Mosmann (14). The assay is based on the tetrazolium salt 3-(4,5 dimethylthiazol-2-yl)2,5diphenylt etrazoliumbromide (MTT), (Sigma, Milan, Italy) a pale yellow substrate that is cleaved by active mitochondria to produce a dark blue formazan product. Briefly, PBMC were seeded in 96-microwell plates at $2x10^4$ per well, then treated with live or killed *H. pylori* for 24 or killed + live *H. pylori* for 44 hours. MTT diluted in saline solution was added to the cells and incubated for 4 hours, then acid propan-2-ol (0.04M HCl in propan-2-ol) (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.

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

RESULTS

The effects of different concentrations of live *H. pylori* on cytokine release by PBMC are reported in Fig. 1. The results demonstrate that the production of GRO- α and TNF- α was dose-dependent. In particular, a significant differential stimulation was evident at a concentration of $1x10^9$ CFU/mL. In a second series of experiments, we examined how treatment with killed *H. pylori* or/and live *H. pylori* infection may differentially influence the *in vitro* cytokine release by PBMC. Results reported in Fig. 2 show that infection with live *H. pylori* triggers PBMC to release marked amounts of GRO-α compared with those induced by killed *H. pylori* (1972 ± 414 pg/mL vs 642 ± 121 pg/mL; $p<0.05$).

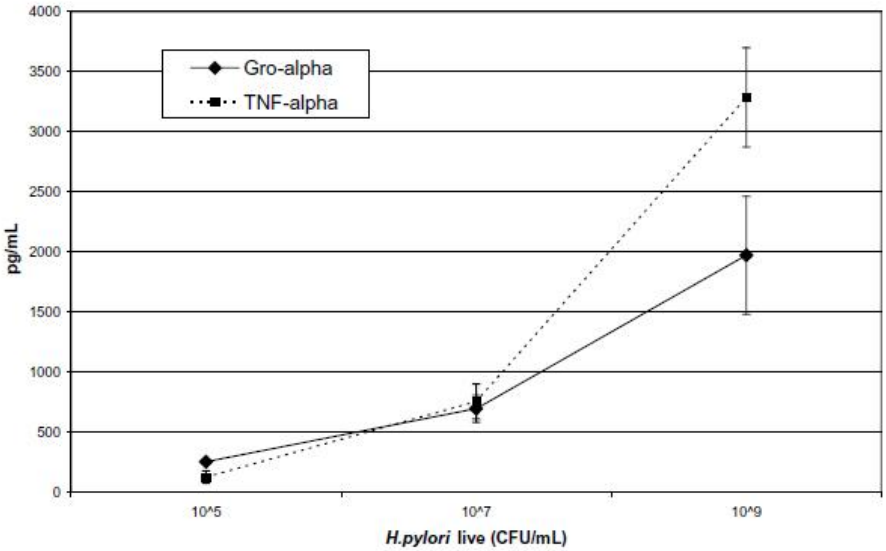

Fig. 1. *Effects of Helicobacter pylori concentration on GRO-alpha and TNF-alpha production by PBMC. Results represent the means of five experiments using PBMC of 5 different donors. Data are shown as mean ± standard deviation.*

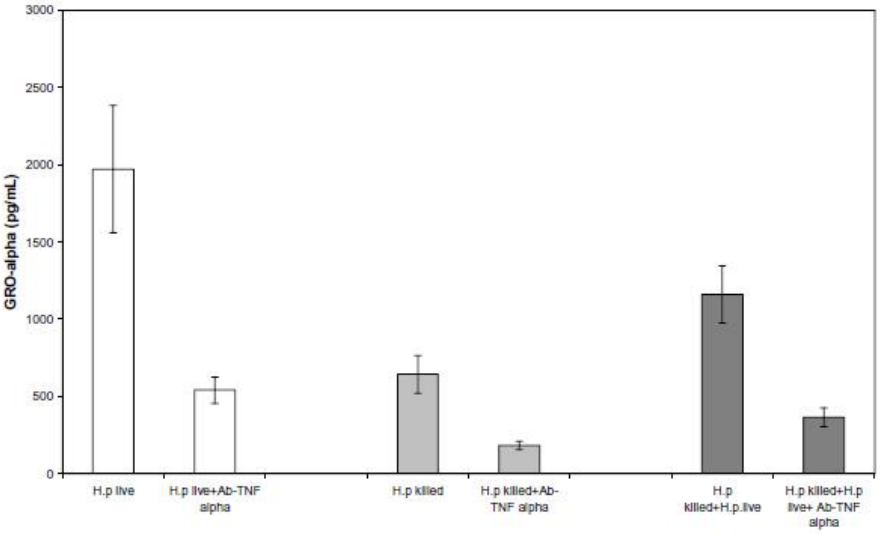

Fig. 2. *Production of GRO-alpha by PBMC after treatment with live, killed and killed+live Helicobacter pylori in presence or not of monoclonal antibodies vs TNF-alpha. Results represent the means of 5 experiments using PBMC of 5 different donors. Data are shown as mean ± standard deviation.*

The combined treatments induced a down-regulation of GRO- α production compared to live *H. pylori* (1161 ± 185 pg/mL vs 1972 ± 14 pg/mL; $p<0.05$). In order to verify whether these results were influenced by the incubation time and additional treatment, the cell viability was analyzed by MTT test. It was found that the different incubation times (24 and 44 hours) as well as the treatment with killed and live *H. pylori* did not significantly influence the cell viability (data not shown).

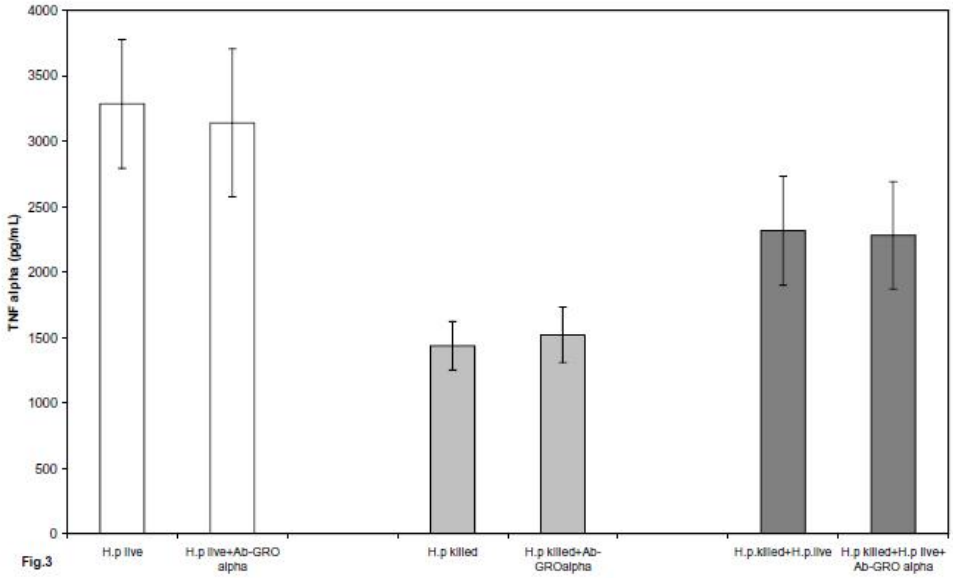

Fig. 3. *Production of TNF-alpha by PBMC after treatment with live, killed and killed+live Helicobacter pylori in presence or not of monoclonal antibodies vs GRO-alpha. Results represent the means of 5 experiments using PBMC of 5 different donors. Data are shown as mean ± standard deviation.*

Furthermore, as shown in Fig. 3, similar behaviour was seen for TNF–α. In particular, whereas live *H. pylori* infection induced marked levels of TNF- α compared with killed *H. pylori* (3285 ± 92 pg/mL vs 1436 ± 186 pg/mL; $p<0.05$), the combined effect (killed + live) resulted in higher levels of TNF- α compared to the treatment with killed *H. pylori* (2317 ± 417 pg/mL vs 1436 ± 186 pg/mL; $p<0.05$), but lower when compared with live *H. pylori* (2317 ± 417 pg/mL vs 3285 ± 492 pg/mL; $p<0.05$).

To verify a possible correlation between TNF- α and GRO- α we added monoclonal anti-TNF-α or anti-GRO-α antibodies to PBMC in all experimental conditions. As shown in Fig. 2 the addition of monoclonal Ab-TNF- α determined a down-regulation of GRO-α production. In particular, the addition of anti-TNF- α to PBMC respectively incubated with live, killed or killed+live *H. pylori*, decreased the levels of GRO- α produced by these cells (540 ± 86 pg pg/mL; respectively $p<0.05$). /mL vs 1972 ± 414; 180 ± 27 pg/mL vs 642 ± 121; 364± 61 vs 1161 ± 185.

These results demonstrate that the strong increase in GRO-α expression in PBMC infected with live *H. pylori* was, at least in part, dependent on the presence in supernatants of TNF- α. On the contrary, as shown in Fig. 3, the addition of monoclonal antibodies anti-GRO-α did not influence the TNF-α release. This finding demonstrates that TNF-α production is not supported by GRO-α. Furthermore, as shown in Fig. 2 and 3, the combined treatment with killed+live *H. pylori* produced the same effect, though to a lesser extent, on TNF-α and GRO-α release observed with killed *H. pylori*.

DISCUSSION

Few studies have investigated the expression of GRO-α and its role in the outcome of the *H. pylori* infection. Such knowledge is critical to fully understanding the relationships of *H. pylori* infection with gastric carcinoma.

Evidence for *in vivo* GRO-α induction in *H. pylori* infection has been reported by Suzuki *et al.*, who demonstrated increased mucosal levels of this chemokine in infected individuals, as well as decreased levels after eradication of infection (15). It is generally agreed that PBMC are important sources of chemokines and other proinflammatory mediators. In particular, it was shown that several chemokines are an essential component of the primary innate

immune response to *H. pylori* infection (4, 16-17).

In this paper we report our experimental data that further characterize the chemokine response induced in PBMC *in vitro* treated with live or killed *H. pylori*. Our results demonstrate that treatment with live *H. pylori* triggers PBMC to release marked amounts of GRO-α compared with those induced by killed *H. pylori* In light of the results obtained with GRO-α and in order to better understand its role during *H. pylori* infection, we made a pre-treatment of PBMC with killed *H. pylori* ,and after 20 hours we infected the same cells with live *H. pylori*. Our findings demonstrate that the combined treatments induced a down-regulation of GRO- α production compared to live *H. pylori*. Furthermore a similar behaviour was seen for TNF–α.

To verify a possible correlation between TNF-α and GRO-α we added monoclonal anti-TNF-α or anti-GRO-α antibodies to PBMC in all experimental conditions. The addition of monoclonal Ab-TNF-α determined a down-regulation of GRO-α production On the contrary, the addition of monoclonal antibodies anti-GRO-α did not influence the TNF-α release. These results demonstrate that the strong increase in GRO-α expression in PBMC infected with live *H. pylori* was, at least in part, dependent on the presence in supernatants of TNF-α. On this basis, we can speculate that TNF-α induced by *H. pylori* may exert a pathogenic effect not only by itself but also by supporting GRO-α production. Our results are in agreement with those of other Authors which demonstrate that TNF–α is important for the up-regulation of GRO-α production by stimulated human endothelial cells (18).

In previous studies, we reported that treatment of peripheral blood mononuclear cells with killed *H. pylori* promoted the release of anti-inflammatory cytokines such as IL-10 and IL-4, thus subverting immunological disorders accounted for by a shift from a Th-2 to a Th-1 type cytokine profile determined by live *H. pylori* infection (11, 19).

These data indicate that treatment with killed *H. pylori* may alter the balance of cytokines in the environment of cells, preventing potentially harmful effects of high levels of TNF-a and GRO-a representative of the deleterious Th1 immune response usually associated with *H. pylori* infection. In fact, these *in vitro* results demonstrate that pre-treatment with killed *H. pylori* plays a protective role during *H. pylori* infection decreasing inflammatory response supported by Th-1 cytokines and chemokines.

Taken together our results suggest that treatment of PBMC with killed *H. pylori* could subvert the environment of cytokine patterns responsible for the inflammatory process and marked recruitment of monocytes and lymphocytes in gastric mucosa during *in vivo H. pylori* infection. Therefore, it could be speculated that treatment with killed *H. pylori* could represent an innovative therapeutical approach during *H. pylori* gastric infection.

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REFERENCES

1. **Isaacson P.G.** 1994. Gastrointestinal lymphoma. *Hum. Pathol. 25: 1020.*
2. **Blaser M..J.** 1998. *Helicobacter pylori* and gastric diseases. *British. Med. J. 316:1507.*
3. **Wu C.Y., C.J. Wang, C.C. Tseng, H.P. Chen, M.S. Wu, J.T. Lin, H. Inoue and G.H. Chen.** 2005. *Helicobacter pylori* promote gastric cancer cells invasion through a NF-kappaB and COX-2-mediated pathway. *World J. Gastroenterol. 11:3197.*
4. **Eck M., B. Schmausser, K. Scheller, A. Toksoy, M. Kraus, T. Menzel, H.K. Muller-Hermelink and R. Gillitzer.** 2000. CXC chemokines Gro(alpha)/IL-8 and IP-10/MIG in *Helicobacter pylori* gastritis. *Clin. Ex. Immunol. 122:192.*
5. **Sieveking D., H.M. Mitchell and A.S. Day.** 2004. Gastric epithelial cell CXC chemokine secretion following *Helicobacter pylori* infection *in vitro*. *J. Gastroenterol. Hepatol. 19:982.*
6. **Gasperini S., F. Calzetti, M.P. Russo, M. De Gironcoli and M.A Cassatella.** 1995. Regulation of GRO alpha production in human granulocytes. *J. Inflamm. 45:143.*
7. **Persson-Dajotoy T., P. Andersson, A. Bjartell, J. Calafat and A. Egesten.** 2003. Expression and production of the CXC chemokine growth-related oncogene-alpha by human eosinophils. *J. Immunol.*

170:5309.

8. **Geiser T., B Dewald., M.U. Ehrengruber, I. Clark-Lewis and M. Baggiolini.** 1993. The interleukin-8-related chemotactic cytokines GRO-α, GRO-$_{β,}$ and GRO-γ activate human neutrophil and basophil leukocytes. *J. Biol. Chem. 268:15419.*
9. **Richmond A. and H.G. Thomas.** 1986. Purification of melanoma growth stimulatory activity. *J. Cell. Physiol. 129:375.*
10. **Strieter R.M., P.J. Polverini, S.L., Kunkel, S.D. Arenberg, M.D. Burdick, J. Kasper, J. Dzuiba, J. Van Damme, A. Walz and D. Marriott.** 1995. The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *J.Biol. Chem. 270:27348.*
11. **Stassi G., A. Arena, A. Speranza, D. Iannello and P. Mastroeni.** 2002. Different modulation by live or killed *Helicobacter pylori* on cytokine production from peripheral blood mononuclear cells. *New Microbiol. 25:247.*
12. **Boyum A.** 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest. 21:77.*
13. **Haeberle H.A., M. Kubin, K.B. Bamford, R. Garofano, D.J. Graham, F. El-zaatari, R. Karttunen, S.E. Crowe, V.E. Reyesand and P.B Ernst.** 1997. Differential stimulation of interleukin-12 and interleukin-10 by live and killed *Helicobacter pylori in vitro* and association of interleukin-12 production with gamma interferon- producing T cells in the human gastric mucosa. *Infect. Immun. 65:4229.*
14. **Mosmann T.** 1989. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods 65:55.*
15. **Suzuki H., M. Mori, A.A. Sakaguchi, S. Miura and H. Ishii.** 1998. Enhanced levels of C-X-C chemokine, human GROalpha, in *Helicobacter pylori*-associated gastric disease. *J. Gastroenterol. Hepatol. 13:516.*
16. **Kusugami K., T. Ando, M. Ohsuga, A. Imada, M. Shinoda, T. Konagaya, K. Ina, N. Kasuga, A. Fukatsu, S. Ichiyama, T. Nada and M. Ohta.** 1997. Mucosal chemokine activity in Helicobact Mucosal chemokine activity in *Helicobacter pylori* infection. *J. Clin. Gastroenterol. 25:203.*
17. **Yamaoka Y., M. Kita, T. Kodama, N. Sawai, T. Tanahashi, K. Kashima and J. Imanishi.** 1998. Chemokines in the gastric mucosa in *Helicobacter pylori* infection. *Gut 42:609.*
18. **Wen D.Z., A. Rowland and R. Derynck.** 1989. Expression and secretion of gro/MGSA by stimulated human endothelial cells. *EMBO J. 8:1761.*
19. **Stassi G., A. Arena, A. Speranza, D. Iannello, M. Miceli and P. Mastroeni.** 2003. Treatment of PBMC with killed *Helicobacter pylori* subverts the environment of inflammatory cytokines. *New Microbiol. 26:227.*