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Immunobiological Activities of *Helicobacter pylori* Porins

MARIA ANTONIETTA TUFANO,[1]* FABIO ROSSANO,[1] PIERGIORGIO CATALANOTTI,[1] GIORGIO LIGUORI,[2] CIRO CAPASSO,[1] MARIA TERESA CECCARELLI,[2] AND PAOLO MARINELLI[2]

Istituto di Microbiologia[1] and Istituto di Igiene,[2] Facoltà di Medicina e Chirurgia, Seconda Università di Napoli, Naples, Italy

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Studies were carried out on some biological activities of *Helicobacter pylori* porins in vitro. We extracted and purified a porin with an apparent molecular mass of 30 kDa. Human polymorphonuclear leukocytes preincubated with *H. pylori* porins showed a decrease of chemotaxis, of adherence to nylon wool, and of chemiluminescence. Used as chemotaxins in place of zymosan-activated serum or as chemotaxinogens in place of zymosan, the porins induced polymorphonuclear leukocyte migration. Human monocytes and lymphocytes cultivated in the presence of *H. pylori* porins released cytokines. Release of the various cytokines studied was obtained with differentiated kinetics and at various porin concentrations. Starting only 3 h after culture, tumor necrosis factor alpha is released quickly, reaching a peak at 18 h, at a porin concentration of 1 μg/ml/10^6 cells. Interleukin-6 (IL-6) appears later, with a peak at 10 μg/ml/10^6 cells, while IL-8 is released after 6 h of culture, with a peak at 24 h, at a porin concentration of 10 μg/ml/10^6 cells. Lymphocytes stimulated by *H. pylori* porins release gamma interferon after 18 h of culture at higher concentrations of porins (20 μg/ml/10^6 cells). Granulocyte macrophage colony-stimulating factor is released from 6 to 48 h at a concentration of 1 μg/ml/10^6 cells, while both IL-3 and IL-4 are released after 18 h of culture at different porin concentrations (0.1 and 1 μg/ml/10^6 cells, respectively). Our results lead us to think that during *H. pylori* infection, surface components, porins in particular, are able to induce a series of chain reactions ranging from the inflammatory to the immunological responses.

Helicobacter pylori is a microaerophilic bacterium implicated as a causal agent of gastritis and associated with duodenal ulcers in humans (4, 25, 32). The mechanisms by which *H. pylori* infection may cause gastritis are unknown but probably include cytotoxin production, degradation of physiologic defenses against acid-pepsin damage, adherence to epithelial cells, and urease activity (31).

As with all other gram-negative bacteria, the cell envelope of *H. pylori* contains lipopolysaccharide (LPS). Recently Muotiala et al. (26) tested *H. pylori* LPS for its ability to induce mitogenicity in mouse spleen cells, pyrogenicity in rabbits, and toxic lethality in galactosamine-sensitized mice. Compared with other enterobacterial LPS activity, mitogenicity and pyrogenicity of *H. pylori* LPS were 1,000-fold lower and lethal toxicity was 500-fold lower.

Our previous research projects dealing with the immunobiologic activity of porins from *Salmonella typhimurium* pointed out their role as determinants of pathogenicity (2, 5, 7, 11–13, 41, 45). Comparative studies have shown that under the same experimental conditions, porins are more active than LPS for some kinds of biologic activities.

The study of these surface molecules appears to be of interest, as is the role that they play in the gastrointestinal pathology of *H. pylori*. In fact, the direct action of intrinsic virulence factors and the release of mediators may both be responsible for pathogenic phenomena ranging from erosive and ulcerating actions at the mucosal level to ischemic necrosis and loss of vascular tone.

Among the mediators most involved in inflammation and tissue damage, tumor necrosis factor alpha (TNF-α) (8), interleukin-6 (IL-6) (15, 28), and IL-8 (20) are known to modulate the production of acute-phase proteins by hepatocytes, chemokinetics, chemotaxis, and the aggregation and release of lysosomal enzymes from neutrophils. The production of HCl by parietal cells of the gastric mucosa is modulated by mediators such as histamine. Some cytokines, such as IL-3 and granulocyte macrophage colony-stimulating factor (GM-CSF) (19, 21, 23, 37), favor the release of histamine by mast cells. On the other hand, IL-4 plays a fundamental role in promoting the production of immunoglobulin E.

In an attempt to verify the role played by other determinants of pathogenicity of *H. pylori*, we carried out a study on the interference of porins in the pathogenic mechanisms which permit *H. pylori* to induce gastric damage. To do so, we investigated the roles played by these proteins which allow the invasiveness of *H. pylori*, modify the functional activity of polymorphonuclear leukocytes (PMNs), and induce the release of some cytokines from human lymphocytes-monocytes.

MATERIALS AND METHODS

Bacteria and preparation of porins. *H. pylori* was isolated on *Campylobacter* agar (BBL, Cockeysville, Md.) at 37°C for up to 5 days in a microaerophilic atmosphere from gastric biopsies at Servizio di Virologia e Microbiologia, Facoltà di Medicina e Chirurgia, Seconda Università di Napoli, Naples, Italy, and identified by conventional methods and confirmed by Api-Campy (BioMérieux, Marcy l'Étoile, France); cultures were stored in milk and frozen. Stocks were recultivated on the same medium, and cultures were collected. The method described by Nurminen (30) and previously used by us to extract and purify *S. typhimurium* SH5014 porins was used. One gram of envelope was treated with Triton X-100 in 0.01 M Tris-HCl (pH 7.5) containing 10 mM EDTA; after addition of trypsin (10

* Corresponding author. Mailing address: Istituto di Microbiologia, Facoltà di Medicina e Chirurgia, Seconda Università di Napoli, Larghetto Sant'Aniello a Caponapoli, 2, I-80138 Naples, Italy. Phone: +39 +81 5665667. Fax: +39 +81 5665664.

mg/g of envelope), the pellet was dissolved in sodium dodecyl sulfate (SDS) buffer (4% [wt/vol] in 0.1 M sodium phosphate [pH 7.2]) and applied on an Ultragel ACA34 column equilibrated with 0.25% SDS buffer. The fraction enriched in protein, identified by A_{280}, was extensively dialyzed and checked by gel electrophoresis by the Laemmli method (22). Traces of LPS in our preparation, identified by means of gel electrophoresis and staining with silver nitrate, as described by Tsai and Frash (40) and by means of the *Limulus* amoebocyte lysate assay (*Limulus* test; BioWhittaker, Walkersville, Md.) (39), were neutralized by polymyxin B (PB; Sigma, St. Louis, Mo.), incubating porins (20 μg/ml) with PB (5 μg/ml) at room temperature for 1 h as described previously (13, 43), as specified by Blanchard et al. (3) and Rifkind and Palmer (33). A negative *Limulus* test was obtained when porins plus PB were used as a control. The bioactivity of the porin preparation was tested by measuring the release of ^{51}Cr from human PMNs purified as described below (42). Briefly, 2.5×10^6 PMNs were incubated for 1 h at 37°C with 250 μCi of $Na^{51}CrO_4$ (specific activity, 300 to 500 mCi of Cr per g; New England Nuclear), washed three times with Eagle's modified Dulbecco medium (Biochrom KG, Berlin, Germany), and resuspended with 1 ml of the same medium. After incubation, in the absence or in the presence of higher concentrations (50 μg/ml) of porins than the noncytotoxic ones used in our tests, the cells were centrifuged and the amount of ^{51}Cr released into the supernatant was calculated. The radioactivity in the supernatant, plus the value for a sample extracted from the cell pellet treated with 5% Triton X-100, was assumed to represent 100% radioactivity (6). This value was corrected for any spontaneous release of radioactivity (5%). After a 1-h incubation with $Na^{51}CrO_4$, the porin preparation used in this study induced 43 to 45% ^{51}Cr release.

Preparation of human leukocyte populations. To exclude possible variance, leukocyte populations were obtained for each test series from a buffy coat of a blood sample from a consenting healthy volunteer through the courtesy of the Centro Trasfusionale, Facoltà di Medicina e Chirurgia, Seconda Università di Napoli. Buffy coat, diluted threefold with phosphate-buffered saline (PBS), was applied on Ficoll-Hypaque gradient (Monopoly Resolving Medium; Flow Laboratories, Irvine, Scotland) and centrifuged for 30 min at 300 × *g* at 4°C. Mononuclear cells and granulocytes were then isolated by centrifugation on Ficoll-Hypaque gradient, density of 1.077 (Biochrom), for 30 min at 300 × *g* at 4°C. Finally, mononuclear cells washed twice with PBS were resuspended in RPMI 1640 (Biochrom) containing 10% heat-inactivated fetal calf serum (FCS) and were incubated in six-well plastic trays (Falcon; Becton Dickinson Labware, Franklin Lakes, N.J.) at 2×10^6 to 2.5×10^6 cells per well for 2 h at 37°C in 5% CO_2–95% air. Nonadherent cells were removed, washed, and incubated in different wells (0.8×10^6 per well).

PMNs, obtained from the pellet of the Ficoll gradient, were washed three times with PBS and resuspended in RPMI 1640 with 10% inactivated FCS added. The cell viability of each of the three cell populations was evaluated by the trypan blue exclusion test. At least 96% of the adherent mononuclear cells were monocytes, as determined by adherence, staining with α-naphthylbutyrate (nonspecific esterase), Pappenheim staining (morphology), and uptake of colloidal carbon (9); at least 93% ± 4% of the nonadherent mononuclear cells and 91% of the polynuclear leukocytes were, respectively, lymphocytes, as shown by flow cytofluorometry analysis (FACS IV; Becton Dickinson) of stained cells, and neutrophil granulocytes, as determined by May-Grünwald-Giemsa panoptic staining.

Evaluation of PMN chemotaxis. A modification (43) of the under-agarose assay of Nelson et al. (27) was used to test the chemotaxis of untreated and treated PMNs. In this procedure, *H. pylori* porins at 1, 5, and 10 μg/ml/10^6 PMNs, nontoxic concentrations for PMNs, as checked by the trypan blue exclusion test, were assayed for 30 min at 37°C. Then 3×10^5 PMNs suspended in RPMI 1640 with 10% inactivated FCS were placed in the center of three equally spaced wells cut into the agarose plate. An equivalent amount (10 μl) of zymosan-activated homologous serum was placed in the outer well, and the inside well received RPMI 1640. Stimulus was obtained by incubating 200 μl of a zymosan suspension in PBS with 1.8 ml of pooled homologous normal serum (final concentration, 5 mg/ml) for 30 min at 37°C; the supernatant was used as the chemoattractant. In some tests, porins or *S. typhimurium* LPS (Difco Laboratories, Detroit, Mich.) (final concentration, 10 μg/ml) were used as chemotaxinogens in place of zymosan, and in other tests porins were used alone as chemoattractants instead of classic stimulus. As a control, we also used porin- or LPS-activated serum or LPS plus PB (5 μg/ml). The agarose plates were incubated for 2 h at 37°C in 5% CO_2–95% air and fixed with methanol and 3.7% formaldehyde, the agarose was removed, and the cells were stained by the May-Grünwald-Giemsa method. Finally, the migrated cells were counted by using an eyepiece grid under ×40 magnification, and the migration index was calculated as $[(A - B) \times (C - D)]/100$, where A is the number of PMNs migrated toward the chemoattractant site, B is the number of random-migrated PMNs, C is the maximum distance of chemotactic migration, and D is the distance of random migration.

Evaluation of PMN adherence to nylon wool. PMN adherence was measured by using the MacGregor technique as modified by Krause et al. (16, 43). Briefly, 40 mg of nylon wool was packed to a height of 1.5 cm in a tuberculin syringe. Then 0.5 ml of untreated and treated PMN suspension in RPMI 1640 (5×10^6 PMNs per ml) was allowed to percolate through the nylon column. The number of PMNs in the postelution samples was determined by microscopic counts; the results were expressed as percentage of neutrophil adherence to nylon.

Evaluation of the metabolic burst of PMNs activated by a phagocytic stimulus (zymosan). The respiratory burst of stimulated PMNs was evaluated with a luminol-enhanced chemiluminescence assay (34). Briefly, the chemiluminescence mixture consisted of 400 μl of PBS, 200 μl of a 10^6/ml untreated and treated PMN suspension in RPMI 1640, 50 μl of a 10^{-4} M/ml luminol solution (Sigma), and 200 μl of zymosan-activated pooled homologous normal serum (as described for chemotaxis, using the suspension) at half concentration (final concentration, 2.5 mg/ml). Chemiluminescence measurements were made every 5 min over a 45-min period, under constant agitation at 37°C between measurements, by a microprocessor-controlled Beckman LS 7500 scintillation counter. Chemiluminescence was expressed as peak activity (10^3 cpm/2×10^5 cells) rather than as an integral value, since results are entirely comparable when calculated by either method.

Kinetics of cytokine release by human leukocytes. Human monocytes and lymphocytes obtained as described above were further cultivated, at 37°C in 5% CO_2–95% air, in RPMI 1640 containing 10% inactivated FCS and glutamine.

H. pylori porins were added to the culture at concentrations indicated in single assays in a volume of 10 μl. The tests always included a standard challenge with *S. typhimurium* LPS at 10 μg/ml/10^6 cells for monocytes, while concanavalin A (ConA) at 10 μg/ml/10^6 cells was used for lymphocytes. In some tests, as a control, porins and LPS were preincubated with PB (5 μg/ml). After 3, 6, 18, 24, and 48 h, the cell viability was

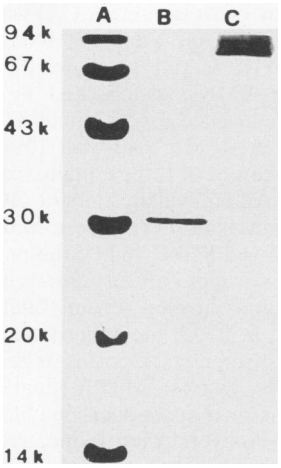

FIG. 1. Protein patterns of purified *H. pylori* porins (10 μg of protein). SDS-polyacrylamide (12%) gel stained with Coomassie blue. The molecular mass markers, given in kilodaltons, migrated in lane A. Lane B, sample heated for 5 min at 100°C before being applied to the gel; lane C, sample sonicated and unheated.

checked, and the culture supernatants were collected and stored at −20°C until they were assayed for cytokine activity. IL-3, IL-4, IL-6, IL-8, gamma interferon (IFN-γ), TNF-α, and GM-CSF assays were carried out with monoclonal antibodies by an immunoenzymatic method (Genzyme Corp., Cambridge, Mass.).

Statistics. The experiments were carried out in triplicate in three independent series, and the results are expressed as the mean ± standard deviation (SD). Comparisons between tests were done by Student's *t* test, with statistical significance considered to be $P < 0.05$.

RESULTS

Purity of porin preparation. The protein (10 μg) profile of our preparation was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 1). SDS-PAGE showed one band with molecular mass of 30 kDa in a sample heated for 5 min at 100°C in SDS and porin trimers in a sample sonicated and unheated in SDS. The majority of bacterial porins formed trimers resistant to denaturation in SDS buffer below 60°C (17, 24), trypsin resistant (14, 18), and peptidoglycan associated. This protein appeared to be porin-like. Gel filtration in the presence of detergent (SDS) eliminated LPS from porin preparations (1, 35). The controls carried out on SDS-PAGE and subsequent silver nitrate staining showed that the first fraction eluted did not contain any LPS. The detection limit of the gel system was 1 ng, using pure LPS of known concentration. The *Limulus* test (sensitivity, 0.125 endotoxin unit/ml, corresponding to 10 pg of LPS per ml) showed the presence of LPS at 10 pg/10 μg of porins. As described previously (13, 43), we used porins plus PB to neutralize the biological activity of LPS. A negative *Limulus* test was obtained when porins (20 μg/ml) plus PB were used as controls. SDS present in traces in a final preparation after dialysis (0.01 μg/20 μg of porins per ml) (43) was used as a control. No modification was noted in our experimental tests (data not shown). Our experiments also called for porin trimers treated with SDS at 100°C, with subsequent inactivation of the trimers to monomers and loss of biological activity (data not shown).

TABLE 1. Effects of *H. pylori* on human granulocyte chemotaxis

PMNs	Chemoattractant(s)	Migration index[a]
Untreated	Zymosan-activated serum[b]	0.789 ± 0.035
	Porin-activated serum[b]	0.621 ± 0.021
	Porin-activated serum + PB[c]	0.598 ± 0.019
	Porins, 1 μg/ml	0.185 ± 0.018
	Porins, 5 μg/ml	0.374 ± 0.025
	Porins, 10 μg/ml	0.605 ± 0.028
	Porins, 20 μg/ml	0.474 ± 0.025
	LPS-activated serum[b]	0.398 ± 0.022
	LPS-activated serum + PB[c]	0.038 ± 0.002
	LPS 5 μg/ml	0.152 ± 0.016
	Porins (20 μg/ml) + PB[c]	0.467 ± 0.026
	LPS (5 μg/ml) + PB[c]	0.000
	PB (5 μg/ml)	0.000
Treated with porins at[d]:		
1 μg/ml/10^6 cells	Zymosan-activated serum	0.121 ± 0.010
5 μg/ml/10^6 cells	Zymosan-activated serum	0.035 ± 0.002
10 μg/ml/10^6 cells	Zymosan-activated serum	0.081 ± 0.0087

[a] Mean of three independent experiments carried out in triplicate ± SD ($P < 0.05$).
[b] For details, see Material and Methods.
[c] Porins and LPS were incubated with PB (5 μg/ml) for 1 h at room temperature.
[d] PMNs were incubated with porins (1, 5, and 10 μg/ml/10^6 cells) for 30 min at 37°C and then washed three times prior to the assay. These concentrations proved to be not toxic in preliminary trypan blue exclusion tests.

***H. pylori* porins affect human PMN chemotaxis.** The effects of porin-induced chemotaxis are reported in Table 1. With porin (10 μg/ml)-activated serum, the PMN migration index was very close to the one obtained by classic chemotactic stimulus (zymosan-activated serum). Porins also acted as chemotaxins at concentrations of between 1 and 20 μg/ml: the PMN migration index was higher than when LPS was the chemoattractant. Polymyxin B neutralized the effect of LPS chemotaxis but not that of porin chemotaxis. Human PMNs treated for 30 min at 37°C with 1, 5, and 10 μg of *H. pylori* porins per ml showed a significant decrease ($P < 0.05$) of the chemotaxis induced by the classic chemotactic stimulus with respect to the untreated PMNs. The maximum decrease occurred at a porin concentration of 5 μg/ml/10^6 PMNs.

***H. pylori* porins decrease human PMN adherence to nylon wool.** The treatment of human PMNs with porins at a concentration of 5 μg/ml/10^6 cells for 30 min at 37°C induced a decrease of adherence to the nylon wool of about 13% with respect to the untreated cells. Treatment with higher or lower concentrations of porins did not modify the adherence of human PMNs to the nylon wool (Fig. 2).

***H. pylori* porins decrease human PMN chemiluminescence.** Human PMNs treated for 30 min at 37°C with 1, 5, and 10 μg of *H. pylori* porins per ml showed a significant decrease ($P < 0.05$) of the metabolic burst with respect to the untreated cells. The effect is dose dependent, with a peak at a porin concentration of 5 μg/ml/10^6 cells; Fig. 3 shows data for the chemiluminescence at 20 min, when the peak is reached.

Human monocytes stimulated with *H. pylori* porins release TNF-α, IL-6, and IL-8. The TNF-α production test was performed by stimulating human monocytes (0.5×10^6 to 0.8×10^6) with porins at concentrations ranging from 0.01 to 20 μg/ml/10^6 PMNs for 3, 6, 18, 24, and 48 h. TNF-α release was obtained 3 h after stimulus and increased progressively until 18 h, when it started to decrease; it was dose dependent, with the peak seen at the concentration of 1 μg/ml/10^6 cells. The

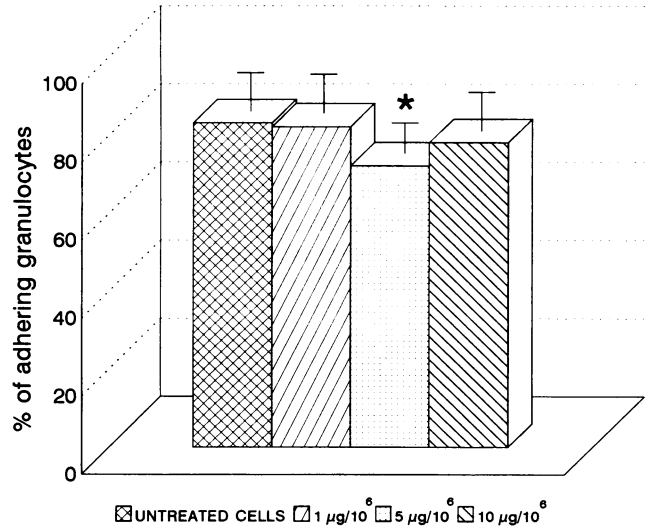

FIG. 2. Adherence to nylon wool of human PMNs treated with *H. pylori* porins. Each value represents the mean of three experiments carried out in triplicate. The bars indicate SD. *, $P < 0.05$. PMNs were treated at 37°C for 30 min with porins (1, 5, and 10 μg/ml/10^6 cells). See Materials and Methods for further details.

maximum release, at 18 h, was 43-fold over that of the untreated cells but represented 67.9% of the quantity produced by monocytes stimulated with LPS (10 μg/10^6 cells) (Table 2).

In tests for IL-6 and IL-8 production, the stimulus was limited to 0.1 to 20 μg/ml/10^6 cells. As shown in Table 2, IL-6 release appeared later (18 h) and was obtained at higher concentrations of porins (10 μg/ml/10^6 cells). At this time, the release was about 26-fold over that of the untreated cells and was found to be 57.7% of that produced by LPS-stimulated

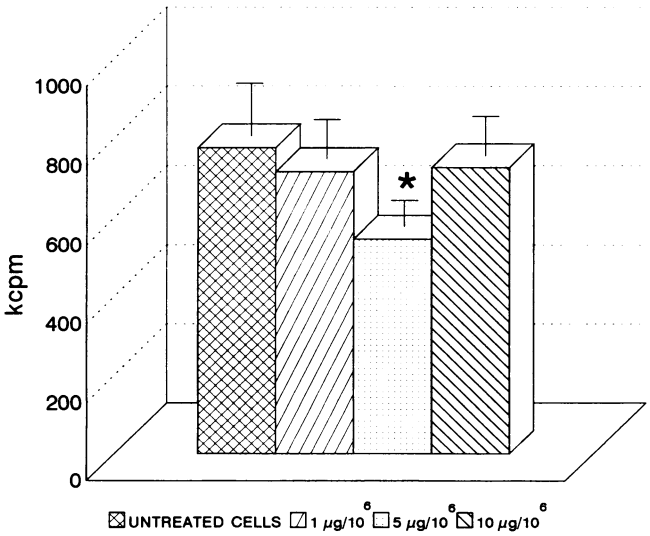

FIG. 3. Chemiluminescence of human PMNs treated with *H. pylori* porins. Each value represents the mean of three experiments carried out in triplicate. The bars indicate SD. *, $P < 0.05$. PMNs were treated at 37°C for 30 min with porins (1, 5, and 10 μg/ml/10^6 cells). Data reported refer to the chemiluminescence at 20 min, when the peak is reached. See Materials and Methods for further details.

monocytes. The amount of IL-6 produced by porin-stimulated monocytes decreased thereafter.

IL-8 release occurred starting after 6 h, at a concentration of 10 μg/ml/10^6 cells; after 18 h, the release was about sixfold over that of the untreated cells and even higher than that produced by LPS-stimulated monocytes (Table 2). After 24 h, the IL-8 release diminished progressively.

In all monokine assays, the preincubation of porins with PB (5 μg/ml) did not nullify the effects of porin stimulation on cytokine release. Preincubation of LPS with PB inhibited LPS-induced cytokine release (data not shown).

Human lymphocytes stimulated with *H. pylori* porins release IFN-γ, GM-CSF, IL-3, and IL-4. Lymphokine production tests were performed by stimulating human lymphocytes (0.8×10^6) with porins at various concentrations for 3, 6, 18, 24, and 48 h. In the test for IFN-γ production, the stimulus concentrations ranged from 0.1 to 20 μg/ml/10^6 cells. IFN-γ was present and was found to be dose dependent. Release was greatest after 18 h, and the porins, at a concentration of 20 μg/ml/10^6 cells, induced IFN-γ release eightfold over that of the untreated cells but twofold less than that by ConA-stimulated lymphocytes (10 μg/ml/10^6 lymphocytes) (Table 3).

GM-CSF release by lymphocytes was investigated by using porins at concentrations ranging from 0.1 to 20 μg/ml/10^6 cells. In the stimulated lymphocyte supernatants, GM-CSF was present and was found to be dose dependent. Release was evident starting from 6 h, and maximum production occurred at 1 μg/ml/10^6 cells. Until 48 h, no significant variation was seen in the production of this lymphokine; the maximum amount of GM-CSF was found to be about fivefold over that of the untreated cells and about 28.9% of the quantity produced following stimulation of lymphocytes with ConA (Table 3).

In Table 4 are shown data on the kinetics for 48 h of IL-3 release by human lymphocytes stimulated with porins at concentrations ranging from 0.01 to 20 μg/ml/10^6 cells. IL-3 production occurred after 18 h, with a peak at 0.1 μg/ml/10^6 cells; at this time the amount of cytokine produced was 29-fold over that of the untreated lymphocytes and about 67.4% of that produced by ConA stimulation. The amount of IL-3 produced by porin-stimulated lymphocytes diminished thereafter.

Tests for the kinetics of IL-4 release were carried out by using as the stimulus porins at concentrations ranging from 0.1 to 20 μg/ml/10^6 cells. This lymphokine too reached maximum release at 18 h, with a peak at a porin concentration of 1 μg/ml/10^6 cells. At this time and this porin concentration, cytokine production was 15-fold over that of the untreated cells and a little more than half of that produced by stimulating lymphocytes with ConA (Table 4).

In all lymphokine assays, the preincubation of porins with PB (5 μg/ml) did not nullify the effects of porin stimulation on cytokine release (data not shown).

DISCUSSION

Epithelial lesions with irregularities of the luminal profile and vacuolar lesions often, in the absence of inflammatory intraepithelial cells, appear to be a consequence of a direct histolytic action of *H. pylori*. More serious epithelial lesions of the micropapillar and erosive types normally coexist with a granulocytic intraepithelial infiltration, suggesting that, in addition to the cytotoxic action directed by *H. pylori*, the proteolytic activity of granulocytic origin can play a role in pathogenesis (47).

H. pylori porins are important components of the inflammatory response. Our experiments have shown that these proteins

TABLE 2. Kinetics of cytokines (TNF-α, IL-6, and IL-8) by human monocytes treated with *H. pylori* porins

Time (h)	Release[a]								
	In untreated cells	At porin concn (μg/ml/10^6 cells) of:							With LPS (10 μg/10^6 cells)
		0.01	0.05	0.1	1	5	10	20	
					TNF-α (pg/ml)				
3	1 ± 0.05	1 ± 0.04	3 ± 0.10	648 ± 31[b]	1,043 ± 51[b]	162 ± 8[b]	101 ± 5[b]	76 ± 3[b]	1,184 ± 58
6	9 ± 0.41	8 ± 0.42	13 ± 0.61	985 ± 45[b]	1,435 ± 69[b]	316 ± 13[b]	185 ± 9[b]	96 ± 4[b]	2,202 ± 101
18	44 ± 2.13	47 ± 2.16	48 ± 2.31	1,317 ± 63[b]	1,897 ± 81[b]	432 ± 22[b]	201 ± 10[b]	193 ± 8[b]	2,794 ± 118
24	41 ± 2.42	40 ± 1.93	47 ± 2.52	1,231 ± 67[b]	1,688 ± 86[b]	333 ± 15[b]	190 ± 9[b]	145 ± 6[b]	2,537 ± 107
48	30 ± 1.15	33 ± 1.21	34 ± 1.21	1,115 ± 58[b]	1,563 ± 77[b]	215 ± 11[b]	135 ± 7[b]	101 ± 5[b]	2,101 ± 99
					IL-6 (pg/ml)				
3	1 ± 0.08	ND	ND	33 ± 1	35 ± 1	45 ± 2	48 ± 2	47 ± 2	714 ± 31
6	9 ± 0.9	ND	ND	38 ± 1	38 ± 1	47 ± 3	50 ± 2	49 ± 2	954 ± 41
18	43 ± 4.1	ND	ND	239 ± 11[b]	536 ± 12*	726 ± 31[b]	1,125 ± 63[b]	994 ± 48[b]	1,954 ± 87
24	41 ± 4	ND	ND	208 ± 9[b]	498 ± 22*	589 ± 23[b]	854 ± 39[b]	761 ± 34[b]	1,704 ± 74
48	31 ± 3.8	ND	ND	192 ± 7[b]	332 ± 12*	451 ± 21[b]	716 ± 31[b]	612 ± 28[b]	1,598 ± 64
					IL-8 (ng/ml)				
3	0.6 ± 0.03	ND	ND	0.5 ± 0.01	0.5 ± 0.02	0.9 ± 0.04	1.2 ± 0.03	0.9 ± 0.03	1.1 ± 0.05
6	2.1 ± 0.18	ND	ND	1.8 ± 0.04	2.3 ± 0.11	2.7 ± 0.11	4.2 ± 0.18[b]	2.2 ± 0.11	2.3 ± 0.12
18	2.7 ± 0.21	ND	ND	2.7 ± 0.12	3.1 ± 0.14	11.3 ± 0.43[b]	17.3 ± 0.62[b]	13.5 ± 0.61[b]	14.3 ± 0.68
24	2.9 ± 0.21	ND	ND	2.8 ± 0.12	3.2 ± 0.13	10.7 ± 0.39[b]	16.4 ± 0.59[b]	12.5 ± 0.59[b]	13.4 ± 0.61
48	3.1 ± 0.25	ND	ND	1.9 ± 0.08	2.9 ± 0.12	5.9 ± 0.24	11.9 ± 0.44[b]	9.4 ± 0.48[b]	10.7 ± 0.45

[a] Mean of three independent experiments carried out in triplicate ± SD. ND, not determined.
[b] $P < 0.05$, porin-stimulated versus untreated cells.

modulate the main functions of the granulocytes, acting on the chemokinetics and chemotaxis, and could in vivo promote the recruitment of these inflammatory cells from the circulation and their accumulation in the tissues. The inhibition of migration observed when PMNs are preincubated with porins and then subjected to the classical chemoattractants (serum plus zymosan) can most likely be attributed to the modification of the physicochemical characteristics of the membranes with overall reduction in the receptor expression, as previously demonstrated with *S. typhimurium* porins (43). *H. pylori* porins, at a concentration of 5 μg/10^6 cells, reduce the chemiluminescence of PMNs, suggesting that in vivo, porins can interfere with intracellular killing of *H. pylori*, favoring invasiveness.

The porins of this bacterium also act through the release of mediators in the inflammatory process. The elevated production of cytokines with inflammatory activities, such as TNF-α, IL-6, and IL-8, could account for the notable presence of PMNs and of the histolytic action of *H. pylori*, even higher when accompanied by granulocytic infiltrations inside the epithelium. Even if polynuclear cell elements seem to be predominant within the histologic framework of *H. pylori* infection, other cell elements, such as T lymphocytes and plasmacytes, are present in considerable numbers (47). Our experimental data indicate that *H. pylori* porins are able in vitro to induce synthesis both of IL-6, a factor of proliferation from B cells, and of cytokines released by T lymphocytes. Among the latter is GM-CSF, whose release in our tests is fairly quick, starting after only 6 h of culture of the lympho-

TABLE 3. Kinetics of cytokines (IFN-γ and GM-CSF) by human lymphocytes treated with *H. pylori* porins

Time (h)	Release (pg/ml)[a]						
	In untreated cells	At porin concn (μg/ml/10^6 cells) of:					With ConA (10 μg/10^6 cells)
		0.1	1	5	10	20	
					IFN-γ		
3	1 ± 0.04	1 ± 0.04	2 ± 0.05	2 ± 0.06	8 ± 0.44[b]	2 ± 0.05	20 ± 0.83
6	2 ± 0.09	1 ± 0.03	2 ± 0.05	6 ± 0.10[b]	13 ± 0.51[b]	11 ± 0.43[b]	37 ± 1.15
18	11 ± 0.44	10 ± 0.41	14 ± 0.67	17 ± 0.65[b]	62 ± 2.65[b]	88 ± 2.98[b]	159 ± 5.98
24	10 ± 0.42	8 ± 0.32	10 ± 0.43	16 ± 0.59[b]	58 ± 2.14[b]	72 ± 2.71[b]	173 ± 6.67
48	9 ± 0.38	7 ± 0.29	9 ± 0.31	14 ± 0.51[b]	43 ± 1.89[b]	61 ± 1.58[b]	171 ± 6.24
					GM-CSFC		
3	0.80 ± 0.05	1.03 ± 0.04	1.21 ± 0.04	1.25 ± 0.04	0.86 ± 0.03	0.78 ± 0.02	9.06 ± 0.37
6	2.31 ± 0.14	2.67 ± 0.11	9.82 ± 0.36[b]	6.79 ± 0.19[b]	4.18 ± 0.18[b]	3.21 ± 0.11	33.49 ± 2.81
18	2.11 ± 0.14	2.54 ± 0.09	10.36 ± 0.41[b]	8.21 ± 0.28[b]	5.08 ± 0.19[b]	4.02 ± 0.15[b]	35.86 ± 3.01
24	1.91 ± 0.09	2.01 ± 0.07	9.71 ± 0.31[b]	7.89 ± 0.20[b]	3.86 ± 0.15[b]	3.61 ± 0.11[b]	31.54 ± 2.98
48	1.68 ± 0.06	1.28 ± 0.05	7.83 ± 0.21[b]	6.48 ± 0.16[b]	2.43 ± 0.11	1.76 ± 0.06	29.34 ± 2.87

[a] Mean of three independent experiments carried out in triplicate ± SD.
[b] $P < 0.05$, porin-stimulated versus untreated cells.

TABLE 4. Kinetics of cytokines (IL-3 and IL-4) by human lymphocytes treated with *H. pylori* porins

Time (h)	Release[a]								
	In untreated cells	At porin concn (μg/ml/10^6 cells of:							With ConA (10 μg/10^6 cells)
		0.01	0.05	0.1	1	5	10	20	
					IL-3(pg/ml)				
3	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
18	2 ± 0.01	4 ± 0.02	6 ± 0.02[b]	58 ± 2.13[b]	5 ± 0.23[b]	3 ± 0.01	3 ± 0.02	1 ± 0.01	86 ± 3.58
24	3 ± 0.01	4 ± 0.02	5 ± 0.02	49 ± 1.89[b]	3 ± 0.18	2 ± 0.01	3 ± 0.01	2 ± 0.01	82 ± 3.11
48	3 ± 0.01	4 ± 0.02	4 ± 0.02	38 ± 1.65[b]	1 ± 0.02	1 ± 0.01	1 ± 0.01	1 ± 0.01	78 ± 2.89
					IL-4(ng/ml)				
3	0	ND	ND	0.02	0.01	0.01	0.01	0	0
6	0	ND	ND	0.02	0.02	0.03	0.02	0	0
18	0	ND	ND	0.03	0.15 ± 0.01[b]	0.09 ± 0.01[b]	0.05 ± 0.01	0.02	0.23 ± 0.02
24	0	ND	ND	0.03	0.14 ± 0.02[b]	0.09 ± 0.01[b]	0.05 ± 0.01	0.02	0.20 ± 0.01
48	0	ND	ND	0.03	0.12 ± 0.02[b]	0.07 ± 0.01[b]	0.06 ± 0.01	0.01	0.19 ± 0.01

[a] Mean of three independent experiments carried out in triplicate ± SD. ND, not determined.
[b] $P < 0.05$, porin-stimulated versus untreated cells.

cytes in the presence of porins. IL-3 and IL-4 as well peaked at 18 h of cultivation. Among the various mediators released by lymphocytes, IL-3 and IL-4 are of particular interest in *H. pylori* infection. Histamine is one of the fundamental mediators which modulate HCl production, capable of penetrating the damaged gastro-duodenal mucosa (46). The mast cells, present at the mucosal level, can produce and release large quantities of histamine if they are properly stimulated. Some cytokines, such as IL-3 and GM-CSF, can promote the release of histamine by mast cells (19, 21, 23, 37).

Of course, caution must be taken in attempts to attribute any pathophysiological significance to in vitro phenomena; however, with reference to our experimental results, we can speculate that porins contribute to *H. pylori* virulence through their capacity to interfere with the functions of PMNs directly and induce the inflammatory process through the production of mediators. TNF-α is the most precocious of the ones tested, being released after only 3 h of incubation; the effects on the vascular endothelium could be the cause of ischemic necrosis and the loss of vasal tone. GM-CSF production, seen in vitro 6 h after stimulus, increases the inflammatory process and the infiltration of granulocytes, whose metabolic burst is reduced, favoring the invasiveness of *H. pylori*. IL-6 is released even later (after 18 h) and increases the inflammation. IL-3 release appears later also, and *in vivo* it stimulates histamine release, inducing a greater secretion of HCl by parietal cells of the gastric mucosa. IL-4 and IFN-γ modulate immunoglobulin isotype selection and expression of major histocompatibility complex class II, inducing the specific immune response. In addition, IFN-γ activates the phagocytes to produce reactive intermediates from O_2 and nitrogen (NO) (extremely harmful to tissues) capable of supporting chronic histolytic phenomena and at the same time capable of microbicidal effects. Concerning the secretion of IL-4 and IFN-γ induced by porins, it is important to note that porins determine the optimum production of these cytokines at different concentrations. Indeed, IFN-γ is produced maximally by lymphocytes in response to porins at 20 μg/ml/10^6 lymphocytes, while IL-4 is secreted in response to half that quantity of stimulus. This fact probably is related to different sensitivities of the two lymphocyte subsets (T_H1 and T_H2) responsible for the production of IFN-γ and IL-4, respectively. Several different factors, including the dose of antigen (10), have been proposed to determine whether a T-cell response will be predominantly T_H1- or T_H2-like. The biological effects of *H. pylori*, including cytokine release, are not modified by preincubation of the porins with PB, which stops the biological activity of lipid A; although this activity is lower than that of enterobacterial LPS, it could nonetheless interfere with the results obtained. We can deduce that the concentration of LPS present in the porin preparation cannot induce cytokine production by itself.

Furthermore, our results, as well as those of other authors, show that porins derived from various microorganisms have similar immunobiologic activities (38, 44).

Because of the importance of porins in pathogenesis and their role as protective antigens (29, 36), further studies will be of great interest to determine the in vivo role of *H. pylori* porins, to identify the molecules responsible for induction of protective immunity in the host, and to formulate strategies for the effective stimulation of gastric system-associated immunity, with an aim toward developing immunization strategies against the pathological effects of *H. pylori*.

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