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Partial Characterization of a Cell Proliferation-Inhibiting Protein Produced by *Helicobacter pylori*

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Despite the induction of an immunological reaction, *Helicobacter pylori*-associated gastritis is a chronic disease, suggesting that this microbe can evade the host immune defense. Previous studies by our group showed that *H. pylori* suppresses the in vitro proliferative response of human mononuclear cells to mitogens and antigens. Here we demonstrate that the antiproliferative activity of *H. pylori* also affects the proliferation of various mammalian cell lines (U937, Jurkat, AGS, Kato-3, HEP-2, and P388D1). This effect is detectable in the first 16 h of incubation and maximal between 24 and 48 h. In addition, the presence of *H. pylori* significantly diminished the protein synthesis of cells in the first 6 h of incubation, comparable to the results with cycloheximide and diphtheria toxin. The urease enzyme, the *cagA* gene product, and the vacuolizing cytotoxin of *H. pylori* were excluded as causative agents of the antiproliferative effect by using isogenic knockout mutant strains. The inhibitory effect was not due to a lytic activity of this bacterium. The results reported here indicate that the responsible factor is a protein with an apparent native molecular mass of 100 ± 10 kDa. Our work implicates the presence of a protein factor in *H. pylori* (termed PIP [for proliferation-inhibiting protein]) with antiproliferative activity for mammalian cells, including immunocompetent and epithelial cells. Thus, it is reasonable to presume that this property may contribute to the pathogenesis of *H. pylori*-induced diseases. It may be involved on the one hand in immune response evasion and on the other hand in the suppression of epithelial repair mechanisms.

The discovery of the gastropathogenic gram-negative bacterium *Helicobacter pylori* has revolutionized our current understanding of a number of gastroduodenal diseases in humans. Today there is agreement that chronic type B gastritis, a histologically defined inflammation of the gastric mucosa, as well as gastric and duodenal ulcerations, is due directly or indirectly to an *H. pylori* infection of the stomach (2, 5, 24, 40). Furthermore, there is increasing evidence that *H. pylori* is also involved in the generation of gastric MALT lymphoma and gastric adenocarcinoma (15, 20, 32, 42).

Infection with *H. pylori* occurs early in life, possibly through fecal-oral or oral-oral transmission, and persists for life (4). This bacterium does not invade the mucosal epithelium but colonizes the overlaying mucus layer. Thus, the local inflammation induced by infection seems to be due to a transport of bacterial products, especially proinflammatory factors, into the mucosal tissue (3). The inflammation consists of a remarkable local cellular infiltration with polymorphonuclear leukocytes, macrophages, and lymphocytes (13, 21, 39, 43). During infection, the level of specific local as well as systemic antibodies against *H. pylori* is also increased (35, 40, 43, 44). Nevertheless, the infection is not eliminated and this bacterium can persist in the mucosa, implying chronic active gastritis, a condition that usually persists for life (40, 41). Therefore, investigations of the mechanisms of bacterial resistance to the immune response are important for understanding the pathogenesis of *H. pylori*-induced diseases. The inability of the immune response to eradicate this bacterium may be due in part to immune response-evasive mechanisms which enable the development of a stable host-parasite relationship. Previous studies by our group (22, 23) and others (1, 7) have revealed that *H. pylori* suppresses the in vitro proliferative response of human peripheral blood mononuclear cells to mitogens and antigens. Thus, it is reasonable to presume that this capability to inhibit the cellular immune response is also involved in limiting the effectiveness of the immunological host response in vivo. To further investigate the effects of *H. pylori* on cell proliferation, we used different mammalian cell lines as targets. We found that the proliferation of leukocytic cells as well as epithelial cells was inhibited, causing us to suspect that in addition to the suppression of the host cellular immune response, *H. pylori* can alter the repair activity of gastric mucosal epithelial cells in vivo, thus promoting the generation of lesions. Furthermore, we report here a partial functional and physicochemical characterization of the responsible protein factor.

MATERIALS AND METHODS

Bacteria. Seven of the 16 *H. pylori* strains used were clinical isolates from the area of Bochum, Federal Republic of Germany (FRG). The cytotoxin-producing reference strain 60190 and the cytotoxin-negative reference strain Tx30a were kindly provided by N. Lehn (Munich, FRG). Strain N6 and the isogenic urease-negative mutant strain N6 *ureB* (16) were kindly provided by Agnès Labigne (Institut Pasteur, Paris, France). Strain P 12 and the isogenic *vacA* (P 14) and *cagA* (P 17) knockout mutants (36) were kindly provided by Rainer Haas (Max Planck Institut, Tübingen, FRG). NCTC 11637 and NCTC 11639 were obtained from the Public Health Laboratory Service (London, United Kingdom). Except for N6 and N6 *ureB*, bacteria were usually grown in brain heart infusion broth with 10% horse serum under conditions previously described (17). N6 and N6 *ureB* were grown on blood agar base 2 plates, supplemented with 10% horse blood and the following antibiotics: vancomycin (10 mg/liter), polymyxin B (2,500 U/liter), trimethoprim (5 mg/liter), amphotericin B (4 mg/liter), and for N6 *ureB* kanamycin (20 mg/liter).

Preparation of SE. After bacteria were harvested by centrifugation (10,000 × *g*, 20 min), soluble extract (SE) was prepared in phosphate-buffered saline (PBS; pH 7.4) by sonication with a Branson sonifier (model W 185) at 20-s intervals (35% pulses) for 5 min as previously described (22). Unbroken cells and the membrane fraction were sedimented at 48,000 × *g* for 90 min at 4°C. The obtained supernatant was designated the SE. SE was used in all experiments on

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TABLE 1. Effects of an SE of *H. pylori* on the proliferation of different cell lines[a]

SE (μg/ml)	Proliferation (% of control)[b]					
	U937	Jurkat	AGS	Kato-3	HEp-2	P388D1
1.88	90.2 ± 5.9	97.5 ± 3.0	65.3 ± 13.0	ND[c]	99.0 ± 4.0	85.4 ± 2.6
3.75	83.2 ± 6.0	91.7 ± 4.6	48.4 ± 11.3	ND	102.2 ± 2.3	83.0 ± 9.8
7.5	71.1 ± 4.9	85.3 ± 5.2	31.2 ± 6.4	ND	95.8 ± 2.0	67.5 ± 7.4
15	57.5 ± 3.6	73.9 ± 8.8	15.7 ± 2.7	96.5 ± 5.7	87.1 ± 9.1	61.5 ± 2.9
30	36.0 ± 6.4	45.3 ± 9.4	11.9 ± 2.3	97.2 ± 6.2	73.6 ± 4.0	54.2 ± 1.4
60	29.0 ± 6.5	16.9 ± 6.9	9.4 ± 2.5	81.7 ± 7.5	49.4 ± 2.1	18.5 ± 5.4
120	16.9 ± 3.9	1.9 ± 1.0	9.8 ± 9.4	51.7 ± 13	41.9 ± 2.4	5.4 ± 2.1
240	2.6 ± 0.9	0.6 ± 0.2	ND	20.5 ± 5.3	15.5 ± 4.2	1.1 ± 0.4

[a] The IC_{50}s of SE for U937, Jurkat, AGS, Kato-3, HEp-2, and P388D1 cells were 18.3 ± 2.4, 28.6 ± 5.9, 3.4 ± 1.1, 130 ± 25.4, 67.9 ± 5.3, and 27.8 ± 3.7 μg/ml, respectively.
[b] Data are the means ± standard errors of the means of four experiments (six experiments with U937 cells and five experiments with Kato-3 cells).
[c] ND, not determined.

the basis of its protein content, determined by the bicinchoninic acid method (37) with bovine serum albumin as a standard.

Cell lines. Cell cultures of the following types were kindly provided by the following persons: U937 (human monocyte-like lymphoma cells), Carsten Mahnke (Department of Cell Biology, University of Münster, Münster, FRG); P388D1 (mouse lymphoid macrophages), Frank Falkenberg (Department of Medical Microbiology and Immunology, University of Bochum, Bochum, FRG); and HEp-2 (human larynx carcinoma cells), Hermann Werchau (Department of Medical Microbiology and Virology, University of Bochum). Jurkat (human lymphoblastic T cells), AGS (human gastric adenocarcinoma cells), and Kato-3 (human gastric carcinoma cells) cells were purchased from the European Collection of Animal Cell Cultures (Salisbury, United Kingdom). Cell lines were cultured in 250-ml plastic T flasks at 37°C and 5% CO_2 in appropriate culture media, as recommended by the European Collection of Animal Cell Cultures. All media were supplemented with 2 mM L-alanyl-L-glutamine (Gibco BRL, Eggenstein, FRG), penicillin-streptomycin, and 10% heat-inactivated fetal calf serum (Gibco BRL). Cells were subcultured twice weekly. Prior to cells being tested, cell viability was checked by trypan blue dye exclusion.

Determination of the antiproliferative activity. Unless stated otherwise, cells were plated in triplicate at 2×10^4 cells per well in 96-well flat-bottom microculture plates in 200 μl of supplemented culture medium in the presence or absence of serially diluted SE for 48 h. To assess DNA synthesis, cells were labeled for an additional 16 h with 0.5 μCi of [^{3}H]thymidine (NEN DuPont, Bad Homburg, FRG). Incorporation was measured in an LKB liquid scintillation counter after the harvest of cells on glass fiber filters. The antiproliferative activity was usually expressed as the 50% inhibitory concentration (IC_{50}), that is, the concentration necessary to achieve 50% inhibition of cell proliferation relative to controls cultured in medium alone.

Protein synthesis assay. To determine protein synthesis activity, 10^5 U937 cells were incubated in the presence and absence of serially diluted SE in 200 μl of leucine-free RPMI culture medium (Gibco BRL) supplemented with 1 μCi of [^{3}H]leucine (NEN DuPont). After 6 h, the incorporated radioactivity was monitored by a protocol described by Chang et al. (6) and based on trichloroacetic acid precipitation and standard liquid scintillation spectrometry.

Viability testing. Cells (10^5/ml) were incubated in 50-ml plastic T flasks for 48 h in the presence and absence of SE. After being harvested, cells were checked for cytotoxic effects with thiazolyl blue (MTT; Sigma, Deisenhofen, FRG) as described by Mosmann (30) with slight modifications. Briefly, 10^5 cells were incubated in the presence of MTT (0.25 mg/ml) for 4 h and lysed by the addition of 10% sodium dodecyl sulfate (SDS) in 0.01 M HCl overnight before the optical density at 550 nm (OD_{550}) (reference, 690 nm) was measured with a microplate reader.

Protease treatment. For digestion of protein, SE was treated with proteases at 37°C for 4 h in 0.1 M Tris-HCl buffer (pH 8.5) by using trypsin (Boehringer Mannheim) with 1/20 of the protein content by weight or pronase (Boehringer Mannheim) at a concentration of 0.12 mg/ml.

Isoelectric focusing. SE was prepared in bidistilled water, heat treated (56°C, 30 min), and centrifuged. Fifty milliliters of this extract containing 2 to 3 mg of protein per ml and 2% ampholytes with a pH range of 3 to 10 (Bio-Rad, Munich, FRG) was applied to a Bio-Rad Rotofor apparatus and run for 6 h under the conditions recommended by the manufacturer. At the end of the run, fractions were harvested and the pH of each fraction was measured with a pH meter. Afterwards, fractions were neutralized by the addition of 10× PBS (Gibco), concentrated, and sterile filtered before they were used in a proliferation assay with U937 cells as targets. The pI of the proliferation-inhibiting activity was calculated from the pH curve.

Size exclusion chromatography. SE was prepared in buffer containing 50 mM sodium phosphate and 400 mM NaCl (pH 7.4), heat treated at 56°C for 30 min, and centrifuged (48,000 × *g*, 20 min). The supernatant was concentrated up to 16 mg/ml by using a 10-kDa ultrafiltration membrane (Amicon Inc., Beverly, Mass.). Five hundred microliters of this concentrate was resolved on a Pharmacia (Uppsala, Sweden) Superdex-200 10/30 column by using the Pharmacia-LKB fast protein liquid chromatography system at a flow rate of 0.25 ml/min with the buffer described above. The column eluate was monitored for UV A_{280}. Fractions (1 ml) were concentrated with Microseps-10K microconcentrator devices (Filtron, Northborough, Mass.), sterile filtered, and monitored for antiproliferative and urease activities. The molecular mass of the native antiproliferative factor was calculated from molecular weight standards (Pharmacia), including blue dextran, to determine the void volume. The standard proteins were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and RNase (13.7 kDa).

Urease assay. Urease activity was quantitated spectrophotometrically by the method of Mobley et al. (29) with slight modifications. Briefly, samples (10 to 50 μl) were added to cuvettes (1-cm path length) containing 3 mM sodium phosphate buffer with 10 mM urea and 7 μg of phenol red per ml in a total volume of 2 ml. OD_{556} was monitored over time, and ΔE was calculated from the linear portion of the curve (0.15 to 0.5 OD units).

Assay for vacuolating cytotoxic activity. Vacuolating cytotoxic activities were determined in 30-fold-concentrated bacterium-free culture supernatants with HEp-2 cells as the indicator system (22). Serially diluted samples (1:4 to 1:128) were incubated with HEp-2 cells for 24 h in 96-well flat-bottom microculture plates. Cell vacuolation was quantitated spectrophotometrically by a neutral red uptake assay described previously (12). Strains were defined as cytotoxin positive if the net OD_{550} was higher than 0.1. Additionally, the results were verified by visual inspection of cells by light microscopy (11).

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Lugtenberg et al. (26). Proteins were resolved in the gel by using Coomassie brilliant blue R-250. The molecular weight standards (Pharmacia) included phosphorylase *b* (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400).

RESULTS

Susceptibilities of various mammalian cell lines to the PIP activity of *H. pylori*. The susceptibilities of various cell types to the proliferation-inhibiting protein (PIP) activity of *H. pylori* are given in Table 1. The proliferation of all cell lines tested was inhibited by the addition of SE in a dose-dependent manner. For the purpose of comparing the effects of SE on different cell types, the IC_{50} of SE was calculated. The degree of inhibition ranged from an IC_{50} of 3.4 μg/ml for AGS cells to 130 μg/ml for Kato-3 cells, indicating the different susceptibilities of cells to PIP. Nevertheless, proliferation inhibition was not correlated to the type (leukocytic or epithelial) nor to the species origin (human or mouse) of the cells.

Screening of various *H. pylori* strains for PIP activity. To study whether there is strain variability with respect to PIP activity, SEs of various *H. pylori* strains were added to U937 cells in serial dilution and the IC_{50}s were calculated. The PIP activities (IC_{50}s) of 16 strains and their urease, *cagA*, and *vacA* statuses are shown in Table 2. All strains inhibited the proliferation of U937 cells to a similar degree, with the IC_{50} ranging from 10.7 to 44.0 μg/ml. There was no relation between the inhibitory activities of strains and their urease, *cagA*, or *vacA*

TABLE 2. Comparison of the PIP IC_{50}s of different *H. pylori* strains

Strain	Status[a]			PIP IC_{50} (μg/ml)[b]
	Urease	*cagA*[c]	Cytotoxin[d]	
008	+	+	− (22)	15.3 ± 2.8
030	+	+	ND[e]	15.1 ± 2.6
040	+	+	ND	25.9 ± 2.6
072	+	+	ND	13.2 ± 0.7
105	+	+	+ (22)	15.1 ± 1.5
133	+	+	+ (22)	23.0 ± 3.1
8981	+	+	− (22, 36)	16.7 ± 1.8
NCTC 11637	+	+	+ (36)	28.2 ± 4.8
NCTC 11639	+	−	ND	27.8 ± 3.8
60190	+	+	+ (22, 26)	14.2 ± 1.2
Tx30a	+	−	− (22, 26)	44.0 ± 5.8
N6	+	+	+ (16)	13.8 ± 3.1
N6 *ureB*	−	+	+ (16)	10.7 ± 2.0
P 12	+	+	+ (36)	15.7 ± 2.4
P 14	+	+	− (36)	9.9 ± 0.5
P 17	+	−	+ (36)	11.3 ± 1.3

[a] +, present; −, absent.
[b] PIP activities were detected with U937 cells as targets. Data are the means ± standard errors of the means of four different experiments.
[c] The presence of *cagA* was determined by PCR using primers based on the published DNA sequence (9).
[d] References are noted parenthetically. The cytotoxin is VacA.
[e] ND, not determined.

status. Furthermore, the inhibitory activity was independent on whether the strain was grown on solid or liquid medium. PIP activity was also detectable in the cell-free supernatant (30-fold concentrated) of *H. pylori* log-phase cultures. For eight strains tested, the titer inducing 50% inhibition of U937 proliferation ranged from 10 to 278 (medium control, <10), with a median value of 26.2 (data not shown).

Functional characteristics of PIP. To exclude lethal effects of SE as the cause of the observed proliferation inhibition, the viability of U937 cells was checked after 48 h of incubation in the presence of SE at various concentrations. The results in Table 3 indicate that proliferation inhibition was not correlated to lytic effects of SE, as determined by trypan blue exclusion and mitochondrial dehydrogenase activities (MTT turnover). Furthermore, proliferation inhibition was reversible. This was shown by using U937 cells after 48 h of incubation in the presence of up to 120 μg of SE per ml. After harvesting and washing cells, the ability of these cells to incorporate [^{3}H]thymidine was determined. The [^{3}H]thymidine uptake of SE-incubated cells was comparable to that of control cells (data not shown). In order to determine the time course of proliferation inhibition, the influence of SE on DNA synthesis was measured after different incubation periods. As shown in Fig. 1, SE-mediated proliferation inhibition was obvious in the first 16 h of incubation and maximal between 24 to 48 h, depending on the concentration used. In another experiment (Fig. 2), the addition of SE to the culture diminished the protein synthesis of U937 cells in the first 6 h of incubation, a result analogous to those with known inhibitors of protein synthesis such as diphtheria toxin and cycloheximide. The mean concentration of SE necessary to achieve 50% inhibition of protein synthesis was 43.4 μg/ml.

TABLE 3. Influence of an *H. pylori* SE on the viability of U937 cells after 48 h of incubation[a]

SE (μg/ml)	Dead cells (%)[b]	MTT turnover (OD_{550})	Proliferation (cpm)[c]
0 (control)	0	1.668	157,350
15	6	1.562 (94)	56,646 (36)
60	13	1.347 (81)	23,602 (15)
240	27	1.124 (67)	14,161 (9)

[a] Data are representative of three different experiments. Parenthetical data are percentages of control values.
[b] Determined by trypan blue exclusion.
[c] Proliferation was measured in a parallel assay by [^{3}H]thymidine incorporation as described in Materials and Methods.

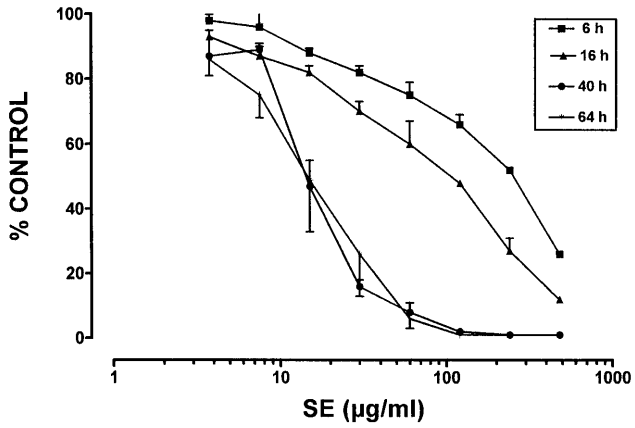

FIG. 1. Kinetics of proliferation inhibition. A total of 5×10^4 (6 and 16 h) or 2×10^4 (40 and 64 h) U937 cells were incubated for the intervals indicated in the presence or absence of serially diluted SE. [^{3}H]thymidine was added at the start of culture (6 and 16 h) or for the last 16 h of incubation (40 and 64 h). Data are the means ± standard errors of the means of four different experiments.

Physicochemical characteristics of PIP. The effects of protease, heat, and pH treatments on PIP activity are summarized in Table 4. PIP activity was sensitive to trypsin as well as pronase and was inactivated by incubation at 70°C for 30 min, while incubation at 56°C for 30 min or at 37°C for 72 h had no or only weak effects. PIP activity was stable over a broad pH range. After pretreatment at pH 2 and pH 12, PIP activity

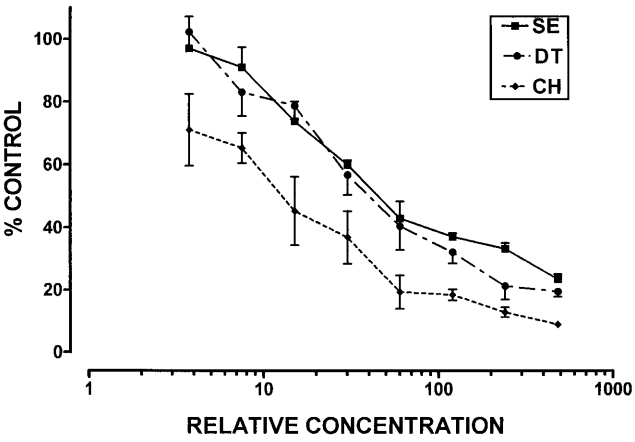

FIG. 2. Influence of the SE of *H. pylori*, diphtheria toxin (DT), and cycloheximide (CH) on the protein synthesis of U937 cells in the first 6 h of incubation. Samples were serially diluted twofold. SE, diphtheria toxin, and cycloheximide were used within different concentration ranges. The SE concentration ranged from 3.75 to 480 μg/ml, the diphtheria toxin concentration ranged from 7.8 to 1,000 ng/ml, and the cycloheximide concentration ranged from 15.5 to 2,000 ng/ml. Data are the means ± standard errors of the means of four different experiments.

TABLE 4. Effects of protease, heat, and pH on the proliferation-inhibiting activity of SE

Treatment	PIP IC_{50}[a]
Protease:	
None	21.4 ± 5.7
Trypsin	>480
Pronase	>480
Heat:	
Unheated	16.6 ± 6.9
56°C for 30 min	20.6 ± 7.5
70°C for 30 min	>480
37°C for 72 h	56.0 ± 12
pH:	
2	67.5 ± 7.6
4	7.7 ± 1.6
6	9.1 ± 2.5
8	7.9 ± 1.9
10	10.5 ± 2.3
12	70.6 ± 13.5

[a] Data are the means ± standard errors of the means of three experiments.

decreased but was still detectable. Furthermore, PIP activity was precipitable with >50% ammonium sulfate (data not shown). By using the crude SE and ampholytes with a pH range from 3 to 10 in a Bio-Rad Rotofor system, two peak activities with isoelectric points of 5.35 ± 0.1 and 6.03 ± 0.1 were found. The molecular mass of PIP was estimated by fractionation of the SE with a calibrated Superdex-200 column. The elution profile and a plot of the peak activity are shown in Fig. 3A. SDS-PAGE analysis of the fractions containing PIP activities revealed rather heterogenic protein profiles (Fig. 3B), foiling the identification of a corresponding protein band. The PIP activity-containing fractions had neither urease activity nor vacuolizing effects on HEp-2 cells. The molecular mass of partially purified PIP was calculated to be approximately 100 ± 10 kDa.

DISCUSSION

A major characteristic of *H. pylori*-associated gastritis is the long-term persistence of this microbe in the gastric mucosa (4, 40) despite specific local and systemic immune responses (35, 40, 43, 44). As the immunological host defense is unable to clear the infection, bacterial immune response-evasive mechanisms have to be discussed as the responsible cause for the chronicity of infection. The ability of *H. pylori* to suppress the in vitro proliferative response of human mononuclear cells to mitogens and antigens, clearly demonstrated by us (22, 23) and others (1, 7), represents one way that *H. pylori* may alter the host immune reaction.

One purpose of this study was to demonstrate that the antiproliferative activity of *H. pylori* is not restricted to mononuclear cells but also exerts its effects on other cell types. This was investigated by the use of six different cell lines. The results show that all cell lines tested were susceptible to PIP activity in general but with different susceptibilities, as indicated by different IC_{50}s. The murine monocytic line P388D1 was also inhibited, excluding the restriction of PIP activity to human cells. While the inhibition of cell lines of leukocytic origin confirms previous results obtained with activated human mononuclear cells (22, 23), the proliferation inhibition of epithelial cell lines indicates a possible relationship between PIP activity and the epithelial damage observed in *H. pylori*-associated diseases. Concordantly, Hirose and coworkers reported recently that a water-soluble extract of *H. pylori* inhibited cell proliferation and retarded the restoration of isolated rabbit gastric epithelial cells in vitro (18). Furthermore, Nakajima et al. also observed proliferation inhibition of the gastric epithelial cell line Kato-3 in the presence of whole *H. pylori* cells (31). Thus, in addition to participation in immune response evasion, PIP may disturb the normal restoration process of the gastric epithelium. Reports concerning cell proliferation in gastric mucosa colonized by *H. pylori* are quite inconsistent at present. Increased (27) as well as normal (8) mucosal cell proliferation was observed in biopsy specimens in cases of *H. pylori* infection. Even if the proliferation index is increased because of *H. pylori* infection, this does not contradict our in vitro observations per se. Increased cell proliferation could be regarded as a protective reaction of the epithelium toward the cytopathic effects induced by *H. pylori* infection. This restoration process may be retarded in the presence of PIP. Thus, the proliferation index would be increased compared with that of normal mucosa, but it would not be sufficient to protect the epithelium

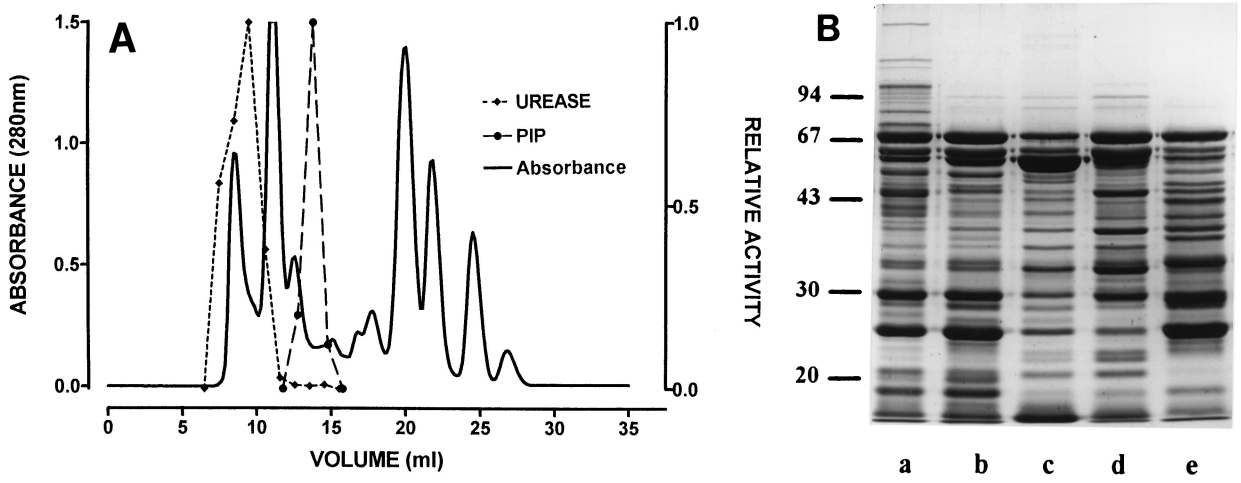

FIG. 3. (A) Size exclusion chromatography of *H. pylori* cytoplasmic proteins on a Superdex-200 10/30 column. For details, see Materials and Methods. (B) SDS-PAGE (12% polyacrylamide) analysis of fractions after size exclusion chromatography. Lanes: a, SE; b, SE after heat treatment (56°C, 30 min), as used for chromatography; c through e, fractions containing PIP activities (see the activity peak in panel A). The migrations of marker proteins of known molecular masses (in kilodaltons) are shown on the left.

from cytopathic events. Nevertheless, the influence of *H. pylori* on gastric mucosal cell proliferation in vivo mainly remains unclear, making further investigation necessary.

The capability to inhibit the proliferation of mammalian cells can be regarded as a constitutional characteristic of *H. pylori* because all strains tested showed this activity with comparable IC_{50}s. There was no relation between the inhibitory activity and the urease, *cagA*, or *vacA* status of any strain, which was especially obvious when using isogenic knockout mutant strains. PIP also differs from the vacuolizing cytotoxin (VacA) and urease in molecular weight. These proteins are reported to have native molecular masses of ≥972 (10) and 550 (19) kDa, respectively, in contrast to the native molecular mass of 100 ± 10 kDa for the proliferation-inhibiting activity. Furthermore, partially purified PIP had neither urease activity nor vacuolizing effects on HEp-2 cells. Although the cytopathic effects of *H. pylori* have been associated with the expression of vacuolizing cytotoxin (10, 25) and urease (12, 28, 38), our results exclude these virulence factors as responsible agents for the observed antiproliferative effects of *H. pylori*. In log-phase cultures of *H. pylori*, PIP activity was detectable only in rather small amounts compared with that of the SE. Thus, PIP seems to be predominately located within the cytoplasm. Nevertheless, PIP may be released into the environment under certain circumstances, for example, because of autolysis, as recently suggested by Phadnis and colleagues (34) for surface association of urease and HspB of *H. pylori*. Although the mode of action of PIP is not yet clear in detail, we have shown that this proliferation inhibition is not due to lytic effects. Furthermore, the effects of PIP were reversible and DNA fragmentation, one hallmark of apoptosis (14), was not observed (data not shown). The proliferation inhibition induced by *H. pylori* is a dose- and time-dependent phenomenon. In addition to an early influence on cell proliferation, protein synthesis was inhibited in the presence of PIP in the first 6 h of culture. Whether the protein synthesis apparatus of mammalian cells is the primary target for PIP as it is for diphtheria toxin (33) cannot be concluded from the present data. In contrast to diphtheria toxin, the inhibition of DNA and protein synthesis induced by *H. pylori* is not followed by cell lysis; thus, the effects promoted by PIP rather resemble those of cycloheximide (6). Cell growth, as well as the progress of the cell cycle, is regulated by feedback controls that can arrest the cell cycle at various checkpoints. A hypothetical explanation for the nonlethal and reversible antiproliferative effects of *H. pylori* is the induction of such a cell cycle arrest. For example, with Jurkat cells it was demonstrated that an anti-CD3 antibody-driven activation resulted in a transient, reversible inhibition of DNA synthesis, with an arrest at the G_1/S interface without inducing cell death (45). This hypothesis is currently under investigation.

We conclude that *H. pylori* produces a soluble protein (PIP) with a native molecular mass of approximately 100 kDa and two isoelectric points (5.35 and 6.03) that causes the inhibition of mammalian cell proliferation. The present results let us assume that PIP contributes to the survival of this bacterium in the gastric mucosa via immunosuppression. In addition, PIP may alter the repair activity of the gastric mucosal epithelium. Together with direct bacterial cytotoxicity and inflammatory cell aggression, this may predispose the patient to peptic ulcer disease. Nevertheless, at present the significance of PIP for the pathogenesis of *H. pylori*-induced diseases has to remain theoretical. To understand the role of PIP in vivo, further purification of PIP, cloning of the coding gene, construction of isogenic PIP-negative mutants, and use in an animal model are required.

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REFERENCES

1. **Benz, J., H. Hasbach, M. Brenden, S. Eidt, G. Fatkenheuer, M. Schrappe, J. Geisel, H. Goossens, and G. Mauff.** 1993. Humoral and cellular immunity in HIV positive and HIV negative Helicobacter pylori infected patients. Int. J. Med. Microbiol. Virol. Parasitol. Infect. Dis. **280:**186–196.
2. **Blaser, M. J.** 1990. *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. J. Infect. Dis. **161:**626–633.
3. **Blaser, M. J.** 1992. Hypotheses on the pathogenesis and natural history of *Helicobacter pylori*-induced inflammation. Gastroenterology **102:**720–727.
4. **Blaser, M. J.** 1993. *Helicobacter pylori*: microbiology of a 'slow' bacterial infection. Trends Microbiol. **1:**255–260.
5. **Buck, G. E.** 1990. *Campylobacter pylori* and gastroduodenal disease. Clin. Microbiol. Rev. **3:**1–12.
6. **Chang, M. P., J. Bramhall, S. Graves, B. Bonavida, and B. J. Wisnieski.** 1989. Internucleosomal DNA cleavage precedes diphtheria toxin-induced cytolysis. J. Biol. Chem. **264:**15261–15267.
7. **Chmiela, M., T. Wadström, R. Rappuoli, and W. Rudnicka.** 1995. The inhibition of antigen- or mitogen-induced response of T cells by *H. pylori* bacteria and their products. Gut **37**(Suppl. 1)**:**A32. (Abstract.)
8. **Chow, K. W., S. Bank, J. Ahn, J. Roberts, M. Blumstein, and V. Kranz.** 1995. *Helicobacter pylori* infection does not increase gastric antrum mucosal cell proliferation. Am. J. Gastroenterol. **90:**64–66.
9. **Covacci, A., S. Censini, M. Bugnoli, R. Petracca, D. Burroni, G. Macchia, A. Massone, E. Papini, Z. Xiang, N. Figura, et al.** 1993. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. Proc. Natl. Acad. Sci. USA **90:**5791–5795.
10. **Cover, T. L., and M. J. Blaser.** 1992. Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. J. Biol. Chem. **267:**10570–10575.
11. **Cover, T. L., C. P. Dooley, and M. J. Blaser.** 1990. Characterization of and human serologic response to proteins in *Helicobacter pylori* broth culture supernatants with vacuolizing cytotoxin activity. Infect. Immun. **58:**603–610.
12. **Cover, T. L., W. Puryear, G. I. Perez-Perez, and M. J. Blaser.** 1991. Effect of urease on HeLa cell vacuolation induced by *Helicobacter pylori* cytotoxin. Infect. Immun. **59:**1264–1270.
13. **Dixon, M. F., J. I. Wyatt, D. A. Burke, and B. J. Rathbone.** 1988. Lymphocytic gastritis—relationship to *Campylobacter pylori* infection. J. Pathol. **154:** 125–132.
14. **Duvall, E., and A. H. Wyllie.** 1986. Death and the cell. Immunol. Today **7:**115–119.
15. **The Eurogast Study Group.** 1993. An international association between *Helicobacter pylori* infection and gastric cancer. Lancet **341:**1359–1362.
16. **Ferrero, R. L., V. Cussac, P. Courcoux, and A. Labigne.** 1992. Construction of isogenic urease-negative mutants of *Helicobacter pylori* by allelic exchange. J. Bacteriol. **174:**4212–4217.
17. **Geis, G., H. Leying, S. Suerbaum, U. Mai, and W. Opferkuch.** 1989. Ultrastructure and chemical analysis of *Campylobacter pylori* flagella. J. Clin. Microbiol. **27:**436–441.
18. **Hirose, M., S. Watanabe, X. E. Wang, R. Ohkura, T. Murai, and N. Sato.** 1995. A water extract of *Helicobacter pylori* inhibited gastric epithelial restoration in vitro. Gastroenterology **108**(Suppl.)**:**A115. (Abstract.)
19. **Hu, L. T., and H. L. Mobley.** 1990. Purification and N-terminal analysis of urease from *Helicobacter pylori*. Infect. Immun. **58:**992–998.
20. **Hussell, T., P. G. Isaacson, J. E. Crabtree, and J. Spencer.** 1993. The response of cells from low-grade B-cell gastric lymphomas of mucosa-associated lymphoid tissue to *Helicobacter pylori*. Lancet **342:**571–574.
21. **Kirchner, T., A. Melber, W. Fischbach, K. L. Heilmann, and H. K. Müller-Hermelink.** 1990. Immunhistological patterns of the local immune response in *Helicobacter pylori* gastritis, p. 213–222. *In* P. Malfertheiner and H. Ditschuneit (ed.), *Helicobacter pylori*, gastritis and peptic ulcer. Springer-Verlag, Berlin.
22. **Knipp, U., S. Birkholz, W. Kaup, K. Mahnke, and W. Opferkuch.** 1994. Suppression of human mononuclear cell response by *Helicobacter pylori*: effects on isolated monocytes and lymphocytes. FEMS Immunol. Med. Microbiol. **8:**157–166.
23. **Knipp, U., S. Birkholz, W. Kaup, and W. Opferkuch.** 1993. Immune suppressive effects of *Helicobacter pylori* on human peripheral blood mononuclear cells. Med. Microbiol. Immunol. **182:**63–76.
24. **Lee, A., J. Fox, and S. Hazell.** 1993. Pathogenicity of *Helicobacter pylori*: a perspective. Infect. Immun. **61:**1601–1610.
25. **Leunk, R. D., P. T. Johnson, B. C. David, W. G. Kraft, and D. R. Morgan.** 1988. Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. J. Med. Microbiol. **26:**93–99.

26. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the "major outer membrane protein" of *Escherichia coli* K12 into four bands. FEBS Lett. **58:**254–258.
27. **Lynch, D. A. F., N. P. Mapstone, A. M. T. Clarke, P. Jackson, M. F. Dixon, P. Quirke, and A. T. R. Axon.** 1995. Cell proliferation in the gastric corpus in *Helicobacter pylori* associated gastritis and gastric resection. Gut **36:**351–353.
28. **Megraud, F., V. Neman Simha, and D. Brugmann.** 1992. Further evidence of the toxic effect of ammonia produced by *Helicobacter pylori* urease on human epithelial cells. Infect. Immun. **60:**1858–1863.
29. **Mobley, H. L., M. J. Cortesia, L. E. Rosenthal, and B. D. Jones.** 1988. Characterization of urease from *Campylobacter pylori*. J. Clin. Microbiol. **26:**831–836.
30. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods **65:**55–63.
31. **Nakajima, N., H. Kuwayama, and Y. Arakawa.** 1995. Effects of *Helicobacter pylori* and neutrophils on gastric epithelial cell proliferation. Gastroenterology **108**(Suppl.)**:**A175. (Abstract.)
32. **Parsonnet, J., G. D. Friedman, D. P. Vandersteen, Y. Chang, J. H. Vogelman, N. Orentreich, and R. K. Sibley.** 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. N. Engl. J. Med. **325:**1127–1131.
33. **Perentesis, J. P., S. P. Miller, and J. W. Bodle.** 1992. Protein toxin inhibitors of protein synthesis. Biofactors **3:**173–184.
34. **Phadnis, S. H., M. H. Parlow, M. Levy, D. Ilver, C. M. Caulkins, J. B. Connors, and B. E. Dunn.** 1996. Surface localization of *Helicobacter pylori* urease and a heat shock protein homolog requires bacterial autolysis. Infect. Immun. **64:**905–912.
35. **Rathbone, B. J., J. I. Wyatt, B. W. Worsley, S. E. Shires, L. K. Trejdosiewicz, R. V. Heatley, and M. S. Losowsky.** 1986. Systemic and local antibody responses to gastric *Campylobacter pyloridis* in non-ulcer dyspepsia. Gut **27:**642–647.
36. **Schmitt, W., and R. Haas.** 1994. Genetic analysis of the *Helicobacter pylori* vacuolating cytotoxin: structural similarities with the IgA protease type of exported protein. Mol. Microbiol. **12:**307–319.
37. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150:**76–85.
38. **Smoot, D. T., H. L. Mobley, G. R. Chippendale, J. F. Lewison, and J. H. Resau.** 1990. *Helicobacter pylori* urease activity is toxic to human gastric epithelial cells. Infect. Immun. **58:**1992–1994.
39. **Stolte, M., and S. Eidt.** 1989. Lymphoid follicles in antral mucosa: immune response to *Campylobacter pylori*? J. Clin. Pathol. **42:**1269–1271.
40. **Taylor, D. N., and M. J. Blaser.** 1991. The epidemiology of *Helicobacter pylori* infection. Epidemiol. Rev. **13:**42–59.
41. **Telford, J. L., A. Covacci, P. Ghiara, C. Montecucco, and R. Rappuoli.** 1994. Unravelling the pathogenic role of *Helicobacter pylori* in peptic ulcer: potential new therapies and vaccines. Trends Biotechnol. **12:**420–426.
42. **Wotherspoon, A. C., C. Doglioni, T. C. Diss, L. Pan, A. Moschini, M. de Boni, and P. G. Isaacson.** 1993. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of *Helicobacter pylori*. Lancet **342:**575–577.
43. **Wyatt, J. I., and B. J. Rathbone.** 1988. Immune response of the gastric mucosa to *Campylobacter pylori*. Scand. J. Gastroenterol. Suppl. **142:**44–49.
44. **Wyatt, J. I., B. J. Rathbone, and R. V. Heatley.** 1986. Local immune response to gastric Campylobacter in non-ulcer dyspepsia. J. Clin. Pathol. **39:**863–870.
45. **Zhu, L., and C. Anasetti.** 1995. Cell cycle control of apoptosis in human leukemic T cells. J. Immunol. **154:**192–200.

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