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Cytokine Expression in Pediatric *Helicobacter pylori* Infection

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Helicobacter pylori **infection is one of the most common gastrointestinal infections worldwide and almost invariably causes chronic gastritis in the infected host. A predominant Th1 profile has been demonstrated in *H. pylori*-infected mucosa from adults, but no previous study has evaluated in situ cytokine expression in children. We therefore examined expression of proinflammatory, anti-inflammatory, and regulatory cytokines by immunohistochemistry in cryopreserved antral biopsy specimens from 10 *H. pylori*-infected and 10 uninfected children and correlated expression of cytokines with histology scores. Concomitant expression of interleukin-8 (IL-8), gamma interferon (IFN-γ), IL-4, transforming growth factor β, and tumor necrosis factor alpha was seen in 8/10 *H. pylori*-infected cases and in 5/10 noninfected cases; all *H. pylori*-infected subjects showed staining for at least two of the cytokines. The proportion of epithelial cytokine-specific staining did not differ significantly between the groups, either in surface or glandular epithelium. Furthermore, no significant differences were noticed between intraepithelial or lamina propria lymphocyte staining in the groups. There was, however, a tendency of higher numbers of IFN-γ- and IL-8-positive cells in the *H. pylori*-infected group. IFN-γ and IL-8 lamina propria lymphocyte expression correlated significantly with antrum chronic inflammation, but there was no correlation between histology scores and epithelial cytokine expression. When the same techniques were used, the cytokine response appeared to be smaller in *H. pylori*-infected children than in adults, and there was no clear Th1 dominance. These results therefore suggest a different mucosal immunopathology in children. It remains to be determined whether the gastric immune response is downregulated in children with *H. pylori* infection and whether this is relevant to the outcome of infection.**

Helicobacter pylori infection is one of the most common gastrointestinal infections worldwide and the main cause of chronic gastritis, gastric mucosal atrophy, peptic ulcer, and some forms of gastric cancer (21, 24, 29, 30, 42). Although *H. pylori* infection almost invariably causes chronic gastritis, major complications will develop only in a minority of infected subjects, predominantly in the adult host (21, 24). Epidemiological evidence of *H. pylori* acquisition during childhood (25, 44) and the rare occurrence of peptic ulcer or gastric atrophy in children (17, 18) suggests that the gastric mucosal damage resulting from the infection might be progressive, through childhood until adulthood. During this time, bacterial determinants, the nature of the host immune responses, and exposure to potential environmental factors all appear to influence outcome (20).

H. pylori induces a strong and complex immune response in the gastric mucosa, both humoral and cellular (11, 19), which nevertheless fails to clear the infection and may even contribute to immunopathology. The exact mechanisms by which the *H. pylori*-induced immune response contributes to gastrointestinal mucosal damage are still not clear.

A predominant Th1 T-cell response, associated with elevated levels of expression of proinflammatory cytokines gamma interferon (IFN-γ) and interleukin-2 (IL-2) and with lower levels of expression of regulatory cytokines IL-4 and IL-10, has been clearly demonstrated in *H. pylori*-infected mucosa from adult subjects suffering from gastritis and peptic ulcers (6, 28, 32). Additionally, several studies investigating IL-8, a neutrophil chemotactic factor mainly secreted by epithelial cells, have shown an association between the levels of this cytokine and *H. pylori* infection (3, 10, 12, 23). At this time, it is not known whether this Th1 cell-mediated immune response is protective or whether it contributes to the pathogenesis of *H. pylori*-associated diseases.

Considering that *H. pylori*-associated gastritis is usually mild in children, with low lymphocyte and neutrophil infiltration (4, 5), and if pediatric infection is viewed as an earlier stage of the *H. pylori*-induced inflammatory response, a different immunopathology and different patterns of cytokine expression in children could be anticipated compared to those of adults. There is, however, a paucity of information regarding local immune responses in the gastric mucosa from children, regardless of *H. pylori* infection status. Only a few studies have so far evaluated the local cytokine profile in children, with somewhat conflicting results, but they most consistently show that *H. pylori* infection induces production of proinflammatory cytokines and a Th1 response, similar to studies in adults (8, 9, 26, 31, 34, 41). Most of the previous pediatric studies have focused on detection of cytokine mRNA in gastric biopsy specimens or on quantification of protein supernatants in gastric biopsy homogenates or gastric juice specimens, using enzyme-linked immunosorbent assays (ELISAs) (8, 26, 31, 34, 41). However, these

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TABLE 1. Clinical data for the 20 *H. pylori*-infected and noninfected patients

Patient	Age (yr)	ELISA serology	Helicoblot 2.0 serology	Genotype				
				vacA	*cagA*	*cagE*	*cag* PAI	*oip* status
H. pylori-infected patients								
1	12.6	+	+	s2m2	−	NP	NP	NP
2	10.3	NP[a]	+	s2m2	−	−	Absent	Off
3	11.8	+	+	s2m2	−	+	Mixed	Off
4	8.1	+	+	s2m2	−	−	Absent	Off
5	11.0	+	+	s2m2	−	−	Absent	Off
6	10.7	NP	NP	s2m2	−	−	Absent	Off
7	10.4	NP	NP	s2m2	−	−	Absent	Off
8	7.3	−	NP	s2m2	−	−	Absent	Off
9	9.3	NP	NP	s2m2	−	−	Absent	Off
10	7.3	NP	NP	s2m2	−	−	Absent	Off
Noninfected patients								
1	5.8	+	+					
2	9.6	−	−					
3	11.5	+	+					
4	16.7	−	−					
5	12.0	−	−					
6	9.7	−	NP					
7	10.7	−	NP					
8	7.7	−	NP					
9	7.6	NP	NP					
10	18.8	−	NP					

[a] NP, not performed.

methods do not reflect the in vivo situation as accurately as the immunohistochemistry approach does, allowing the identification, localization, and quantification of cytokine-producing cells.

To our knowledge, there are no previous pediatric studies evaluating in situ expression of different cytokines in gastric mucosa from *H. pylori*-infected children by immunohistochemistry. We therefore examined the gastric expression of proinflammatory, anti-inflammatory, and regulatory cytokines by epithelial cells, as well as by lamina propria and intraepithelial lymphocytes, in *H. pylori*-infected and uninfected children by immunohistochemistry. This expression was correlated with gastric mucosal inflammatory cell infiltration and with *H. pylori* density of colonization and the presence of virulence factors.

MATERIALS AND METHODS

Clinical samples. Twenty children and adolescents of European origin (10 infected with *H. pylori* and 10 not infected with *H. pylori*) referred for endoscopy with upper gastrointestinal symptoms (mostly recurrent abdominal pain), suggestive of organic disease and severe enough to require endoscopic evaluation, were included in the study. Informed consent from the parents and approval from the local Faculty and Hospital Ethics Committees, were obtained. The mean age of *H. pylori*-positive subjects was 9.9 years (range, 7.3 to 12.6 years); the mean age of *H. pylori*-negative subjects was 11.0 years (range, 5.8 to 18.8 years). Exclusion criteria were treatment with antisecretory, antimicrobial, or anti-inflammatory medication, for the 3 months preceding the endoscopy. Subjects with peptic ulcer or severe organic disease were also excluded.

Sampling of mucosal biopsy specimens, specimen collection, and evaluation. Upper endoscopy was performed under general anesthesia. Endoscopically, a mild to moderate erythema was present in all cases, and antral nodularity was evident in 13/20 cases (9 of these were *H. pylori*-positive cases). Biopsy specimens were systematically taken from the duodenum (one or two), gastric antrum (four), and gastric body (one). One of the four antral biopsy specimens was immediately snap-frozen in isopentane previously cooled in liquid nitrogen, subsequently embedded in OCT compound (Tissue-Tek; Miles, Inc., Elkhart, Ind.), and processed for immunohistochemistry as described below. One antral biopsy specimen was fixed in 4.5% buffered formalin and embedded in paraffin; 2-μm sections were stained with hematoxylin and eosin. A modified Giemsa stain was used for *H. pylori* identification, and gastritis was evaluated according to the updated Sydney system (16) by an experienced histopathologist who was unaware of the patient's *H. pylori* status or clinical condition. Accordingly, the chronic inflammation score (mononuclear cell [MNC] infiltration), activity score (polymorphonuclear cell infiltration), and *H. pylori* density score were determined separately and graded from 0 to 3 (for none, mild, moderate, and severe, respectively). Duodenal inflammation was evaluated in similarly treated biopsy specimens from all cases according to Whitehead criteria (46). The two additional antral biopsy specimens were used for urease test (in-house test) and culture, respectively. The antral biopsy specimens for culture were put into sterile saline solution and processed within 3 h, according to a protocol previously described (35). Briefly, biopsy specimens were ground with a tissue homogenizer (Ultra Turax; Labo Moderne, France) and inoculated onto a selective medium (bioMérieux) and a nonselective medium, Mueller-Hinton agar (Oxoid, United Kingdom) supplemented with 10% horse blood (Probiológica, Portugal). Plates were incubated at 37°C in a microaerobic atmosphere obtained with a gas-generating system (CampyGen CN 35; Oxoid) for up to 14 days of incubation. Identification of *H. pylori* was performed according to conventional tests: colony and gram stain morphology, catalase, oxidase, and hydrolysis of urea.

Diagnosis of *H. pylori* infection. *H. pylori* status was assessed according to conventional biopsy-based criteria. Allocation to *H. pylori*-positive or *H. pylori*-negative group, was based on positivity of urease, histology, and culture, or on negativity of all three tests, respectively.

Serology. Serum samples were obtained for determination of anti-*H. pylori*-specific immunoglobulin G (IgG) antibodies by an ELISA (Roche) in 14/20 patients and by Western blotting (Helicoblot 2.0; Genelabs Diagnostics) in 10/20 patients (Table 1). ELISA results were in accordance with *H. pylori* status in four/five *H. pylori*-positive cases and in seven/nine *H. pylori*-negative cases. Helicoblot 2.0 results were positive in five/five *H. pylori*-positive cases and negative in three/five *H. pylori*-negative cases. Two currently *H. pylori*-negative cases (by culture, histology, and urease), had positive ELISA and Helicoblot 2.0 serology results, suggesting previous *H. pylori* infection.

Genotyping of isolated strains. Analysis of *vacA* and *cagA* genotypes was performed in all *H. pylori*-positive cases using PCR, according to a protocol previously described (39). *cag* pathogenicity island (PAI) status was also evaluated in 9/10 available strains by PCR using specific primers for the *cagE* gene and *cag* PAI empty site (1, 47). The functional status of the *oipA* gene was determined as previously described (47).

Cytokine-specific MAbs. The cytokine-specific monoclonal antibodies (MAbs) used, all mouse anti-human antibodies, were anti-IL-4 (8F12; ImmunoKontact, Bioggio, Switzerland), anti-IL-8 (NAP 11; Bender, MedSystem, Vienna, Austria), anti-tumor necrosis factor alpha (anti-TNF-α) (MAb 1; Pharmingen, San Diego, California), anti-IFN-γ (1-D1K; MABTECH AB, Nacka, Sweden), and anti-transforming growth factor β (anti-TGF-β) (Genzyme Diagnostics, Cambridge, Mass.). All MAbs were of the IgG1 isotype. The MAbs were used at 5 μg/ml, except for anti-TGF-β, which was used at 10 μg/ml. Bovine serum albumin (Sigma, St. Louis, MO.) was applied prior to the primary antibody to block unspecific staining. The specificities of the MAbs were ascertained by preabsorption with recombinant cytokines. An isotype-matched mouse IgG1 antibody (Dako, Denmark) was used as a negative control in each experiment.

Immunohistochemistry. Cytokine expression was assessed in cryopreserved (OCT) antral/antral-body transition biopsy specimens as previously described (32). Briefly, 8-μm-thick sections were mounted on glass slides (Superfrost/Plus; Menzel-Glaser, Braunschweig, Germany); after overnight adhesion at room temperature, the sections were fixed with 4% paraformaldehyde in phosphate-buffered saline containing 0.1% saponin, washed, and permeabilized with 0.1% saponin (Sigma, St. Louis, MO.) in phosphate-buffered saline. Endogenous peroxidase activity was blocked with 1% H_2O_2 and 0.02% NaN_3. The tissue sections were subsequently incubated with the cytokine-specific MAbs at 4°C overnight. The detection and amplification were performed using Envision horseradish peroxidase system (Dako, Glostrup, Denmark). A chromogen (diaminobenzidine) was finally applied according to the manufacturer's instructions. The sections were then washed with distilled water, counterstained with Harry's hematoxylin, dehydrated, and mounted with Entellan (Merck). Entire tissue sections were examined using an Olympus IMT2 microscope, excluding lymphoid follicles, since their random distribution in the tissue specimen may otherwise generate less consistent results. However, very few cytokine-containing cells were observed within the follicles. Positive lymphocytes in epithelium or lamina propria were manually enumerated in a section with a magnification of ×300. The total section area was calculated by computer analysis (Metamorph 4.5 r6 software; Universal Imaging Corporation) with a magnification of ×10. Lymphocyte staining in epithelium or lamina propria was expressed as the number of positive cells per square millimeter of mucosa. Only cells with a distinct intracellular (cytoplasmic) staining were included. The epithelial cytokine-stained area was determined by computer analysis, and the results were expressed as a percentage of stained epithelial area to total epithelial area in each section. Surface epithelial area (including foveola, neck, and pit) was evaluated separately from gland epithelium. All evaluations were performed by the same observer, who was unaware of the patient's *H. pylori* status and histology findings. The mean tissue section area was 1.15 mm^2, ranging from 0.50 to 2.4 mm^2. One section per biopsy sample was analyzed for each cytokine, as the use of a single tissue section to represent immunostaining of an entire biopsy sample, had previously been validated in a reference study (32). The same study validated the representativity of expression of cytokines IL-4, TNF-α, and IFN-γ in each antral biopsy specimen, whereas the expression of TGF-β and IL-8 differed substantially in biopsy specimens from different antral regions in each patient. Therefore, the consistency of TGF-β and IL-8 staining in three different antral biopsy specimens per patient was assessed in three *H. pylori*-positive cases and in three *H. pylori*-negative cases at the start of the present study. The mean values for variation between biopsy specimens when the three biopsy specimens from the same subject were compared were 21.6% (range, 15 to 26%) for epithelial expression of IL-8 and 13.6% (range, 0 to 20%) for epithelial expression of TGF-β. The mean values for variation for lamina propria lymphocyte staining were 3.1% (range, 0 to 19%) for IL-8 and 4.5% (range, 0 to 13%) for TGF-β. In the majority of cases, no lamina propria lymphocyte staining could be detected, and in these instances, the sections from the three biopsy samples were all negative. As the numbers of cytokine-producing cells and the proportion of positively stained epithelium in this material showed much less variation than the reference study, only one biopsy specimen per subject was subsequently evaluated.

Statistics. The nonparametric Mann-Whitney U test and Fisher's exact test were used for statistical evaluation of comparisons between the two groups (*H. pylori* positive and negative) and for numerical and categorical variables, respectively. *P* values of ≤0.05 were considered statistically significant. Spearman's rank correlation coefficients were calculated to evaluate correlations between variables. Statistical analysis was performed using SAS V8.2.

RESULTS

Histopathological evaluation. Most *H. pylori*-positive patients showed slight to moderate chronic gastritis. Antrum and corpus inflammation scores were higher in *H. pylori*-positive cases (median antrum score, 2.0 [range, 1 to 2]; median corpus score, 1.0 [range, 1 to 2]) compared to *H. pylori*-negative cases (median antrum score, 1.0 [range, 1]; median corpus score, 1.0 [range, 0 to 1]), with a statistically significant difference in antrum inflammation ($P = 0.007$). Similarly, *H. pylori*-positive cases showed higher degrees of activity (median antrum activity score, 1.0 [range 0 to 2]; median corpus score, 0.0 [range 0 to 1]) than *H. pylori*-negative cases (median antrum activity, 0 [range, 0]; median corpus activity, 0 [range, 0]). Differences in antrum activity were statistically significant ($P < 0.001$). A positive correlation was found between chronic inflammation and activity scores in the antrum ($r = 0.705$ and $P = 0.001$), but not in the corpus, which was on the border of being significant ($P = 0.051$). In *H. pylori*-positive cases, the median *H. pylori* density scores were 2.0 in the antrum (range, 2.0 to 3.0) and 1.0 in the corpus (range, 1.0 to 2.0). No positive correlation was found between *H. pylori* density scores and chronic inflammation or activity scores in either the antrum or corpus. Lymphoid follicles were present in 4/10 *H. pylori*-positive cases and in 1/10 *H. pylori*-negative cases (antrum or corpus). In one *H. pylori*-positive case, hyperplastic and regenerative features of superficial epithelium were observed in the antrum. Duodenal histology showed a slight unspecific inflammation in 9/10 *H. pylori*-positive cases and in 9/10 *H. pylori*-negative cases and was normal in the remaining cases. As duodenal histology was uniform in most cases and no significant differences were found in corpus scores between *H. pylori*-positive and -negative cases, only antrum gastritis scores were considered for subsequent correlation analysis between cytokine expression and histology scores.

TABLE 2. Frequencies of five cytokine positively stained biopsy specimens

Patients	Frequency of cytokine[a]				
	TNF-α	IFN-γ	TGF-β	IL-8	IL-4
H. pylori infected	8/10	9/10	9/10	10/10	10/10
Noninfected	10/10	7/10	10/10	7/10	8/10

[a] Number of biopsy samples with positively stained epithelium and/or lymphocytes per total number of individuals studied.

Frequency of immunostaining for different cytokines. The intracellular cytokine staining was predominantly localized to the cytoplasm of the cells. The cytokines studied could be detected in most of the *H. pylori*-infected and uninfected patients (Table 2). Concomitant expression of all five cytokines was seen in 8 of the 10 *H. pylori*-positive cases and in 5 of the 10 *H. pylori*-negative cases. However, all the uninfected subjects showed staining for at least two of the cytokines studied, in particular, TNF-α, TGF-β, and IL-4.

Cytokine staining of lamina propria and intraepithelial lymphocytes in antral mucosa. When lymphocyte staining (epithelium and lamina propria) was compared between the *H. pylori*-positive and -negative groups, no significant differences were noticed, although there was a tendency towards higher frequencies of cytokine-producing cells in the lamina propria of *H. pylori*-infected patients, especially with regard to IFN-γ and IL-8 (Fig. 1, left panels). The intraepithelial lymphocytes, on the other hand, had a similar pattern of cytokine production in

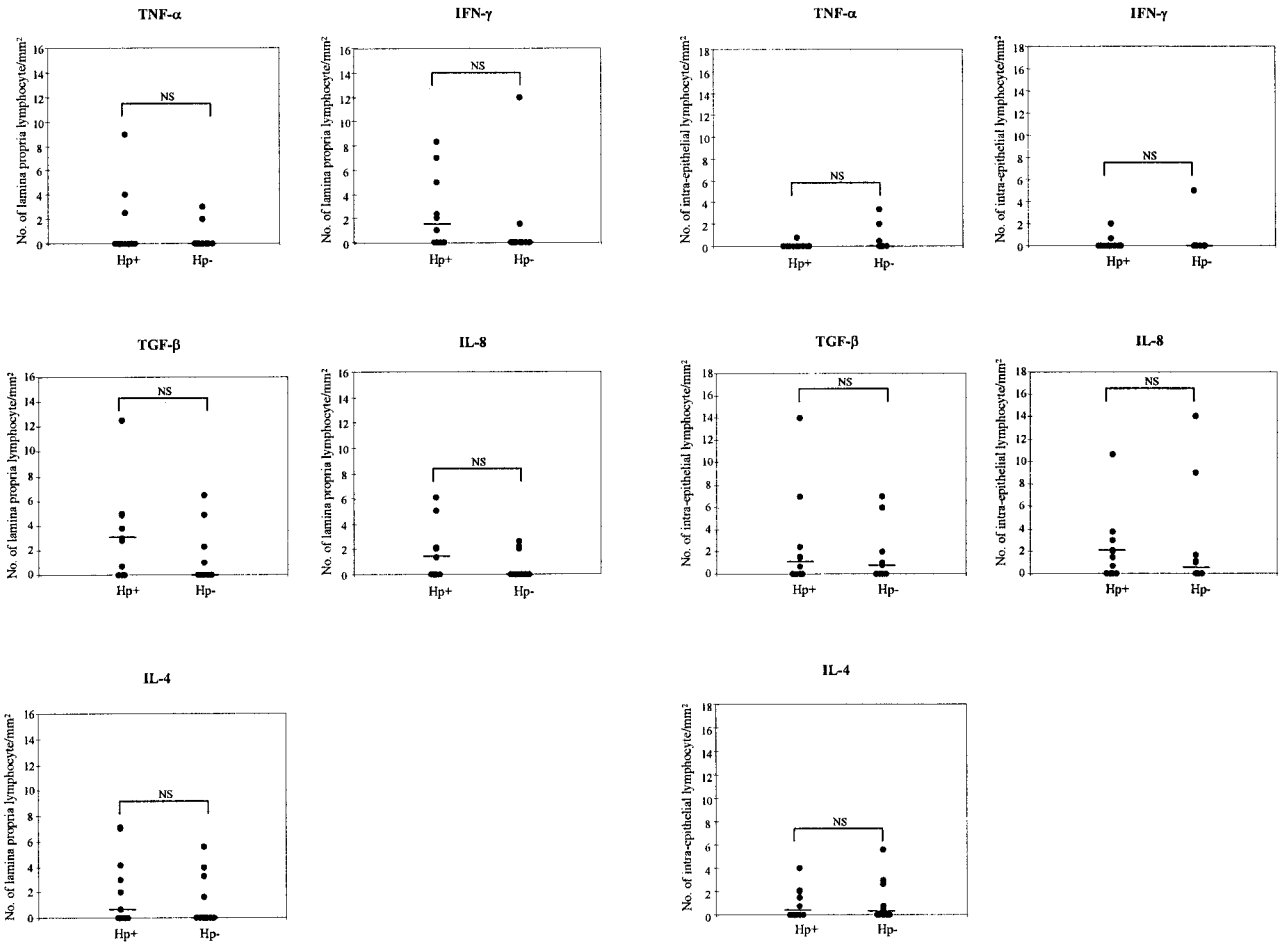

FIG. 1. Comparison of the cytokine staining of biopsy specimens from *H. pylori*-infected (Hp+) and uninfected (Hp-) subjects. The numbers of cytokine-specific stained lamina propria/mm^2 of tissue (left panels) and intraepithelial lymphocytes/mm^2 of tissue (right panels) are given. Each circle represents a biopsy specimen from one individual. Bars represent median values. NS, not significant (Mann-Whitney test).

both patient groups (Fig. 1, right panels). The largest numbers of positively stained lymphocytes located intraepithelially and in the lamina propria were observed for TGF-β and IFN-γ. The positive lymphocytes in the lamina propria were usually observed surrounding the glands or beneath the surface epithelium, particularly TGF-β-containing cells. A significant correlation was found between intraepithelial lymphocyte staining and lamina propria lymphocyte staining for TGF-β ($r = 0.478$ and $P = 0.033$) and for IFN-γ ($r = 0.614$ and $P = 0.004$).

The expression of IFN-γ and IL-8 in lamina propria lymphocytes correlated significantly with antrum chronic inflammation ($r = 0.539$ and $P = 0.014$ for IFN-γ and $r = 0.446$ and $P = 0.048$), but there was no further correlation between the degree of active or chronic antrum inflammation and the staining of the epithelium or lamina propria. Furthermore, the *H. pylori* antrum density score (in the *H. pylori*-positive group) did not correlate with lymphocyte cytokine expression.

Cytokine staining of gastric epithelial cells. In addition to the cytokine staining of lymphocytes, substantial cytokine staining was localized to gastric epithelial cells in the superficial epithelium and antral glands and was seen in *H. pylori*-positive and -negative cases (Fig. 2 and 3). The cytokine staining of epithelial cells showed a large variability between subjects for all the cytokines studied. Positive epithelial staining was especially seen for TGF-β, IL-8, and IL-4 and was localized to both superficial epithelial cells and to antral glands. However, the proportion of the epithelial cytokine-specific staining did not differ significantly between *H. pylori*-positive and *H. pylori*-negative cases for any of the cytokines studied, even though IL-8, IFN-γ, and IL-4 staining was slightly higher in the glandular epithelium of *H. pylori*-positive patients (Fig. 3, right panels). Epithelial staining for IL-8 was always seen in specimens from the infected subjects but was seen in only 6/10 uninfected subjects, in whom the staining intensity was also weaker (Fig. 2). IL-4 was always expressed in *H. pylori*-positive cases, where larger surface staining was observed than in *H. pylori*-negative cases, but without significant differences. IFN-γ staining was usually detected in *H. pylori*-positive cases (9/10), but it was detected in only some *H. pylori*-negative cases (gland, 5/10; surface epithelium, 4/10). The proportions of epithelial staining of the anti-inflammatory cytokine TGF-β did not differ between the *H. pylori*-positive and *H. pylori*-negative subjects. This cytokine was always detected in *H. pylori*-negative subjects and in 9/10 *H. pylori*-pos-

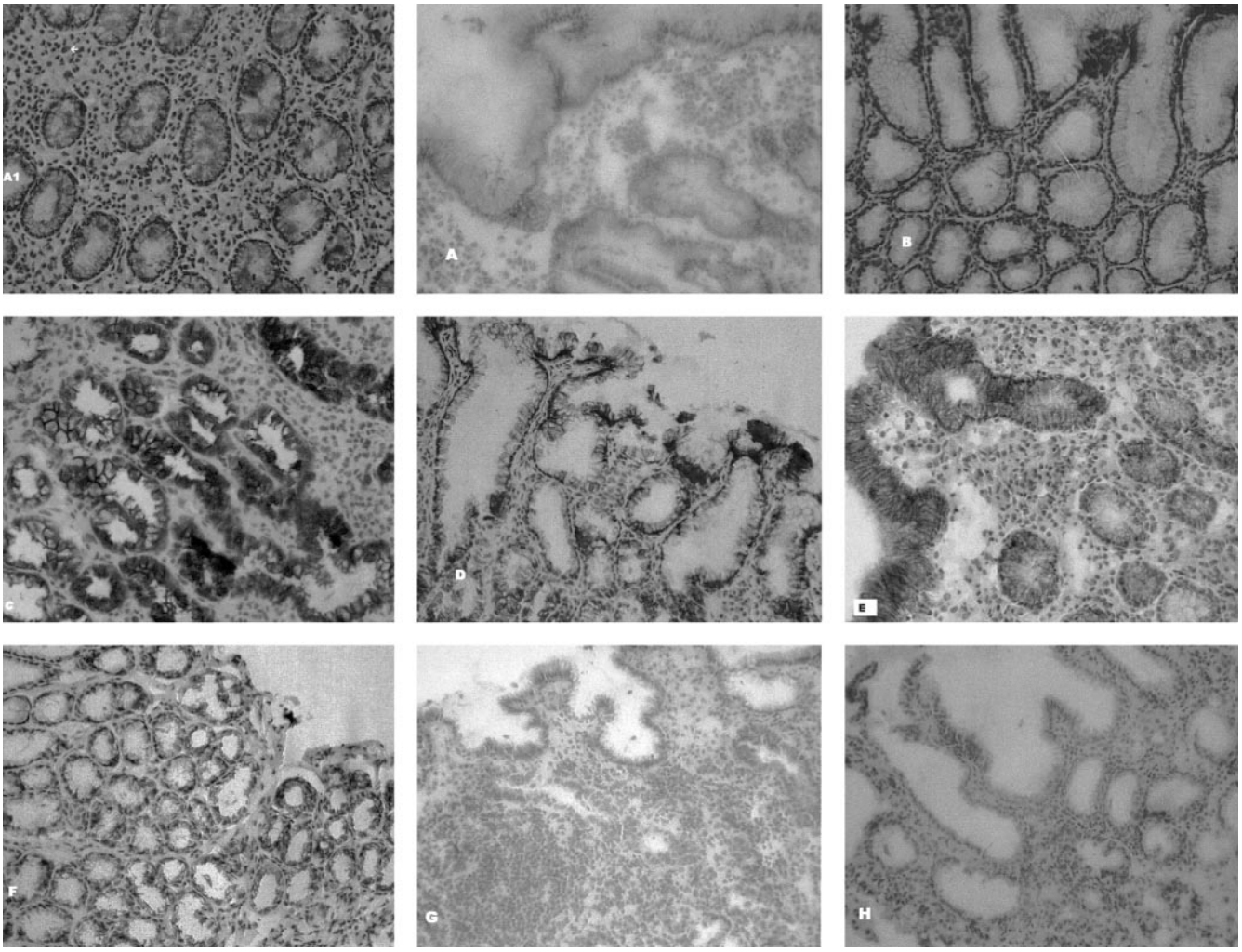

FIG. 2. Microphotographs showing immunohistochemical detection of cytokines in cryopreserved antral tissue specimens from *H. pylori*-infected and uninfected subjects. (A1) Biopsy specimen from an *H. pylori*-infected subject showing IFN-γ staining of MNCs in the lamina propria. Original magnification, ×300. (A and B) Epithelial IFN-γ-specific staining of *H. pylori*-infected and uninfected subjects, respectively. Original magnification, ×300. (C and D) Epithelial IL-8-specific staining of *H. pylori*-infected and uninfected subjects, respectively. Original magnification, ×300. (E and F) Epithelial TGF-β-specific staining of *H. pylori*-infected and uninfected subjects, respectively. Original magnification, ×300. (G and H) Isotype controls from *H. pylori*-infected (G) and uninfected (H) subjects. Original magnification, ×300.

itive cases. TNF-α staining was also similarly found in *H. pylori*-positive (5/10) and *H. pylori*-negative cases (5/10).

The frequency of lamina propria lymphocyte staining correlated significantly with epithelial staining of the epithelial surface and glands, respectively, for TGF-β ($r = 0.564$ and $P = 0.010$; $r = 0.509$ and $P = 0.022$), IFN-γ ($r = 0.638$ and $P = 0.003$; $r = 0.794$ and $P < 0.001$), and TNF-α ($r = 0.584$ and $P = 0.007$; $r = 0.588$ and $P = <0.006$). On the other hand, no correlation was found between antrum histology scores (inflammation and activity) or the colonization density in the *H. pylori*-positive group and epithelial cytokine expression.

Cytokine expression in subgroups of *H. pylori*-negative individuals. Since 2/9 cases in the *H. pylori*-negative group, as determined by biopsy specimen-based criteria, had serological evidence of a previous *H. pylori* infection (specific anti-*H. pylori* ELISA), a subsequent statistical analysis was performed after exclusion of these two *H. pylori*-negative cases (10 *H. pylori*-positive versus 7 *H. pylori*-negative cases). However, very similar results (both for lymphocyte and epithelial cell staining) were found in this subset of individuals, compared to the previous study sample, concerning all the cytokines studied (data not presented).

Genotyping of *H. pylori* strains. Strains were *cagA* negative and harbored the *vacA* s2 (type II genotype). The *oipA* gene was not functional in all of the cases studied. The results obtained for the *cagE* and *cag* PAI empty site corroborate the results for the absence of the *cag* PAI, except in one case, with a mixed infection of *cag* PAI-positive and -negative strains.

DISCUSSION

In the present study and to our knowledge for the first time in a pediatric population, we have evaluated in situ expression

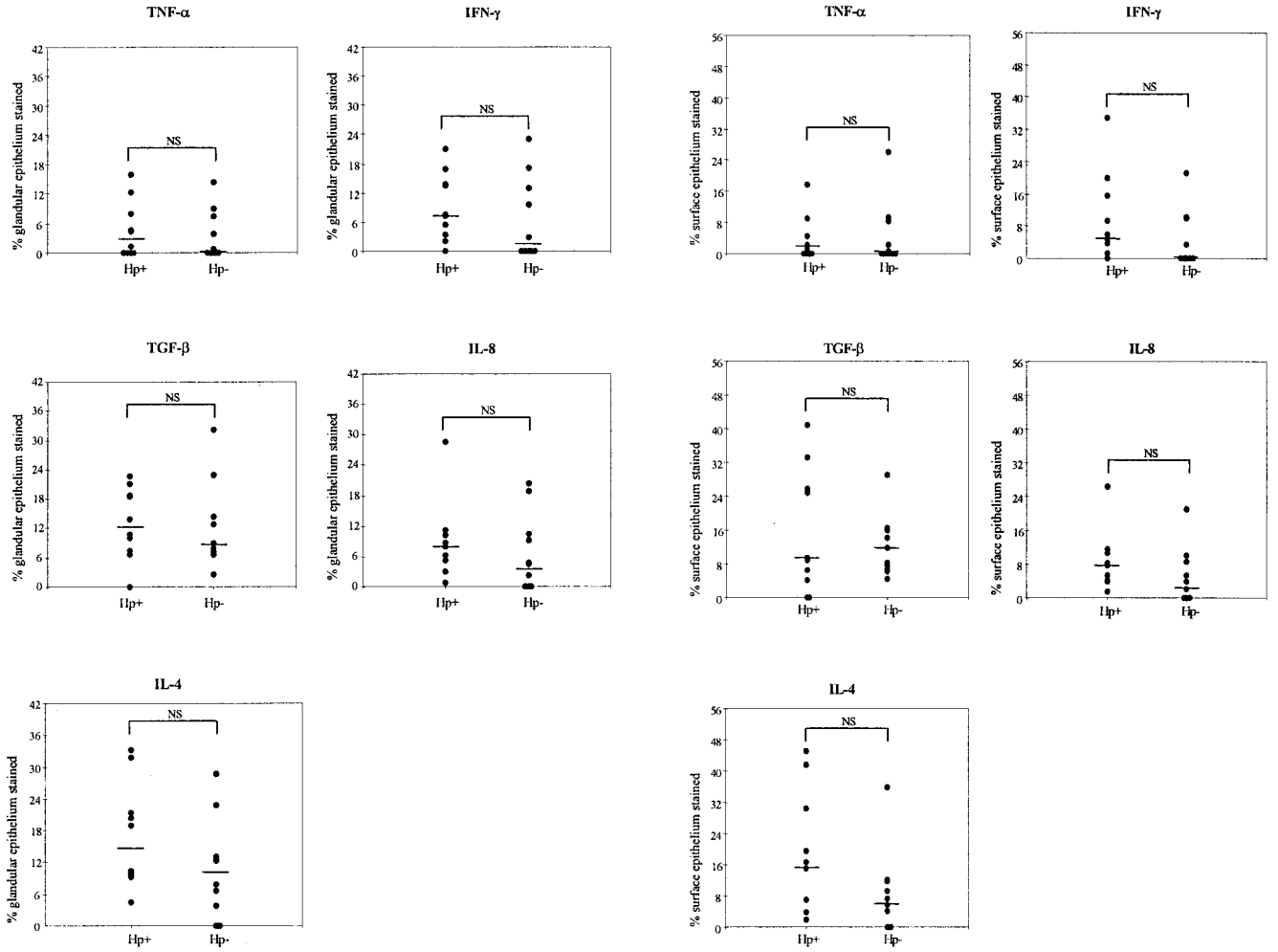

FIG. 3. Epithelial cytokine staining in *H. pylori*-infected (Hp+) and uninfected (Hp-) subjects. Staining of both superficial epithelium (left panels) and epithelial cells from glands (right panels) were expressed as a percentage of the total surface and glandular epithelial area in the section, respectively. Each circle represents a biopsy specimen from one individual. Bars represent median values. NS, not significant (Mann-Whitney test).

of several cytokines in gastric mucosa. Children may be regarded as an interesting natural model for the study of *H. pylori* infection, not only because they are not usually submitted to gastric mucosal noxa, such as alcohol, tobacco, and anti-inflammatory medication, but also because of marked differences from adults regarding clinical course and gastric mucosal histopathology. In particular, children have less intense MNC and polymorphonuclear cell infiltration (4, 5, 20). Considering the shorter duration of *H. pylori* infection in children, gastric mucosal changes may represent an earlier stage of the immunoinflammatory response compared to the adult host, and so a different immunopathology might be anticipated. In the present study, the expression of the cytokines studied in both epithelial cells and lymphocytes from lamina propria and epithelium did not differ significantly between *H. pylori*-positive and -negative cases, and wide variation in each group was observed.

Surprisingly, cytokine expression did not correlate with antrum inflammation scores. Globally, these results do not totally agree with similar adult studies in *H. pylori*-associated gastritis that mostly show a predominant Th1 profile with increased levels of IFN-γ, but not IL-4 and IL-5, in *H. pylori*-infected gastric mucosa (6, 10, 28, 32, 38, 48). Furthermore, D'Elios et al. (15) have shown that T-cell clones generated from antral biopsy specimens of *H. pylori*-infected peptic ulcer patients produce IFN-γ and IL-12 but usually not IL-4 or IL-5 in response to *H. pylori* antigen stimulation. However, recently, Holck et al. (27) found increased numbers not only of cells producing IL-8 (surface epithelium) and IFN-γ (lamina propria MNCs) but also of cells producing IL-10 (lamina propria MNCs) in infected adult patients, compared to uninfected subjects, which may counteract the inflammatory effect of the Th1 response.

The absence of differences between the groups may be at least partially related to the fact that *H. pylori*-negative cases (status according to culture and histology) had some degree of gastritis. Other studies have also demonstrated mild chronic gastritis in a large proportion of uninfected children (9, 34). Even in the absence of clinical evidence of any chronic gastrointestinal disease, another etiology for gastritis (including re-

cent viral infections, common in children) cannot be ruled out. Obviously, only symptomatic children requiring endoscopy were included, as a study design including completely healthy children would be ethically unacceptable, and in the former group, patients with normal mucosa are relatively rare. Moreover, although the possibility of a missed past *H. pylori* infection cannot be totally excluded and it might have accounted for some degree of inflammation in the two/nine *H. pylori*-negative cases with positive serology, subsequent cytokine analysis did not show differences after their exclusion. It is not known how long it would take for the changes in mucosal cytokine levels to normalize after a gastric infection (whether or not due to *H. pylori*). If cytokine levels remain high for a long time, our findings of cytokine expression in some of the *H. pylori*-negative cases could be due to a previous, unrecognized *H. pylori* infection (spontaneous eradication) or to another infection.

Although the potential contribution for cytokine expression of strain-related virulence factors in the *H. pylori* group could not be determined in our study, as the strains were of the type II genotype (nonfunctional *oipA*), the absence of *cagA*-positive strains with a functional *oipA* might be one explanation for the low proinflammatory response compared to those reported for adults (40, 48). The less pathogenic type II genotype is the most frequently found genotype in Portuguese children with nonulcerative gastritis and has been reported in other similar pediatric populations (2, 14, 39).

Previous studies on cytokine responses in *H. pylori*-infected children have yielded somewhat conflicting results (8, 9, 26, 31, 34, 41). However, most studies report increased levels of IL-8 and IFN-γ, as in adults, where elevated levels of IL-8 and Th1 responses are hallmarks of *H. pylori*-induced gastritis. IL-8 is mainly secreted by epithelial cells and is a strong neutrophil attractant, and it is the most extensively investigated cytokine in *H. pylori*-associated disease (3, 23, 32, 38, 48). However, IL-8 was also detected in the epithelium of normal gastric mucosa in some studies (10, 32). It is interesting, though, that the difference in IL-8 production, as measured by immunohistochemistry, is much higher in *H. pylori*-infected and uninfected adults (8) than in children (9; this study). A similar phenomenon is seen when IFN-γ levels in adults and children were compared. Both immunohistochemistry (32; this study) and analysis of secreted cytokines (8) demonstrate lower levels of IFN-γ in children and less pronounced differences in infected and uninfected children than in adults.

Perhaps the most unexpected result of the present study was the presence of IL-4 in both *H. pylori*-infected and uninfected cases, which is in contrast to previous data from adult populations (6, 28, 32). In animal systems, Th2 cell responses comprising IL-4 and IL-5 have been associated with humoral responses and a reduction in bacterial load (36). The fact that children display IL-4 responses in the stomach, regardless of *H. pylori* status, and lower levels of IFN-γ may indicate that children are more prone to mounting a gastric Th0 or Th2 response than adults. The lower gastritis scores in children may also be a reflection of such a skewed Th1/Th2 balance, which may result in a lower risk for developing ulcer disease.

Finally, TGF-β was the sole cytokine observed uniformly in all individuals. This suggests a role as a constitutive homeostatic cytokine also in children, in agreement with previous findings of this cytokine in normal fundic mucosa of adults (37). The inflammatory effects induced by the proinflammatory cytokines might be counteracted by IL-4 but also by other anti-inflammatory cytokines, such as TGF-β, locally produced by immune cells or by gastric epithelial cells. However, TGF-β production in *H. pylori* infection has not yet been extensively studied (32, 37, 43). This type of response could be relevant in trying to neutralize the Th1 response and stimulate Th2 and humoral responses, as it as been clearly demonstrated that children with *H. pylori* infection mount a humoral response, at both the systemic and local levels (7, 13, 45).

It remains to be determined whether the mucosal inflammatory response in the pediatric population is downregulated in response to different infectious agents and whether this is related to the earlier stage of *H. pylori* infection or to age-dependent immunological maturation. Another interesting possibility deserving evaluation is that pediatric *H. pylori* infection could lead to a higher activity of regulatory T cells and thereby suppress proinflammatory responses. This could explain the lower degree of gastritis and higher *H. pylori* colonization rate and may lead to the persistence of infection in children until adulthood. Indeed, the presence of circulating specific regulatory T cells in human *H. pylori* infection has recently been demonstrated (33). Later in the course of the infection, the persistence of *H. pylori* at the surface of the gastric mucosa may eventually favor a predominant Th1-type gastritis, as reported for adults.

Our data are in agreement with previous immunohistochemistry studies showing a substantial contribution of the epithelium to cytokine expression. These data suggest a substantial contribution of the epithelium not only to proinflammatory and regulatory responses but also to anti-inflammatory responses, similar to evidence in the adult host. The positive correlation observed between epithelial cell expression and the number of stained lymphocytes (lamina propria and/or epithelial) for some of the cytokines studied could suggest that at least for some cytokines, such as IFN-γ, produced only by immune cells, the epithelial staining might represent receptor binding of locally produced cytokines, rather than epithelial production. However, IFN-γ was also found in biopsy samples without detectable lymphocyte staining, suggesting the possible involvement of immune cells secreting small amounts of cytokine or positioned at distinct sites in the mucosa. Experiments on gastric epithelial cell lines suggest that IFN-γ may induce class II HLA expression on the epithelium, leading to increased *H. pylori* attachment, and possible epithelial cell apoptosis (22).

In conclusion, the present study indicates similar levels of gastric mucosal cytokine expression in *H. pylori*-positive and -negative pediatric cases, a finding that is in stark contrast to the situation in the adult host. Additionally, we have observed a considerable contribution of gastric epithelium to the antral cytokine response, similar to previous adult studies, suggesting a relevant role of the epithelium in the immunopathogenesis and outcome of infection. Further pediatric studies are warranted to characterize local immunopathology and cytokine responses in children.

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