T Helper 1 Effector Cells Specific for *Helicobacter pylori* in the Gastric Antrum of Patients with Peptic Ulcer Disease

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Chronic antral gastritis following *Helicobacter pylori* (Hp) infection is characterized by a cellular inflammatory infiltrate whose cytokines may represent a host-dependent factor influencing the outcome of the infection. The pattern of cytokines produced by the immunologically active cells in the gastric antrum was analyzed at the mRNA level in antral biopsies from five Hp-infected patients with duodenal ulcer and three Hp-negative dyspeptic controls. T cell clones were generated from parallel antral biopsies of the same Hp-infected patients and assessed for reactivity to Hp Ags, cytokine profile, and effector functions. Antral biopsies from all Hp-infected patients showed IFN-γ, TNF-α, and IL-12, but not IL-4, mRNA expression, whereas no cytokine mRNA signal was found in the mucosa of controls. A total of 24 out of the 163 $CD4^+$ T cell clones (15%) derived from Hp-infected patients proliferated in response to a Hp lysate; 11 clones (46%) also reacted with Cag-A, 2 with Vac-A, and 1 with urease. Upon Ag stimulation, 20 out of the 24 Hp-reactive clones (83%) produced IFN-γ, but not IL-4 or IL-5 (Th1-like), whereas 4 produced IFN-γ, IL-4, and IL-5 (Th0-like). All Hp-specific clones secreted high levels of TNF-α. At low T:B cell ratio, Hp-specific clones expressed Ag-dependent helper function for B cell proliferation and Ig production, whereas at higher T:B cell ratios, 15 Th1 and 2 Th0 clones lysed Ag-pulsed autologous EBV-transformed B cells. Results provide evidence for Hp-specific Th1 effectors in the gastric antrum of Hp-infected patients, where they may play a role in the genesis of either peptic ulcer or Hp-associated gastric B cell lymphoma. *The Journal of Immunology,* 1997, 158: 962–967.

Patients with duodenal ulcer disease invariably harbor *Helicobacter pylori* (Hp)[2] infection of the gastric antrum and an associated chronic antral gastritis, characterized by mucosal infiltration of polymorphonuclear and mononuclear leukocytes. Evidence for a pathogenetic role of Hp infection in ulcer disease comes mainly from clinical observation that removal of Hp speeds up ulcer healing (1, 2), prevents ulcer relapse, and reduces ulcer complications (3). The association of Hp with ulcer disease is not specific, since Hp infection may be present in asymptomatic population, as well as in patients with nonulcer dyspepsia, gastric lymphoma, or carcinoma (4–6).

The more severe pathologies observed in Hp-associated disease, including peptic ulcer, gastric atrophy and cancer, are associated with infection by a subclass of Hp strains that express a 128-kDa immunodominant Ag (Cag-A) and a potent cytotoxin (Vac-A) (7, 8). In a mouse model of Hp infection, these strains cause gastric epithelial necrosis and infiltration of inflammatory cells into the lamina propria (9). In this model, intragastric administration of purified Vac-A results in epithelial erosion but no inflammatory cell infiltration (10). Hence, while the cytotoxin is likely to be responsible for the gastric epithelial erosion observed in Hp-infected patients, other factors are involved in generating and maintaining the inflammatory response. Recent evidence indicates that induction in epithelial cells of the neutrophil chemotactic factor IL-8 may contribute to Hp-associated gastric inflammation (11).

The nature of cytokines produced during the immune response to Hp may represent another, host dependent, factor able to influence the outcome of infection, as well as the type of tissue inflammatory reactions induced. During the effector specific immune response, different patterns of cytokine release are characteristic of certain Th cell subsets, whose polarized forms are termed Th1 and Th2. The former mediates cell-mediated immunity and B cell production of opsonizing and complement-fixing Abs, whereas the latter induces the production of high levels of Abs, including IgE and eosinophilia. In the last few years, IL-12, which is produced by phagocytic cells and other accessory cells in response to bacteria and other pathogens (12), has emerged as a central cytokine in determining the outcome of the effector Th cell response (13). IL-12 induces the rapid production of IFN-γ by NK cells and T cells and, in association with IFN-γ, promotes the differentiation of the naive Th cells into the Th1 phenotype (14–16).

In order to characterize the functional profile of cells participating in the inflammatory reaction induced by Hp infection, we have examined the patterns of cytokines produced by the immunologically active cells in the gastric antral infiltrates of Hp-infected patients with peptic ulcer. The results showed expression of IL-12, IFN-γ, and TNF-α, but not IL-4, in the antral mucosa of these patients. More importantly, a remarkable proportion of $CD4^+$ T cell clones, generated from the gastric mucosa of the same patients, were specific for Hp Ags and most of them exhibited a Th1-like cytokine profile as well as Th1-related effector functions.

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[2] Abbreviations used in this paper: Hp, *Helicobacter pylori*; Cag-A, cytotoxin-associated protein; Vac-A, vacuolating cytotoxin; HSP, 60-kDa heat shock protein; MR, maximum release; SR, spontaneous release; RT-PCR, reverse transcriptase-polymerase chain reaction; EBV-B cells, Epstein-Barr virus-transformed lymphoblastoid B cell lines.

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Materials and Methods

Reagents

Aqueous extract of Hp was prepared by resuspending pelleted bacteria (NCTC11637 strain) in 2 vol and vortexing vigorously for 1 min. Bacteria and cell debris were removed by centrifugation and the protein concentration in the extract was measured using a bacterial cell lysate Ag protein assay reagent kit (Pierce, Rockford, IL). Recombinant Cag-A was expressed and purified from *Escherichia coli* (S. Censini and A. Covacci, unpublished observations). Recombinant Vac-A and recombinant heat shock protein (HSP) were purified from *E. coli* as previously described (17, 18). Urease was purified from Hp as previously described (9). Tetanus toxoid was kindly provided by Istituto Sieroterapico e Vaccinogeno Sclavo (Siena, Italy). Human rIL-2 was kindly provided by Eurocetus (Milano, Italy), human rIL-5 was purchased from Amgen Biologicals (Thousand Oaks, CA). PHA was purchased from Life Technologies (Grand Island, NY) and PMA from Sigma (St. Louis, MO). Staphylococcal enterotoxins SEA, SEB, SED, and SEE were purchased from Serva (Heidelberg, Germany). Anti-CD3, anti-CD4, and anti-CD8 mAb were purchased from Ortho Pharmaceuticals (Raritan, NJ).

Subjects

Eight patients (four males and four females, median age 46 yr, range 36–60) with dyspeptic complaints referred to the Department of Gastroenterology for upper gastrointestinal endoscopy were enrolled in the study. None of the patients had taken antibiotics or bismuth compounds within 3 mo before the study.

Multiple biopsy specimens were obtained during endoscopy from the gastric antrum (2 cm from the pylorus) for 1) histologic examination and Hp detection, 2) rapid urease test, 3) detection of cytokine mRNA expression, and 4) culture of infiltrating lymphocytes. Diagnosis of Hp infection in patients 1, 2, 3, 6, and 7 was based on positive urease test, histologic detection of Hp and serum IgG Ab response to Hp assessed by a commercial ELISA (Helori-test IgG, Eurospital, Trieste, Italy). All these patients were infected with Cag-A$^+$/Vac-A$^+$ Hp strains and showed severe chronic active antral gastritis associated with duodenal ulcer. In contrast, patients 4, 5, and 8, whose gastric histology showed minimal antral inflammation, were defined as Hp-negative because their urease test, histology, and serology provided no evidence for actual or previous Hp infection.

Preparation of mRNA, cDNA, and PCR

The biopsy specimens of antral mucosa were immediately frozen in liquid nitrogen and then homogenized using a mortar and pestle. mRNA was extracted by mRNA direct isolation kit (Qiagen GmbH, Hilden, Germany). The concentration and quality of mRNA samples were estimated by determing the absorbance at 260/280 nm and in all subsequent procedures all the samples and all the reactions were handled and performed under equal circumstances by the use of master mixes. As positive controls, mRNA was also prepared from normal PBMC activated with PHA or LPS. For cDNA synthesis the same amount of mRNA (50 ng) was used and cDNA was synthesized by Moloney murine leukemia virus-reverse transcriptase (MoMuLV-RT) (New England Biolabs, Beverly, MA) and oligo-(dT) primers according to the enzyme supplier's protocol.

cDNA mix of all samples was amplified under equal conditions by a 30-cycle PCR using IFN-γ, IL-4, TNF-α, IL-12 p35, and IL-12 p40 (Stratagene, La Jolla, CA) primers according to the manufacturer's instructions. One-third of the post-PCR sample was analyzed on a 2% Metaphor agarose gel (FMC, Rockland, ME), ethidium bromide stained, and photographed under UV light. β-Actin was assayed in all specimens to verify efficient cDNA synthesis from the extracted RNA. Human IFN-γ, IL-4, TNF-α, IL-12 p35, and IL-12 p40 specific primers were purchased from Stratagene; sense and antisense primers were located at nucleotides 475–503 and 4677–4702 for IFN-γ, 64–86 and 493–519 for IL-4, 174–197 and 504–528 for TNF-α, 296–317 and 688–709 for IL-12 p35, 199–219 and 553–572 for IL-12 p40, and amplifying a 501-, 456-, 355-, 414-, and 373-bp fragment, respectively.

Generation of T cell clones

A biopsy specimen of the antral gastric mucosa from each patient was cultured for 7 days in 2 ml RPMI 1640 medium supplemented with 2 mM L-glutamine, 2×10^{-5} M 2-ME (complete medium), 5% heat-inactivated human serum, and human rIL-2 (50 U/ml) in order to preferentially expand in vivo activated T cells. Mucosal specimens were then mechanically disrupted by repeated pipetting, and single T cell blasts were cloned under limiting dilution (0.3 cells/well) in round-bottom microwell plates containing 10^5 irradiated PBMC (as feeder cells), PHA (0.5% v/v), and IL-2 (20 U/ml), as reported elsewhere in detail (19). Cell surface marker analysis of T cell clones was performed on a Cytoron Absolute cytofluorimeter (Ortho Pharmaceuticals).

Screening assay for Hp Ag specificity of gastric T cell clones

T cell blasts (5×10^4) from all CD4$^+$ and CD8$^+$ clones were screened in triplicate cultures for their responsiveness to PHA and Hp Ags by measuring [^{3}H]TdR uptake after 60 h stimulation with medium alone, PHA (1% v/v), crude Hp lysate (10 μg/ml), and recombinant Cag-A, Vac-A, urease, or HSP (1 μg/ml) in the presence of irradiated autologous PBMC (5×10^4) as APC. Mitogenic index (ratio of mean cpm of stimulated to unstimulated cultures) >5 were considered as positive.

The great majority of Hp-specific T cell clones showed individual patterns of response to superantigens (four staphylococcal enterotoxins: SEA, SEB, SED, and SEE in the presence of allogeneic APC), suggesting a difference in their TCR Vβ-chain expression.

Characterization of the cytokine profile of T cell clones

To induce cytokine production by T cell clones in response to Hp Ags, 10^6 T cell blasts were cocultured in 1 ml complete medium with 5×10^5 irradiated autologous APC and the Hp lysate (10 μg/ml) (19). After 48 h, culture supernatants were collected, filtered, and stored in aliquots at −70°C until used. To induce cytokine production by T cell clones in the absence of APC, 10^6 T cell blasts were stimulated for 36 h with PMA (10 ng/ml) plus anti-CD3 mAb (200 ng/ml), as detailed elsewhere (20). Supernatants were assayed for IFN-γ, IL-4, IL-5, and TNF-α content. The quantitative determinations of IFN-γ or IL-4 and TNF-α were performed by commercial assays (BioSource International, Camarillo, CA, or Quantikine R&D Systems, Minneapolis, MN, respectively). For the measurement of IL-5, the murine LyH7.B13 cell line was used as source of indicator cells, as detailed elsewhere (19). Supernatants showing IFN-γ, IL-4, IL-5, or TNF-α levels 5 SD over the mean levels in control supernatants derived from irradiated feeder cells alone were regarded as positive. T cell clones able to produce IFN-γ, but not IL-4 or IL-5, were categorized as Th1, clones able to produce IL-4 and/or IL-5, but not IFN-γ, were categorized as Th2, and clones producing both IFN-γ and IL-4 or IL-5 were categorized as Th0.

Preparation of B cells and immortalization of B cells with EBV

B cell-enriched suspensions were prepared by a double-step rosetting with neuraminidase-treated SRBC, as described elsewhere (21). Peripheral blood B cell-enriched suspensions usually consisted of 68 to 87% B cells, 9 to 21% monocytes, and <1% T cells. They will be referred to as B cells. To obtain EBV-transformed lymphoblastoid B cell lines (EBV-B cells), B cells were incubated for 48 h with supernatant of the EBV-producing marmoset cell line B95.8 and subsequently expanded in complete medium supplemented with 15% FCS.

Assays for helper function to B cells by T cell clones

The ability of Ag-stimulated T cell clones to induce polyclonal B cell activation was assessed by measuring [^{3}H]TdR uptake by B cells (3×10^4) cocultured for 4 days with irradiated (2000 rad) autologous clonal T cell blasts (3×10^4) in the absence or presence of the specific Hp Ags. The cell culture system used for the induction of Ig synthesis was performed in duplicate tubes by using complete medium supplemented with 10% FCS, as described (21). B cells (5×10^4) were cultured alone or with autologous clonal T cell blasts (5×10^4) in the absence or presence of the specific Hp Ags. After 10 days, culture supernatants were collected and assayed for their Ig content. The immunoradiometric assay used for detecting IgM, IgG, and IgA has been described previously in detail (22).

Assay for cytolytic activity

Cytolytic activity of clones was assessed as reported elsewhere (21). Briefly, T cell blasts were washed three times, resuspended, counted, and divided into two aliquots. One aliquot was tested for cytolytic activity against murine ^{51}Cr-labeled P815 mastocytoma cells in the presence of PHA (1% v/v) (lectin-dependent cytolytic assay) with an E:T ratio of 10:1. After 4 h at 37°C, 0.1 ml supernatant was removed for measurement of ^{51}Cr release. Maximum release (MR) was obtained by treating target cells with 0.1 ml 1 M HCl. Spontaneous release (SR) was determined in control microcultures without effector cells. Specific lysis was calculated according to the formula: % specific lysis = 100 × (experimental release − SR)/(MR − SR). Cultures in which ^{51}Cr release exceeded the mean SR by >5 SD were considered positive for cytolytic activity. To assess the Ag-induced cytolytic activity of T cell clones, autologous EBV-B cells were

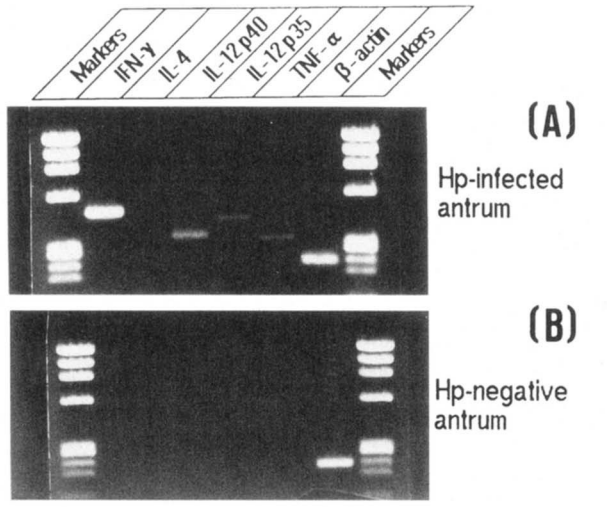

FIGURE 1. Cytokine mRNA expression in the gastric antral mucosa of a representative Hp-infected patient with duodenal ulcer (*A*) and an Hp-negative dyspeptic control (*B*). Biopsy specimens of gastric antral mucosa were homogenized and mRNA was extracted and converted into cDNA. Samples were amplified under equal conditions by a 30-cycle RT-PCR and analyzed on 2% Metaphor agarose gel.

incubated overnight with Hp Ags (20 μg/ml), labeled with ^{51}Cr as detailed elsewhere (21), and incubated with the second aliquot of clonal T cell blasts at an E:T ratio of 10:1. After centrifugation, microplates were incubated for 8 h at 37°C and 0.1 ml supernatant was removed for measurement of ^{51}Cr release. The percent specific lysis was calculated according to the formula reported above.

Results

IFN-γ, TNF-α, and IL-12 mRNA expression in the gastric antral mucosa of Hp-infected individuals

Messenger RNA was extracted from biopsy specimens of gastric antral mucosa of Hp-infected and Hp-negative patients and subsequently amplified by RT-PCR in the presence of appropriate primers. Strong IFN-γ, TNF-α, and IL-12 p40 mRNA expression was found in the antral mucosa of all Hp-infected patients. IL-12 p35 mRNA was also expressed but at lower intensity, whereas IL-4 mRNA signal was consistently negative (Fig. 1). In the same series of experiments, biopsy specimens of the antral mucosa from the Hp-negative dyspeptic controls showed a clear signal for housekeeping genes, such as β-actin, but no cytokine mRNA expression (Fig. 1).

High proportion of Hp-specific CD4$^+$ T cell clones in the clonal progeny of T cells from the gastric antral infiltrates of Hp-infected individuals

Parallel biopsy specimens of antral mucosa from the five Hp-infected patients were cultured for 7 days in IL-2-conditioned medium in order to preferentially expand the in vivo activated T cells present in their antral infiltrates. T cell blasts were recovered and cloned by limiting dilution according to a high efficiency protocol using PHA stimulation in the presence of irradiated feeder cells and IL-2 (23). A total of 163 CD4$^+$ and 81 CD8$^+$ T cell clones were obtained and screened for their ability to proliferate in response to PHA and crude Hp lysate under MHC-restricted conditions. All 81 CD8$^+$ T cell clones proliferated to PHA, but none of them was responsive to the Hp lysate. Likewise, all 163 CD4$^+$ clones derived from the mucosa of Hp-infected donors proliferated to PHA, but 24 of them also showed significant proliferation (mitogenic index >5) in response to Hp lysate (Table I). The proportion of Hp-specific T cell clones in single patients was variable, ranging from 2% (patient 7) to 33% of CD4$^+$ clones (patient 6). No correlation was found between the proportion of Hp-specific T cell clones detected in each patient and the degree of inflammation in the gastric antrum. When assayed for proliferation to Hp lysate in the presence of irradiated allogeneic APC, none of the 24 Hp-specific clones showed mitogenic index higher than 2, hence excluding the possibility that the Hp lysate used in this study contained superantigens.

Table I. *Proliferative response to Hp crude and recombinant Ags of T cell clones derived from the gastric antral mucosa of Hp-infected patients*[a]

Cytokine Profile and T Cell Clone	Mitogenic Index in Response to:				
	Hp lysate	Cag-A	Vac-A	Urease	HSP
Th1					
2.11	264	302	ND	<5	<5
2.39	46	77	<5	<5	<5
2.44	381	428	ND	<5	<5
6.21	28	40	<5	<5	<5
6.23	237	498	<5	<5	<5
6.30	421	716	<5	<5	<5
6.39	119	302	<5	<5	<5
6.40	336	383	<5	<5	<5
6.41	58	437	<5	<5	<5
6.42	481	958	<5	<5	<5
6.62	18	49	<5	<5	<5
1.21	289	<5	<5	<5	<5
1.36	367	<5	<5	<5	<5
2.16	324	<5	<5	<5	<5
3.42	125	<5	<5	<5	<5
3.64	21	<5	<5	<5	<5
6.47	49	<5	<5	<5	<5
6.61	106	<5	<5	<5	<5
6.65	111	<5	<5	<5	<5
7.77	84	<5	<5	<5	<5
Th0					
2.26	108	<5	198	<5	<5
2.46	28	<5	32	<5	<5
2.42	13	<5	<5	37	<5
1.57	18	<5	<5	<5	<5

[a] T cell blasts from each clone (5×10^4) were seeded in triplicate cultures with irradiated autologous PBMC (5×10^4) in the presence of medium alone, crude Hp lysate (10 μg/ml) or Cag-A, Vac-A, urease, and HSP (1 μg/ml). After 60 h [^{3}H]TdR uptake was measured and expressed as mitogenic index.

In order to better define the specificity of the 24 Hp-specific CD4$^+$ clones, their proliferative response to four recombinant Hp Ags was evaluated. Eleven clones (46%) proliferated in response to both crude Hp lysate and even better to Cag-A, but not to Vac-A, urease, or HSP (Table I). Of the other 13 Hp-specific clones, two were specific for Vac-A and one for urease, but they failed to recognize Cag-A or HSP as did the remaining 10 clones, which were responsive only to crude Hp lysate (Table I).

Most Hp-specific T cell clones express the Th1-like cytokine profile

To characterize their cytokine profile, the 24 Hp-specific T cell clones were stimulated for 48 h with the Hp lysate in the presence of irradiated autologous APC or for 36 h with PMA plus anti-CD3 Ab and cytokine (IFN-γ, IL-4, IL-5, and TNF-α) secretion was assessed in culture supernatants. Under both experimental conditions, 20 Hp-specific clones (83%) showed a clear-cut Th1 profile. The results obtained upon stimulation with Hp lysate under MHC-restricted conditions are reported in Table II. A total of 2 out of the 3 clones from patient 1, 4 out of the 7 clones from patient 2, and all clones from patients 3, 6, and 7 (2, 11, and 1, respectively)

Table II. *Ag-induced cytokine production by Hp-specific CD4+ T cell clones derived from the gastric antral mucosa of Hp-infected patients with duodenal ulcer*

Ag Specificity and T Cell Clone	IFN-γ (ng/ml)	IL-4 (ng/ml)	IL-5 (U/ml)	TNF-α (ng/ml)
Cag-A				
2.11	2.8	<0.03	<0.2	3.9
2.39	0.8	<0.03	<0.2	1.4
2.44	1.2	<0.03	<0.2	1.6
6.21	1.8	<0.03	<0.2	2.6
6.23	2.4	<0.03	<0.2	1.5
6.30	2.0	<0.03	<0.2	2.8
6.39	3.4	<0.03	<0.2	8.4
6.40	1.5	<0.03	<0.2	6.6
6.41	0.6	<0.03	<0.2	9.2
6.42	1.7	<0.03	<0.2	2.8
6.62	0.8	<0.03	<0.2	4.4
Hp lysate				
1.21	3.0	<0.03	<0.2	1.7
1.36	6.5	<0.03	<0.2	2.9
2.16	6.7	<0.03	<0.2	1.3
3.42	1.6	<0.03	<0.2	0.9
3.64	2.8	<0.03	<0.2	2.6
6.47	0.6	<0.03	<0.2	5.4
6.61	1.5	<0.03	<0.2	7.7
6.65	0.7	<0.03	<0.2	4.9
7.77	1.9	<0.03	<0.2	1.4
Vac-A				
2.26	9.9	0.3	8.6	0.6
2.46	8.4	0.9	5.0	0.4
Urease				
2.42	5.5	0.7	1.5	1.9
Hp lysate				
1.57	8.5	0.6	2.2	2.8

[a] T cell blasts from each clone (10^6) were cocultured with irradiated autologous APC (5×10^5) in 1 ml in the presence of Hp lysate (10 μg/ml) or Cag-A, Vac-A, or urease (1 μg/ml). After 48 h, cell free supernatants were assayed for their IFN-γ, IL-4, IL-5, and TNF-α content. Mean cytokine levels in culture supernatants of irradiated APC alone were <0.1 ng/ml IFN-γ, <0.03 ng/ml IL-4, <0.2 U/ml IL-5, and <0.15 ng/ml TNF-α.

produced IFN-γ, but not IL-4 or IL-5. Interestingly, all the clones specific for Cag-A were Th1-like, whereas the 2 clones specific for Vac-A and the urease-specific clone produced IFN-γ, IL-4, and IL-5, showing thus a Th0-like profile (Tables I and II). Of note is the fact that all the 24 Hp-specific clones produced high concentrations of TNF-α, particularly upon stimulation with PMA plus anti-CD3 Ab (mean ± SE = 5.5 ± 0.8 ng/ml, range 1.3–12.5). This suggests that the high TNF-α mRNA expression found in fresh antral mucosa of the same patients probably reflects TNF-α production by activated T cells.

Hp-specific T cell clones express Ag-dependent B cell help

The ability of Hp-specific T cell clones to provide B cell help was then investigated. To this end, irradiated T cell blasts were cocultured at 1:1 ratio with autologous B cells in the absence or presence of the specific Ag. B cell proliferation was measured on day 4 and IgM, IgG, and IgA levels were measured in cellfree culture supernatants on day 10. In the absence of Ag, neither B cell proliferation nor increase in IgM, IgG, and IgA production above the spontaneous levels measured in cultures containing B cells alone were observed. In the presence of the specific Ag, all Hp-specific clones provided substantial help for B cell proliferation (mean mitogenic index 19, range 7–42) and IgM, IgG, and IgA synthesis (Fig. 2). No relationship was found between Ag specificity and degree of helper activity by Hp-specific clones, apart from the unique urease-specific clone, which was less efficient than the others in providing help for IgM and IgA synthesis.

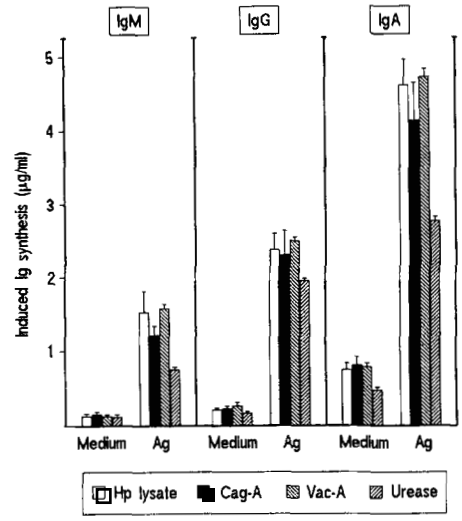

FIGURE 2. In vitro synthesis of IgM, IgG, and IgA induced in autologous B cells by Hp-specific CD4+ T cell clones stimulated with the specific Ag. T cell blasts from each clone (5×10^4) were cultured with autologous B cells (5×10^4) in the presence of the specific Hp Ag. After 10 days, cellfree culture supernatants were assayed for their Ig content by appropriate immunoradiometric assays, as previously described (22). The results represent the mean (± SE) Ig levels induced by T cell clones over the spontaneous Ig production in cultures of B cells alone.

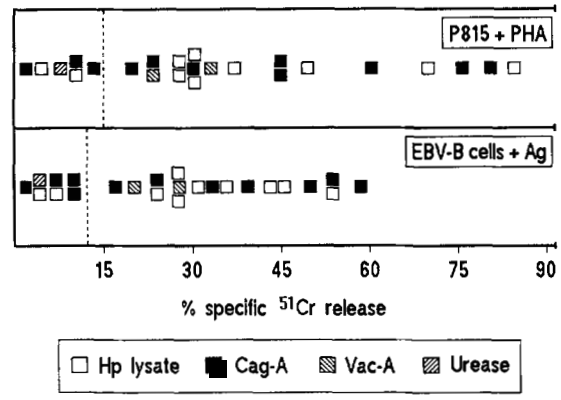

FIGURE 3. Cytolytic activity of individual Hp-specific T cell clones. Cytolytic activity of each clone was tested against ^{51}Cr-labeled P815 target cells at an E:T ratio of 10:1 in the presence of PHA (*upper panel*). The cytolytic activity of the same T cell clones was also tested at an E:T ratio of 10:1 against ^{51}Cr-labeled EBV-transformed autologous B cells pulsed with the specific Hp Ag (*lower panel*). Specific lysis was calculated as described in *Materials and Methods*. The dotted lines indicate 5 SD above the mean spontaneous release of target cells.

Hp-specific T cell clones express Ag-induced cytolytic activity against autologous Ag-presenting B cells

Since under certain experimental conditions activated Th1 clones can express cytolytic activity (21), the cytolytic potential of the Hp-specific clones was assessed in a lectin (PHA)-dependent ^{51}Cr-release assay with P815 murine mastocytoma cells as targets. At an E:T ratio of 10:1, 16 out of the 20 Th1 and 2 out of the 4 Th0 clones specific for Hp Ags showed PHA-induced cytolytic activity (Fig. 3). In a parallel experiment, Ag-pulsed autologous EBV-induced lymphoblastoid B cells were used as target cells for Hp-specific clones. A total of 15 of the 16 Th1 and the 2 Th0 clones that were cytolytic for P815 cells in the lectin-dependent assay also lysed autologous EBV-B cells previously incubated overnight with

the appropriate Hp Ags (Fig. 3), but not the same EBV-B cell targets pulsed with an irrelevant (tetanus toxoid) Ag (mean ± SD ^{51}Cr-release: 5.8 ± 1.2).

Discussion

This study demonstrates that CD4$^+$ T cells specific for Hp are present in the antral inflammatory infiltrates of Hp-infected patients with duodenal ulcer and expands present knowledge in the area of cell-mediated immune response to Hp infection (24–28). Our data are consistent with previous findings showing the predominance of CD4$^+$ vs CD8$^+$ T cells in antral biopsies of the majority of Hp-infected patients, especially in areas with germinal centers (29). They also extend the results reported by Di Tommaso et al. (30), who generated four Hp-specific T cell clones from the antral mucosa of a Hp-infected patient by preliminary in vitro T cell restimulation with Hp Ag. The cloning method used in our study was based on the preliminary expansion of in situ activated IL-2R-expressing T cells by short-term culture of gastric tissue specimens in IL-2-conditioned medium, followed by PHA stimulation of single T cells. This approach has already been successfully used to characterize the functional profile of T cells infiltrating the thyroid of patients with Hashimoto's thyroiditis (31), the retro-orbital tissue of patients with Graves' ophthalmopathy (32), and the bronchial mucosa of patients with allergic bronchial asthma (23). With this cloning method, the proportion of Hp-specific CD4$^+$ T cell clones generated from the gastric mucosa of Hp-infected subjects ranged from 2 to 33%, which is remarkably high compared with the frequency of Hp-specific T cells found in the peripheral blood of the same patients (between 1:1900 and 1:3400; data not shown). A reasonable objection may be that the high proportion of Hp-specific T cell clones merely reflects the IL-2-induced selective expansion of one or few Hp-specific T cells. Such a possibility, however, is unlikely, since the great majority of Hp-specific T cell clones expressed different TCR Vβ rearrangements, as indicated by their individual patterns of response to four different staphylococcal enterotoxin superantigens (data not shown). Thus, it is reasonable to conclude that Hp infection of gastric antrum elicits a powerful local inflammatory response that recruits and activates high numbers of Hp-specific effector Th cells.

When assessed for their cytokine secretion profile, most of Hp-specific T cell clones generated from the gastric mucosa of Hp-infected patients produced IFN-γ and TNF-α, but neither IL-4 nor IL-5, thus exhibiting a clear-cut Th1-like pattern. The possibility that this outcome does not reflect the real functional attitude of effector Th cells in vivo, but it is the result of in vitro artifacts due to the cloning procedure, was considered. However, by using the same cloning protocol, allergen-specific Th0- and Th2-like clones were generated from the bronchial mucosa of grass pollen-sensitive asthmatic patients challenged with the specific allergen in vivo (23). More importantly, the predominance of a Th1-type T cell response found at clonal level was consistent with the mRNA pattern obtained by PCR amplification of cytokine gene transcripts in the antral biopsies of the same Hp-infected patients. Indeed, the expression of mRNA for IFN-γ and TNF-α, in the absence of IL-4 mRNA, strongly supports the possibility that in these patients a Th1-type response is occurring in vivo. Moreover, the constitutive expression of IL-12 mRNA (which is not detectable in the antrum of Hp-noninfected subjects) can provide an explanation for why Hp-specific T cells differentiate into a Th1-type effector cell population. IL-12 is produced by both macrophages and dendritic cells (reviewed in Ref. 13), and its production in response to phagocyte-activating infectious agents has been found to play a central role in the polarization of Th cells into the Th1-like profile (12–15).

The analysis of the fine Ag specificity of Hp-specific clones generated from the antral mucosa of Hp-infected patients showed that the predominant Hp Ag recognized by Th1 clones was Cag-A, the 128-kDa cytotoxin-associated protein (7), whereas other Hp Ags, such as the vacuolating cytotoxin (Vac-A) and urease, were recognized by a minority of gastric clones, all of which expressed a Th0-like profile. It is well known that high titers of anti-Cag-A Abs are detectable in the serum of Hp-infected patients with peptic ulcer (7, 33); moreover, marked spontaneous Cag-A-specific IgA production can be found in culture supernatants of antral biopsies from all Hp-infected subjects with duodenal ulcer (34). The data here reported provide additional evidence that, even at T cell level, Cag-A is the immunodominant Ag for Hp-induced responses. It is also of note that Cag-A Ag is expressed preferentially on the bacterial surface, whereas both Vac-A and urease may be released in vivo as soluble proteins. Such a difference may account for the distinctive cytokine profile of Cag-A- and Vac-A- (or urease)-specific T cell clones. Indeed, surface-bound Ags of microorganisms usually evoke Th1-type responses due to the induction of phagocytosis with consequent production of IL-12 (13), whereas soluble Ags more easily promote unpolarized (Th0-like) responses. Whether such a difference may explain why Cag-A-specific T cells are Th1, whereas Vac-A- and urease-specific T cells are Th0, remains to be determined.

The mechanisms by which the Hp-induced inflammatory reaction in the gastric antrum can exert ulcerogenic effect are still unclear. A direct ulcerogenic effect has been ascribed to the cytotoxin Vac-A, whose oral administration as purified 94-kDa protein caused in mice ulceration and gastric lesions showing similarities to the human pathology (10). Whether Cag-A and other Hp Ags may induce ulcer by a direct mechanism or indirectly through the immune response they elicit in the infected host is still unclear. The results of this study, showing that Hp infection evokes a Th1-type effector immune response at local level, allows us to raise different hypotheses. One possibility is that local IFN-γ production by Th1 cells favors the expression of MHC class II molecules by epithelial cells (29), which in turn enable these cells to present Hp Ag peptides to Th1 clones. This might trigger a cytotoxic response against epithelial cells and/or promote autoimmune-like phenomena. An alternative possibility is that MHC class II-positive epithelial cells, because of their inability to express costimulatory molecules required for full T cell activation (35), can induce anergy in Hp-specific effector T cells, thus hampering an efficient immune response against the pathogen and favoring its persistence and the consequent Hp-related damage to the infected stomach. A third, and more likely, possibility is that cytokines produced by the activated Th1-like cells are themselves responsible for the induction of tissue damage. Th1-derived cytokines can indeed activate macrophages to release proinflammatory cytokines, such as IL-1β, IL-8, and TNF-α, which have been detected in the gastric antrum of Hp-infected patients (36–38). TNF-α, whose effects are in general potentiated by IFN-γ, is able, at least in vitro, to stimulate gastrin secretion by rabbit and canine antral G cells (39, 40). Moreover, TNF-α induces dose-dependent death of, and marked increase of pepsinogen release by, guinea pig gastric chief cells in vitro (41). Whether human gastric epithelial cells are equally susceptible to cytokine-induced functional alterations remains a matter of future investigation.

An unusual complication of gastric Hp infection is the induction of low-grade B cell lymphomas of mucosa-associated lymphoid tissue which, like peptic ulcer, can recede following eradication of

Hp infection (42–44). The present results, showing that Hp-specific clones generated from the gastric tissue of Hp-infected patients act as potent helper cells for B cell proliferation and Ig production, provide convincing explanation for the intense B cell activation in the lymphoid tissue associated with, or newly generated in, the antral mucosa during Hp infection. Such a sustained and chronic Hp-induced T cell-dependent B-cell activation is responsible for the high levels of Hp-specific Abs found in the serum of Hp-infected patients, which decrease only some time after Hp eradication. However, in conjunction with other still undefined alterations, it may also represent an important mechanism leading to uncontrolled B cell proliferation and neoplastic transformation. The direct functional assessment of Th and B cell populations present in mucosa-associated lymphoid tissue lymphomas from Hp-infected patients would help us to better clarify this point.

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