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Blood lymphocyte proliferation, cytokine secretion and appearance of T cells with activation surface markers in cultures with *Helicobacter pylori*. Comparison of the responses of subjects with and without antibodies to *H. pylori*

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SUMMARY

A whole inactivated *H. pylori* bacterium preparation was found to stimulate blood mononuclear cells from both antibody-positive and antibody-negative subjects, but the antibody-positive subjects tended to have lower proliferation responses. The present study was designed to characterize T cell activation further by measuring several components of the response. Eighty-seven subjects (80 dyspeptic patients and seven healthy persons from the laboratory staff) with or without antibodies to *H. pylori* were studied by measuring the DNA synthesis induced by several *H. pylori* concentrations (1–23 μg/ml) and the control stimulants PPD, tetanus toxoid and pokeweed mitogen (PWM). *H. pylori*-induced secretion of interleukin-2 (IL-2), tumour necrosis factor-alpha (TNF-α), interleukin-4 (IL-4), soluble CD8 and IL-2 receptor (IL-2R) molecules and *H. pylori*- and PPD-induced appearances of IL-2R$^+$ and HLA-DR$^+$ T cells were measured in a smaller number of subjects. *H. pylori*-induced DNA synthesis was again lower in the antibody/bacterium-positive subjects, while no differences between the two groups were found in cultures stimulated by unrelated antigens or PWM. Soluble IL-2R and TNF-α were detectable in cultures with *H. pylori* from all subjects, while the amount of IL-2 did not differ from that in the background culture. No differences were found in the amounts of IL-2 or soluble IL-2R between the antibody-positive and negative subjects; while the former tended to secrete more soluble CD8 molecules, a difference which was significant with the smaller *H. pylori* concentration used ($P < 0{\cdot}01$). The numbers of HLA-DR$^+$ and IL-2R$^+$ T cells increased in cultures with *H. pylori* or PPD from all the subjects, the majority of both cells having the CD4 phenotype. Numbers of DR$^+$ and IL-2R$^+$ T cells were similar in the cultures of the antibody-positive and negative subjects, but the respective CD8 subsets were increased in the former. The confirmed decrease in proliferation in the antibody-positive subjects does not seem to be connected with lower IL-2/IL-2R responses but may involve CD8 cell activation.

Keywords *Helicobacter pylori* cytokines DR$^+$ T cells interleukin-2 receptor-positive T cells

INTRODUCTION

Helicobacter pylori, a spiral bacterium, is associated with an active chronic gastritis, especially in the antral part of stomach. The bacterium is recognized by the immune system as shown by the presence of both local and systemic antibody responses (Rauws & Tytgat, 1989). Other reported immunological findings have been related either to local inflammation (Engstrand *et al.*, 1989; Kirchner *et al.*, 1989) or to phagocytosis of the bacterium (Bernatowska *et al.*, 1989).

Since *H. pylori*-associated infection is of prolonged duration if not terminated by effective anti-microbial treatment, it is important to study the whole range of local and systemic reactions to it. We have been interested in T cell responses to this bacterium, a subject which has not been widely investigated. A whole inactivated *H. pylori* bacterium preparation was previously found to stimulate blood mononuclear cells to synthesize DNA and secrete interferon-gamma (IFN-γ) independent of the presence or absence of bacteria/antibodies, but with a tendency for antibody-positive subjects to show a reduction in both functions (Karttunen *et al.*, 1990).

The aim of this work was to investigate which phases of T cell activation are affected, and to compare the responses induced by *H. pylori* with those induced by mitogens or antigens. The DNA synthesis responses induced by different *H. pylori* concentrations were studied, as was the secretion of cytokines and activation molecules: interleukin-2 (IL-2) inter-

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leukin-4 (IL-4), tumour necrosis factor-alpha (TNF-α), IL-2 receptor (IL-2R) and CD8 molecules. The activated T cells were defined by positivity for DR and IL-2R surface markers and their CD4 and CD8 phenotypes were measured in cultures from dyspeptic and healthy subjects. The parameters measured were compared with respect to *H. pylori* antibody status among the subjects.

SUBJECTS AND METHODS

Subjects and samples

Peripheral blood samples and two gastric antral mucosal samples were obtained from 80 dyspeptic patients attending for gastroscopy. Patients with malignant diseases, immunodeficiency or concomitant immunosuppressive or anti-microbial treatment were excluded. Blood samples were also taken from seven healthy laboratory staff.

Characterization of subjects

A touch preparation was made from both mucosal samples and Gram-stained to detect the presence or absence of the *H. pylori* bacterium. This information was used to select bacterium positive and negative samples for the simultaneous lymphocyte cultures. If the samples obtained that day were not of both type, they were discarded, or on seven occasions complemented by a sample from a control subject with the right *H. pylori* antibody status. The sera were stored at −20°C for *H. pylori* antibody measurement, which was performed using an enzyme immunoassay with an acid extract antigen (Kosunen *et al.*, 1989). Of the 87 subjects, 42 were antibody positive (i.e. they had anti-*H. pylori* antibodies of either IgG or IgA class antibodies or both) (age 49 ± 12 years) and 45 were antibody negative (age 44 ± 13 years). The seven control subjects consisted of three antibody-positive and four antibody-negative individuals. Their antibody status was known from an earlier study and on retesting was found to have remained the same. After the antibody testing was performed, it turned out that sampling was successful to the extent that 20 out of 27 culture series (for DNA synthesis measurement) consisted of both positive and negative subjects. Mononuclear cells (MNC) from 84 samples were successfully cultured to measure DNA synthesis. Thirty of them were from subjects with both bacteria and specific antibodies and 36 from subjects with neither. Cytokine measurement and lymphocyte stainings were performed from 48 consecutive subjects. Thirty-two samples (13 positive, 19 negative) were used for supernatant preparation and 16 (seven positive, nine negative) for lymphocyte surface marker stainings.

Stimulants

H. pylori stimulant was prepared by inactivating the bacteria (a mixture of NCTC 11637 and bacteria obtained from five patients) with 1% formalin and washing them with phosphate-buffered saline (PBS). The stimulant was kept stored at −70°C at a protein concentration of 4·6 mg/ml, from which the dilutions were prepared after thawing. PPD (Statens Serum-institut, Copenhagen, Denmark), tetanus toxoid (National Public Health Institute, Helsinki, Finland) and pokeweed mitogen (PWM) (Sigma Chemical Co., St Louis, MO) were used in concentrations of 40 μg/ml, 1 μg/ml and 10 μg/ml, respectively.

Cell cultures for DNA synthesis measurement, supernatant preparation and lymphocyte staining

MNC were separated out by Ficoll–Isopaque gradient centrifugation. RPMI 1640 containing 10% inactivated *H. pylori* antibody-negative male human **AB** serum was used as the culture medium. For DNA synthesis measurement, 1×10^5 MNC were cultured in a total volume of 200 μl for 7 days with five *H. pylori* concentrations (final concentrations 1, 2, 5, 11 and 23 μg/ml), PPD, tetanus toxoid and PWM and with no stimulant (spontaneous cultures). ^{3}H-thymidine (specific activity 74 GBq/mmol) was added for the last 24 h of culture. The median result of triplicate culture wells for each sample/stimulant was taken as the response. For cytokine measurement and lymphocyte staining $1{\cdot}2 \times 10^6$ MNC in a total volume of 0·8 ml were cultured with *H. pylori* (2 and 11 μg/ml), PPD and no stimulant in three parallel tubes, from which the supernatants were collected after centrifugation on days 3, 5 and 7 and stored at −40°C. The cells remaining on day 7 were used for staining (see below).

Measurement of cytokines and soluble IL-2R and CD8 molecules

Cytokine and activation molecules were measured from supernatants cultured with *H. pylori* (2 and 11 μg/ml) or without, and after the following incubation periods: IL-2, 3 days; IL-2R, 5 and 7 days; TNF-α, 5 days; IL-4, 5 days; CD8, 5 and 7 days. Cytokines/soluble activation markers were measured from 13 to 32 subjects. The number of supernatants tested varied depending on the test kit resources and the results, e.g. since no IL-4 was detectable, the number of samples studied was restricted to 13. The following commercial enzyme immunoassay kits were used: TNF-α (TNF ELISA; Endogen, Boston, MA), soluble IL-2R and soluble CD8 molecules (Cellfree IL-2R and Cellfree T8; T Cell Sciences, Cambridge, MA) and IL-4 (Intertest-4; Genzyme, Boston, MA). The tests were done according to the manufacturers' instructions. *H. pylori*-induced and spontaneous supernatants were tested simultaneously. IL-2 was tested using an IL-2-dependent mouse cytotoxic T cell line (CTLL). Five-thousand CTLL cells were cultured with 200-μl volumes of supernatants for 48 h, with radioactive thymidine present for the last 24 h. Several concentrations of standard IL-2 preparation (0·015–4 U/ml; Lymphocult-T-HP; Biotest, Frankfurt, Germany), diluted in the culture medium, were cultured simultaneously. The Ct/min results were converted to units with the help of the standard curve.

Staining of DR^+ and $IL\text{-}2R^+$ T cells

All monoclonal antibodies were supplied by Becton Dickinson (Mountain View, CA): anti-DR (PE conjugated), anti-IL-2R (CD25; PE conjugate) anti-Leu4 (CD3; FITC conjugate), anti-Leu3a (CD4; FITC conjugate) and anti-Leu2a (CD8; FITC conjugate). The MNC (on day 0 and after 7 day cultures with *H. pylori* 11 and 2 μg/ml and PPD) were divided into five tubes and were double-stained according to the following schema: tube no. 1, anti-DR + anti-Leu4; no. 2, anti-DR + anti-Leu3a; no. 3, anti-DR + anti-Leu2a; no. 4, anti-IL-2R + anti-Leu3a; nol 5, anti-IL-2R + anti-Leu2a. The stained cells from the lymphocyte gate were enumerated with FACScan flow cytometer (Becton Dickinson). About 100–5000 cells/tube were counted. The limits for positivity were kept the same for all samples but were adjusted for each antibody separately.

Statistical analysis

Comparisons were performed either using the Mann–Whitney *U*-test or Student's *t*-test.

RESULTS

DNA synthesis responses

The DNA synthesis responses induced by *H. pylori*, control antigens and PWM are shown in Table 1. The responses in cultures with the various *H. pylori* concentrations were lower in the *H. pylori* antibody/bacterium-positive subjects, the differences being significant at concentrations of 11, 2 and 1 μg/ml ($P < 0{\cdot}05$–$0{\cdot}01$). No significant differences were seen between the groups in the responses to PPD, tetanus toxoid or PWM.

Cytokines and soluble IL-2R and CD8

The quantities of *H. pylori*-induced cytokines and soluble activation molecules in culture supernatants from all the subjects are shown in Table 2, which includes the results obtained with *H. pylori* 11 μg/ml and the results of one culture period i.e. 5 days for IL-2R and 7 for CD8. *H. pylori* induced an increase in the amount of TNF-α and soluble IL-2R ($P < 0{\cdot}001$). A tendency for more CD8 molecules to occur in *H. pylori* supernatants than in spontaneous cultures was found ($P < 0{\cdot}1$), but no increases in the amounts of IL-2 or IL-4 were detectable.

The results are grouped according to the antibody status of the subjects in Table 3 (including CD8 values with HP 2 μg/ml). The antibody-positive subjects had more CD8 molecules in their supernatants, a difference which became significant at the lower *H. pylori* concentration (73 *versus* 38 U/ml; $P < 0{\cdot}01$). There was a tendency for the antibody-negative subjects to have more TNF-α secretion ($P < 0{\cdot}1$).

Appearance of HLA-DR$^+$ and IL-2R$^+$ T cells and their phenotypes

The results of cultures with *H. pylori* or PPD are shown for all the subjects in Table 4. The numbers of DR$^+$ and IL-2R$^+$ T cells among the newly separated cells were low (approximately 1–2%). There was a clear increase in both cell types and their CD4 and CD8 counterparts in both *H. pylori* and PPD cultures ($P \leq 0{\cdot}05$–$0{\cdot}001$). Most DR$^+$ and IL-2R$^+$ cells were of the CD4 phenotype.

The results of cultures with *H. pylori* in antibody-positive and negative subjects are shown in Table 5. The numbers of DR$^+$ and IL-2R$^+$ cells with the CD4 phenotype were equally numerous in both groups, while DR$^+$CD8$^+$ and IL-2R$^+$CD8$^+$ cells were increased in the antibody-positive subjects (*H. pylori* 11 μg/ml; $P \leq 0{\cdot}01$).

DISCUSSION

The results of this and an earlier experiment (Karttunen *et al.*, 1990) show that a whole cell preparation of *H. pylori* activates blood T cells as reflected in secretion of the T cell cytokine

Table 1. DNA synthesis responses (ct/min) induced by various *H. pylori* concentrations and control stimulants PPD, tetanus toxoid (TT) and pokeweed mitogen (PWM) in 7-day cultures

	H. pylori (μg/ml)								
	23	11	5	2	1	PPD	TT	PWM	Spontaneous
Antibody/bacterium positive ($n = 30$)									
Median	2983	2118	1463	618	377	7970	3403	17559	187
Range	298–17282	199–18351	396–5383	56–12405	117–13113	1153–26708	50–22592	5399–36423	52–4117
Antibody/bacterium negative ($n = 36$)									
Median	5369	4827	2313	1764	744	5642	1657	19433	217
Range	55–17831	154–24390	435–15229	122–17299	90–7963	421–29725	59–17238	6080–43479	55–1566
P	NS	$<0{\cdot}05$	NS	$<0{\cdot}01$	$<0{\cdot}05$	NS	NS	NS	NS

NS, not significant ($P > 0{\cdot}05$), Mann–Whitney *U*-test.

Table 2. *H. pylori*-induced and spontaneous cytokine secretion and soluble IL-2R and CD8 responses in all subjects (mean ± s.d. values)

	IL-2 (U/ml)	IL-2R (U/ml)	TNF-α (pg/ml)	IL-4 (pg/ml)	CD8 (U/ml)
	3 day	5 day	5 day	5 day	7 day
H. pylori-induced*	0·29 ± 0·23	362 ± 193	554 ± 138	26 ± 21	70 ± 44
Spontaneous	0·23 ± 0·11	147 ± 104	385 ± 20	20 ± 17	51 ± 28
n	32	27	27	13	26
P	NS	$<0{\cdot}001$	$<0{\cdot}001$	NS	$<0{\cdot}1$

NS, not significant ($P > 0{\cdot}1$), Student's *t*-test. **H. pylori* concentration 11 μg/ml.

Table 3. *H. pylori*-induced IL-2 and TNF-α secretion and soluble IL-2R and CD8 responses in subjects positive and negative for *H. pylori* antibodies (mean ±s.d.)

	IL-2 (U/ml)	IL-2R (U/ml)	TNF-α (pg/ml)	CD8 U/ml	
	H. pylori 11 μg/ml	*H. pylori* 11 μg/ml	*H. pylori* 11 μg/ml	*H. pylori* 11 μg/ml	*H. pylori* 2 μg/ml
Positive	0·37±0·19	421±222	501±61	85±52	73±37
Negative	0·24±0·23	315±157	597±167	55±28	38±18
P	NS	NS	<0·1	<0·1	<0·01

NS, not significant ($P>0·1$), Student's *t*-test.

Table 4. Mean±s.d. (%) of DR^+ and $IL\text{-}2R^+$ T cells of different phenotypes, newly separated or cultured with *H. pylori* and PPD (all subjects)

	Newly separated	Cells cultured for 7 days with		
		HP 11 μg/ml	HP 2 μg/ml	PPD
DR^+CD3^+	1·8±1·0	7·6±3·2‡	6·0±2·5‡	10·0±5·3‡
DR^+CD4^+	1·2±0·5	6·1±1·8‡	4·6±1·8‡	9·5±5·8‡
DR^+CD8^+	0·9±0·6	2·2±1·4†	2·6±2·1*	2·1±0·9†
$IL\text{-}2R^+CD4^+$	0·8±0·4	3·4±1·6‡	3·1±1·6‡	7·1±2·9‡
$IL\text{-}2R^+CD8^+$	0±0	0·2±0·2*	0·2±0·2†	0·7±0·3‡
DNA synthesis (ct/min)	—	3772±5445	1491±2590	7276±5343
n	16	10	16	8

The significances of the differences between the newly separated cells and the respective cultures were tested: *$P\leq0·05$; †$P\leq0·01$; ‡$P\leq001$, Student's *t*-test.

Table 5. Mean±s.d. (%) of DR^+ and $IL\text{-}2R^+$ T cells of different phenotypes after 7-day culture with *H. pylori* stimulant in samples from subjects positive and negative for *H. pylori* antibodies

	H. pylori 11 μg/ml		*H. pylori* 2 μg/ml	
	Positive	Negative	Positive	Negative
DR^+CD3^+	10·2±4·8	6·6±1·7	6·4±3·5	5·8±1·5
DR^+CD4^+	7·3±2·3	5·6±1·4	4·4±2·2	4·8±1·5
DR^+CD8^+	3·7±1·5	1·6±0·7†	3·5±2·7	1·9±1·4
$IL\text{-}2R^+CD4^+$	4·9±2·1	2·8±0·9	3·6±1·9	2·7±1·2
$IL\text{-}2R^+CD8^+$	0·4±0·2	0·1±0·1†	0·4±0·3	0·1±0·1*

The significances of the differences between the antibody-positive and negative subjects were tested: *$P\leq0·05$; †$P\leq0·01$, Student's *t*-test.

IFN-γ, expression of activation markers on T cells and shedding of IL-2Rs into supernatants. This activation involves not only the mononuclear cells of subjects with an established contact and serological immunity but also cells of subjects who have neither *H. pylori* bacteria nor antibodies. The lymphocytes of the former subjects in fact proliferate slightly less strongly, a finding which was confirmed by this second experiment.

Lymphocyte proliferation, whether induced by mitogens or antigens, requires T cell growth factor IL-2 and a specific receptor to mediate the effect of IL-2 (review by O'Garra, 1989). We have been studying more closely antigen-induced activation and found that proliferation correlates besides with IL-2/IL-2R responses also with the expression of DR antigens on T cells (Karttunen *et al.*, 1988, Surcel, 1990). Interestingly, there seemed to be a different tendency with *H. pylori*, since the antibody-positive subjects with a weaker proliferative response had higher amounts of DR^+ and $IL\text{-}2R^+$ cells in their *H. pylori* cultures, although the differences were significant only in their CD8 subsets. This result and that for soluble CD8 (the amount of which was higher in cultures from the antibody-positive subjects) indicate that *H. pylori*-induced lymphocyte activation differs somewhat from that occurring in mitogenic or the antigenic reactions we have been studying. They also suggest that an antigen-specific suppressive component may be involved in the MNC cultures of the subjects with antibodies to *H. pylori*.

H. pylori-induced IL-2 was not detected. The reason for this is not known, since we have found positive IL-2 secretion in cultures with nickel ag, which also induces small proliferative responses (median 2384 ct/min; Karttunen *et al.*, 1988).

H. pylori induced a small amount of TNF-α. The subjects without *H. pylori* antibodies tended to secrete slightly more TNF-α in *H. pylori* cultures than the antibody-positive subjects, and thus the secretion resembled the IFN-γ response investigated earlier (Karttunen *et al.*, 1990).

The response of the antibody-negative subjects could suggest mitogenicity of the *H. pylori* preparation. However, it may be explained by wide cross-reactivity with certain ubiquitous bacterial antigens. We have started investigating the stimulation of the antibody-negative subjects in this respect. Preliminary

data on MHC restriction in *H. pylori*-specific T cell lines obtained from *H. pylori* antibody-negative subjects indicate that these responses also resemble antigen-specific ones (Karttunen & Ilonen, 1990). In a study of Gram-negative bacteria-induced lymphocyte stimulation, *Yersinia* and *Salmonella* were found to stimulate the cells of only those subjects who had been infected by those bacteria, respectively. When the stimulation was positive it also was crossreactive to other members of *Enterobacteriae* family. The stimulation resembled *H. pylori*-induced activation with regard to the mean strength and wide individual variation (Vuento *et al.*, 1984).

H. pylori-induced lymphocyte responses are an interesting subject for further immunological investigations, including the reactions of mucosal T cells and reactions to more closely defined bacterial antigens, and the establishment of the nature of the response of antibody-negative subjects. We have found some evidence of antigen-specific suppression, but it is not possible to distinguish this response from the complex background stimulation obtained with the crude whole cell preparation. It may require the availability of peptide antigens with a narrow effect and improved methods for culturing and studying suppressive T cells.

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