INHIBITION OF LYMPHOCYTE PROLIFERATIVE RESPONSES TO *HELICOBACTER PYLORI* BY PLASTIC ADHERENT CELLS

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Abstract. A study was carried out on 49 *H. pylori*-positive and 11 *H. pylori*-negative patients to determine the reactivity of peripheral blood lymphocytes (PBL) to phytohemagglutinin (PHA) and acid glycine extract (AGE) of *H. pylori*, and to identify cells responsible for imunosuppression. Based on response to PHA stimulation, cell-mediated immunity of all patients were competent. In some patients, however, response to AGE of *H. pylori* was suppressed by plastic adherent cells. This study provided evidence of the presence of plastic adherent suppressor cells which suppressed PBL response to AGE of *H. pylori* but not to PHA suggesting that immunosuppression is antigen specific. There is also an indication that immunosuppression may be species-specific as PBL devoid of plastic adherent cells only responded to stimulation by AGE of *H. pylori* but not that to AGE of *C. jejuni*.

INTRODUCTION

Helicobacter pylori was first isolated in Australia by Warren and Marshall (1983) from gastric antrum of patients with active chronic gastritis. The organism, now accepted as an etiologic agent for active chronic gastritis and peptic ulcer is causally associated with a risk of carcinogenesis (Di-Leo *et al*, 1999).

Multi-drug antimicrobial therapy cure infection in more than 80% of the patients (Graham *et al*, 2000) but duodenal ulcer relapses. The presence of *H. pylori* infection induces strong immune responses at both local (Perez-Perez *et al*, 1999) and systemic levels (Sunnerstam *et al*, 1999). However, these immune mechanisms appear to be ineffective in eradicating the organism and infection persists.

There may be additional factors associated with *H. pylori* which protect itself against the host defense system. Therefore the present study was undertaken with the objectives: firstly, to determine the reactivity of peripheral blood lymphocytes (PBL) from *H. pylori*-positive and *H. pylori*-negative to phytohemagglutinin (PHA) and *H. pylori* antigen; and secondly to identify cells responsible for imunosuppression.

MATERIALS AND METHODS

H. pylori was isolated from a 65 year old Chinese female, histologically confirmed with active chronic gastritis. *Campylobacter jejuni* was obtained from Veterinary Research Institute, Ipoh, Malaysia. *H. pylori* was grown on Eugon agar (BBL, Cockeysville, MD, USA) supplemented with 10% human sera whereas *C. jejuni* was grown on Mueller-Hinton agar (BBL, Cockeysville, MD, USA), both incubated at 37ºC in a 10% CO_2-air atmosphere. Acid glycine extract (AGE) was the antigen used. Briefly, 0.5 g wet weight of a 72-hour culture was harvested from agar medium, washed in phosphate buffer saline pH 7.2 (Behringwerke AG, Marburg, Germany) and shaked (Bellco, speed 5) in 12.5 ml 0.2 M glycine hydrochloride (Sigma Chem, St Louis, USA) pH 2.2 at room temperature. After 15 minutes, suspension was centrifuged at 15,000 rpm (Hermle). Supernatant containing AGE was neutralized with 750 µl

3M sodium hydroxide and dialyzed against distilled water for 24 hours. AGE was lyophilized and stored tightly in glass vial at 4ºC before used.

A total of 60 patients (49 *H. pylori*-negative and 11 *H. pylori*-positive) were enrolled in the study. Informed consent was obtained from all patients. Immediately before endoscopy, 18 ml of blood was withdrawn from each patient. Additionally, a 20 year-old apparently healthy Malay male donated 25 ml of blood for the optimization of PHA (Pharmacia). The "gold standard" for infection was defined as either positive by culture or positive by both urease test and Gram stain (Uyub *et al*, 1994a,b).

RPMI 1640 medium (Flow Labratories, Australia) supplemented with 10% final concentration of heparinized autologous plasma (inactivated at 56ºC for 30 minutes), 20 mM HEPES buffer (Sigma Chem, St Louis, USA), 0.02 M sodium bicarbonate (Sigma Chem, St Louis, USA) and 100,000 units penicillin (Sigma Chem, St Louis, USA) was used as the culture medium. The culture medium without penicillin and autologous plasma was used to wash the cells. Both these media were sterilized by filtration (0.2 µm, Corstar), stored at 4ºC and used within 5 days.

For isolation of PBL, blood was layered over Ficoll-Hypaque (density 1.077 g/ml, Pharmacia) and centrifuged at 400*g* for 40 minutes at 20ºC. The lymphocytes were collected, washed thrice at 4ºC, counted in trypan blue (Sigma Chem, St Louis, MD, USA) using Neubauer hemocytometer, resuspended in the culture medium and adjusted to 10^6 ml^{-1}.

Cultures for PHA and antigen stimulation were established in a U-bottomed microtiter plates with lids (Nunclon) in triplicate wells. Each well contained 10^5 PBL in 200 µl culture medium containing optimal concentration of PHA or the antigen. The plates were incubated for 3 days for PHA stimulation or 5 days for antigen stimulation, both at 37ºC in a humidified 5%CO_2-air atmosphere. To determine the cellular proliferation, 1 µCi [^{3}H]-tritiated thymidine (Amersham, England) was pulsed to each culture well. Cells were harvested on discs of glass fiber filter strips using a cell harvester. The discs were dried at 37ºC for 24 hours and placed in a special glass vial before adding 4 ml of toluene based scintillation fluid containing 2,5-diphynyloxazole and 1,4 bis-2, 4-methyl-5-phenyloxazolyl benzene.

The radioactivity as counts per minute (CPM) in each vial was determined using a liquid scintillation counter (Beckman). Stimulation Index (SI) was calculated by dividing the mean CPM of stimulated culture by mean CPM of unstimulated culture.

Plastic adherent cells (Adh cells) were separated by incubating the PBL in a sterile plastic Petri dish (Nunclon) for 3 hours in a humidified 5%CO_2-air atmosphere at 37ºC. Plastic non-adherent cells (NAdh cells) were collected and washed once. Ten ml of cold (4ºC) washing medium was then added to the Petri dish and incubated for a further 10 minutes at 4ºC. Adh cells were then scrapped off the Petri dish using a rubber policeman (Corstar) and washed once. Viable cells were resuspended in the culture medium after counting in trypan blue (Sigma Chem, St Louis, MD, USA) using a Neubauer hemocytometer.

For co-culture, Adh cells and NAdh cells were remixed and recultured in 200 µl culture medium containing optimal concentration of PHA or antigen incubated, pulsed with 1 µCi [^{3}H]-tritiated thymidine (Amersham, England) and harvested as described.

RESULTS

The optimal concentration of PHA was determined by culturing 10^5 PBL from the healthy donor in 200 µl of culture medium containing 0 µg, 3.2 µg, 6.4 µg and 12.8 µg in triplicate for each concentration. Maximum incorporation of [^{3}H]-tritiated thymidine by PBL was achieved at 3.2 µg PHA per well or at 16 $\mu g.ml^{-1}$ final concentration. This corresponds to a stimulation index of 171.1.

Similarly, initial attempt to determine the optimal concentration of AGE of *H. pylori* was carried out by culturing 10^5 PBL from 30 *H.*

pylori-negative and 6 *H. pylori*-positive in 200 µl of culture medium containing 10 µg.ml^{-1}, 20 µg.ml^{-1}, 50 µg.ml^{-1} and 100 µg.ml^{-1} final concentration in triplicate for each concentration. Table 1 compared the stimulation index of culture stimulated by PHA and AGE of *H. pylori*. PBL from all patients responded to PHA but not to AGE of *H. pylori*. For patient (No.36), AGE of *H. pylori* was also tested at 150 µg.ml^{-1}, 200 µg.ml^{-1} and 250 µg.ml^{-1} with no response.

There may be Adh cells which suppressed PBL responsiveness to AGE of *H. pylori*. Therefore, optimization was then performed

Table 1
Response of PBL from *H. pylori*-negative and *H. pylori*-positive patients to PHA and AGE of *H. pylori*.

Patients				*Helicobacter pylori*	Stimulation index	
No.	Age	Sex	Race		PHA (16 µg.ml^{-1})	AGE (20 µg.ml^{-1})
1	32	m	m	-	77.1	0.9
2	56	m	m	-	3.6	1.2
3	65	f	m	-	5.4	1.5
4	32	m	m	-	22.8	0.6
5	37	f	m	-	148.7	0.7
6	22	m	m	-	29.5	0.2
7	34	f	c	-	40.1	0.6
8	63	m	m	-	7.8	0.04
9	37	f	m	-	14.0	0.5
10	42	f	m	-	71.6	1.0
11	34	m	m	-	34.4	1.3
12	32	f	m	-	11.7	0.2
13	52	f	m	-	34.0	1.3
14	67	m	m	-	57.4	0.9
15	40	m	m	-	53.6	0.3
16	50	m	m	-	148.4	0.7
17	21	f	m	-	68.8	0.4
18	61	m	m	-	30.7	0.9
19	66	f	c	-	8.8	1.0
20	58	f	m	-	53.9	1.0
21	51	f	m	-	108.1	1.3
22	22	f	m	-	7.8	0.2
23	43	m	m	-	81.2	0.4
24	62	f	m	-	89.5	1.2
25	25	f	c	-	91.0	0.6
26	51	m	m	-	2.2	0.1
27	76	m	m	-	4.5	0.2
28	31	f	c	-	36.4	1.2
29	35	f	m	-	2.5	0.4
30	30	f	m	-	60.0	0.3
31	54	f	c	+	24.3	1.0
32	30	m	c	+	35.2	0.7
33	65	m	m	+	76.5	0.3
34	62	m	i	+	36.6	0.4
35	49	f	c	+	85.6	0.3
36	50	m	c	+	50.1	0.5

Table 2
Effect of separating Adh cells on the PBL response to PHA and AGE of *H. pylori*.

Patients					Stimulation index (PHA, 16 μg.ml^{-1})			Stimulation index (AGE, 20 μg.ml^{-1})		
No.	Age	Sex	Race	*H. pylori*	PBL	Nadh	Nadh + Adh	PBL	Nadh	Nadh + Adh
37	53	m	m	-	ND	28.2	ND	ND	0.4	ND
38	60	f	m	-	ND	428.0	ND	ND	1.8	ND
39	70	f	m	-	ND	77.9	ND	ND	0.7	ND
40	33	m	m	-	88.3	63.1	66.0	0.7	2.1	0.9
41	23	f	m	-	115.0	49.6	ND	1.2	1.1	0.7
42	64	m	m	-	102.6	ND	ND	1.0	3.4	ND
43	30	m	m	-	24.2	19.4	ND	0.1	3.3	ND
44	62	f	c	-	21.3	18.0	ND	0.3	0.1	ND
45	49	m	m	-	14.9	ND	ND	0.1	0.3	ND
46	35	f	m	-	82.3	47.2	ND	0.2	0.6	ND
47	65	m	m	-	36.9	11.0	ND	0.5	0.3	ND
48	24	f	m	-	154.0	51.6	56.4	0.8	0.9	1.0
49	54	m	i	-	80.2	74.2	ND	1.0	1.5	ND
50	74	f	m	-	65.4	16.7	ND	0.6	0.2	ND
51	48	f	m	-	43.7	18.3	ND	0.6	0.8	ND
52	39	f	m	-	42.3	33.7	ND	0.4	1.3	ND
53	52	m	m	-	114.7	101.0	ND	0.9	1.2	ND
54	70	f	m	-	8.0	5.1	ND	0.5	0.1	ND
55	64	m	m	-	136.5	22.7	ND	0.7	0.8	ND
56	57	m	i	+	61.0	50.4	ND	1.1	3.5	ND
57	48	m	i	+	61.5	42.4	ND	0.8	0.1	ND
58	37	m	m	+	48.2	41.9	ND	0.8	0.7	1.2
59	51	m	i	+	76.0	29.3	ND	0.5	0.6	0.3
60	57	m	c	+	ND	166.0	ND	ND	2.3	ND

using NAdh cells. PBL were obtained from a patient (No.36) who came for a repeat endoscopy on the 13th week. Optimization was similarly done by culturing 10^5 NAdh cells in 200 μl culture medium containing 2 μg.ml^{-1}, 5 μg.ml^{-1}, 10 μg.ml^{-1}, 20 μg.ml^{-1}, 50 μg.ml^{-1} and 100 μg.ml^{-1} in triplicate for each concentration. Optimal activity of AGE was achieved at 20 μg.ml^{-1} which corresponds to a stimulation index of greater than 2.0.

Subsequently, the effect of separating Adh cells on the PBL response to PHA and AGE of *H. pylori* were carried out. Nineteen *H. pylori*-negative and 5 *H. pylori*-positive were enrolled. Table 2 showed that PBL or NAdh cells from all patients responded to PHA stimulation. Among the *H. pylori*-negative patients, NAdh cells from three patients (No. 40,42 and 43) responded to stimulation by AGE of *H. pylori*. In one of them (patient No.40), the response diminished upon co-culture.

Among the *H. pylori*-positive patients, two different kinds of response to AGE of *H. pylori* were observed. Firstly, PBL or NAdh cells from three patients (No.57,58 and 59) were unresponsive at all. Secondly, PBL from three patients (No.36, 56 and 60) responded only after Adh cells were removed.

A follow-up study was carried out on one patient (No.36) with endoscopy done three times

Table 3
Suppression of PBL response to AGE of *H. pylori* by Adh cells in a follow-up patient infected with *H. pylori*.

Endoscopic session (weeks)	*Helicobacter pylori*	Stimulation index (PHA, 16 μg.ml^{-1})		Stimulation index (AGE, 20 μg.ml^{-1})		
		PBL	NAdh	PBL	NAdh	Nadh + Adh
0	+	50.1	ND	0.5	ND	ND
13	+	23.8	24.8	0.4	2.3	ND
18	+	90.4	50.5	0.6	2.3	0.3

within the 18-week period. Table 3 showed the effect of separating Adh cells on the PBL response to PHA and AGE of *H. pylori*. Response of PBL or NAdh cells to PHA remained intact during the 18-week period. In contrast, only NAdh cells responded to stimulation by AGE of *H. pylori*. Co-culture was done at 18 weeks when the patient was still positive for *H. pylori*. It was found that the response to stimulation by AGE of *H. pylori* diminished suggesting that Adh cells were involved in the immunosuppression.

Responsiveness to AGE of *C. jejuni* were also tested on three patients (No. 40, 41 and 56). PBL or NAdh cells from these patients were unresponsive to stimulation by *C. jejuni* antigen.

DISCUSSION

Lymphocyte proliferation assay was used to study the cellular response of *H. pylori*-positive and *H. pylori*-negative patients to stimulation by PHA, AGE of *H. pylori* and *C. jejuni*. Our finding that PBL or NAdh cells from all *H. pylori*-positive and *H. pylori*-negative patients responded to PHA stimulation suggests that their cell-mediated immunity was competent. However, two groups of patients differing in their response to AGE of *H. pylori* were recognized. In one group we found that PBL or NAdh were unresponsive at all to AGE of *H. pylori*. In parasitic infection, such an unresponsiveness have been reported in *Schistosoma mansonii* infection as being due to the presence of serum factors which block receptor site (Colley *et al*, 1977), or in *Toxoplasma gondii* infection as being due to lower population of responding cells (Sklenar *et al*, 1986; Luft *et al*, 1987) or lack of receptor site in the responding cells (Yano *et al*, 1987). It may also be possible that for some individuals, suppressor cells could only be demonstrated using certain antigens. In another group, our finding that NAdh cells responded to AGE of *H. pylori* and the response diminished upon co-culture suggested that Adh cells suppressed the response and were involved in the immunosuppression. Our follow-up study on a patient over an 18-week period also showed that failure to eradicate *H. pylori* concur with the presence of suppressor cells which suppressed the cellular response to *H. pylori* AGE stimulation. This observation provided further evidence that in infected individuals *H. pylori* induced suppressor cells resulting in a state of immunosuppression.

The mechanism of immunosuppression is not elucidated in this study. But pathogens have long been known to use immunosuppression as an adaptive mechanism to evade the host immune system (Bloom, 1979). Kartunnen (1991) reported that decreased response to *H. pylori* was due to suppressor T-cells. Lutton *et al* (1995) reported that *H. pylori* inhibited histamine release from human basophils indicating that the organism exerts an inhibitory effect on cells of the immune system that contributes to its presence within the gastric mucosa. Fan *et al* (1995) reported reduced antigen specific T-cell proliferative responses to *H. pylori* seen in patients with *H. pylori* infection is due to monocyte prostaglandin E sub (2)

production. Knipp *et al* (1996) reported a proliferation-inhibiting protein with antiproliferative activity against mammalian cells including immunocompetent and epithelial cells which may be involved in immune response evasion and suppression of epithelial repair mechanisms. According to Ferrero (1997), *H. pylori* is able to restrict or even modulate the human immune system so as to ensure a persistent infection in the host.

Incubating PBL in a plastic Petri dish have been demonstrated to remove monocytes (Luft *et al*, 1987) suggesting that suppressor Adh cells are macrophages. Therefore immunosuppression may involve Adh cells or macrophage-lymphocytes interaction. Since autologous sera were used in this study for cell culture, it is also possible that interaction of cell and serum factors may activate suppressor cells for immunosuppression. In addition our finding that immunosuppression is antigen specific may suggest that antibody or antibody-antigen complexes playing a role in activating the immunosuppression.

We also found that NAdh cells from the three *H. pylori*-negative patients responded to stimulation by *H. pylori* antigen. There have been reports that *H. pylori* distribution is patchy in the antrum (Goodwin *et al*, 1986; Sorensen *et al*, 1991). Therefore in truly infected patients *H. pylori* could have been inevitably missed for culture, urease test and Gram staining which were used to confirm the infection and were taken as negative. They may be truly *H. pylori*-positive cases.

Overall, immunosuppression observed here was antigen specific, a similar state to that observed in chronic *S. mansoni* infection (Ottesen, 1979) or in acute *T. gondii* infection (Yano *et al*, 1987). This is in contrast to non-specific immunosuppression which have been shown experimentally in *Meriones unguiculatus* infected by *Dipetalonema viteae* (Khairul Anuar *et al*, 1982) or in mice infected by *Plasmodium berghei* (Yadav, 1989). Induction of specific immunosuppression confers a survival advantage for the chronic existence of pathogen in the host. In contrast to non-specific immunosuppression which is disadvantageous to the host, specific immunosuppression favor the host to tolerate or respond minimally to the pathogen. Therefore the host defense mechanism may be directed for other purposes. Our finding that NAdh cells were only responsive to *H. pylori* antigen and not *C. jejuni* antigens may suggest species specificity for immunosuppression.

In conclusion, the study provided evidence of the presence of plastic adherent suppressor cells which suppressed PBL response to AGE of *H. pylori* but not to PHA. Immunosuppression is therefore antigen specific. There is an indication that immunosuppression may be species-specific as Nadh cells only responded to AGE of *H. pylori* but not to AGE of *C. jejuni*.

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