Lymphocyte Proliferative Response to Helicobacter pylori Antigens in H. pylori–Infected Patients

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Specific cellular immune response to *Helicobacter pylori* detected by modified lymphocyte transformation test in the patients

M. Hybenova[1*], P. Hrda[1,2], B. Potuznikova[1], E. Pavlik[1], V. Stejskal[1], J.Dosedel[3], I. Sterzl[1,2]

[1]Institute of Immunology and Microbiology, First Faculty of Medicine, Charles University in Prague;

[2]Institute of Endocrinology, Prague, Czech Republic;

[3]Hospital of the Sisters of Order of St.Charles Boromeo in Prague, Czech Republic

Corresponding author:

Monika Hybenova, MD

Institute of Immunology and Microbiology,

First Faculty of Medicine, Charles University

Studničkova 7, 128 00 Prague 2, Czech Republic

TEL: +420 224968452

EMAIL: hybenova@yahoo.com

ABSTRACT

Helicobacter pylori (Hp) has been implicated in development of gastric and extra-gastric diseases such as autoimmune thyroiditis (AT). It causes persistent life-long infection despite local and systemic immune response.

In present study, we have determined specific cellular immune response to *Hp* antigens in two groups of *Hp* infected patients using modified lymphocyte transformation test, LTT-MELISA, before and after eradication therapy in comparison with healthy controls, group C (n=15). Group A (n=21) created patients with autoimmune thyroiditis and group B (n=13) patients without AT.

In comparison with healthy *Hp* negative controls, immune reactivity to majority of *Hp* antigens was significantly lower in group B before eradication therapy. In group B, significant increase of immune reactivity was observed in certain *Hp* antigens after successful eradication. The same observations in immune reactivity were shown in group A but without significance.

Our results indicate that *Hp* might cause inhibition of the specific cellular immune response in *Hp* infected patients with or without autoimmune diseases such as AT, which can be abrogated by successful eradication of *Hp*. LTT appears to be a good tool for detection of immune memory cellular response in patients with *Hp* infection.

Abbreviations

Hp	*Helicobacter pylori*
aHp	autologous *Helicobacter pylori* strain
hHp	heterologous *Helicobacter pylori* strain
CagA	cytotoxin associated gene A product (antigen)
VacA	vacuolating toxin A
HpAg	*Helicobacter pylori* antigen
AT	autoimmune thyroiditis
TPO	thyroid peroxidase
Tg	thyreoglobulin
PWM	pokeweed mitogen
LTT	lymphocyte transformation test
MELISA	memory lymphocyte immunostimulation assay
cpm	counts per minute
SI	stimulation index
TLR	Toll-like receptor

Helicobacter pylori (Hp) infection affects half of the world population and plays a causative role in the development of serious gastric diseases. *Hp* is a gram-negative, micro-aerophilic, spiral bacterium colonizing gastric mucosa. The infection is acquired usually in childhood and may persist a lifetime, unless treated. Although the majority of infections causing chronic gastritis are asymptomatic, the presence of *Hp* is associated with increased risk for the development of gastro-duodenal ulceration, gastric adenocarcinoma and MALT lymphoma (Sanders and Peura 2002; Suerbaum and Michetti 2002; Makola *et al.* 2007; Kandulski *et al.* 2008a).

Hp induces local and systemic immune response involving both innate and adaptive immunity. Despite of a cellular and humoral immune response, the host organism is often not able to eliminate the *Hp* infection. The inability of the host to clear the infection and pronounced inflammatory response leads to persistent infection and tissue damage. During the *Hp* infection, the lymphocytes are predominantly differentiated to Th1 subtypes that are associated with cytotoxic reaction responsible for damage of gastric mucosa rather than elimination of the infection (Portal-Celhay and Perez-Perez 2006; Suarez *et al.* 2006; Velin and Michetti 2006; Robinson *et al.* 2007). The inability to eliminate the infection may be due to bacterial virulence determinants and immune-evasive strategies as well as an

inappropriate host immune response. *Hp* LPS, compared with other gram-negative bacteria, has been described as a poor TLR activator of the innate immune response and *Hp* flagellin as well (Muotiala *et al.* 1992; Bliss *et al.* 1998; Gewirtz *et al.* 2004). The pathogen-recognition molecule Nod1-mediated interaction appears to be more important for induction of the inflammatory response than those mediated by TLR-4 and TLR-5, especially in cagA positive *Hp* strains (Viala *et al.* 2004; O'Keeffe and Moran 2008). Part of *Hp* strains posseses cytotoxin-associated gene pathogenicity island (cag-PAI) encoding a type IV bacterial secretion system through which a CagA protein, the most important *Hp* virulence factor, is translocated into gastric epithelial cells to induce pro-inflammatory cytokine IL-8 (Crabtree *et al*. 1994; Blaser and Atherton 2004). *Hp* cagA positive strains are associated with severe gastric inflammation and higher risk of adenocarcinoma (Blaser *et al.* 1992; Kuipers *et al.* 1995; Parsonnet *et al.* 1997). *Hp* can evade also adaptive immune response.

Carcinogenesis, immunosuppression

Long lasting inflammatory response may cause an accumulation of genetic defects in epithelial cells, altered cell growth regulation resulting in carcinogenesis. *Hp* has been classified as carcinogen I class by the World Health Organization (Logan *et al.* 1994). About 1% *Hp* infected individuals develop gastric adenocarcinoma and in a few percent, infection leads to MALT lymphoma. It was also described that *Hp* could act in pathogenesis of oropharyngeal carcinogenesis (Akbayir *et al.* 2003; Nurgalieva *et al.* 2005; Kizilay *et al*. 2006; Pavlik *et al.* 2007). However, the exact mechanism of carcinogenesis has not yet been fully understood. The immunosuppression can be mediated by *Hp* VacA or induction of T regulatory cells (Gebert *et al.* 2004; Lundgren *et al.* 2003). *Hp* CagA has been suggested as a direct mutagen (Hatakeyama *et al.* 2009).

Autoimmunity

The chronic inflammation can lead to autoimmune immunopathological reactivity. The most important mechanism by which *Hp* induces gastric autoimmunity is molecular mimicry, cross-reaction between antigens expressed both on *Hp* and on gastric parietal cells in proton pump, H+, K+ -ATPase (D'Elios *et al.* 2004; Bergman *et al.* 2005).

Besides gastric disorders, the *Hp* etiology is discussed in connection with the development of different extra-gastric diseases such as vascular, skin and autoimmune diseases such as autoimmune thyroiditis (Martin de Argila *et al.* 1995; Realdi *et al.* 1999; Tsang and Lam 1999; De Koster *et al.* 2000; Nilsson *et al.* 2005; Solnic *et al.* 2006).

In several studies, increased prevalence of *Hp* infection in patients with AT has been observed and confirmed not only by higher anti-*Hp* IgG levels but also positive urea breath tests (De Luis *et al.* 1998). Further, a strong positive correlation between the titers of anti-TPO antibodies and anti-*Hp* IgG levels was demonstrated (Bertalot *et al.* 2004). It was shown that monoclonal antibodies against the specific *Hp* antigen CagA react with thyroid follicular cells and that cagA-positive *Hp* carries a gene for endogenous peroxidase (Figura *et al.* 1999). The cross-reaction between antigens of the Lewis blood groups, Lewis X and Y, which are expressed on *Hp* LPS as well as on gastric epithelium on membrane H+, K+ ATP-ase pump and the thyroid gland, was observed (Bertalot *et al.* 2004). Thus, it is possible that the mechanism of molecular mimicry, a structural or sequential similarity, between the *Hp* and the host, may be one of the pathogenetic mechanisms in AT (Tomer *et al.* 1993).

LTT

Lymphocyte transformation test (LTT) has been used as a diagnostic method in different allergic and autoimmune diseases (Stejskal *et al.* 1996; Pichler *et al.* 2004; Prochazkova *et al.* 2004; Sterzl *et al.* 2006). However, only a few studies have recommended this test as standardized technique for the diagnosis of infectious diseases (Valentine-Thon *et al.* 2007; Prasad *et al.* 2008; Nyati *et al.* 2010). Modified lymphocyte transformation test, LTT-MELISA had been developed and validated as a reproducible, sensitive, specific, and reliable method for detecting metal sensitivity (Stejskal *et al*. 1994; Valentine-Thon and Schiwara 2003). In this validated format, it was also evaluated for improvement of Lyme borreliosis diagnosis in clinically and serologically ambiguous cases (Valentine-Thon *et al.* 2007). In modified form, LTT-MELISA could be suitable to follow the memory lymphocyte response of the immune system to infectious agents such as *Helicobacter pylori*.

MATERIALS AND METHODS

Participants in the study were selected from the patients of the Department of Clinical Immunology and Allergology, First Faculty of Medicine, Charles University and General University Hospital in Prague and the Institute of Endocrinology in Prague. A group of healthy volunteers represented mainly students of the First Faculty of Medicine, Charles University. All participants have been asked to confirm the inform consent.

Based on detection of organ specific anti-thyroid antibodies, the clinical state and *Hp* positivity, probands were divided into three groups:

Group A: 21 with *Hp* infection and AT

Group B: 13 with *Hp* infection without AT

Group C: 15 healthy individuals without AT and *Hp* infection

Diagnosis of AT was based on clinical, ultrasound findings and positivity of antibodies against thyroid peroxidase (TPO) and/or thyreoglobulin (Tg). For detection of *Hp* infection the patients were tested for presence of anti-*Hp* antibodies (IgM, IgG and IgA) followed by urea breath test or examination of *Hp* antigen in stool. *Hp* positive patients underwent gastroscopy with biopsies for culture and genotyping of *Hp* and endoscopic evaluation of gastric inflammation as well. In all patients with verified *Hp* infection, we have measured specific cellular immune responses to different *Hp* antigens in comparison with healthy controls. The laboratory testing was performed prior *Hp* eradication and approximately 3 and 6 months after eradication therapy. Urea breath test was used as therapy efficiency control.

The mean age of patients in group A was 47.7 years, in group B 42 years and 24.5 years in group C. Group A consisted of 18 women and 3 men, group B of 9 women and 4 men, group C of 12 women and 3 men.

Autoantibodies against TPO and Tg were detected in sera by ELISA (kit AescuLab). The stool assay was performed using the test ImmunoCard STAT HpSA (Meridian Diagnostics, Inc, USA). Quantitative determination of anti-*Hp* antibodies IgG, IgA and IgM in sera was carried out by commercial kit (EIA – H.pylori, Test-Line, Czech Republic) and IgG antibodies against *Hp* CagA protein were detected with commercial kit (H.pylori 120 CagA, ELISA Test-Line, Czech Republic). *Hp* DNA isolation and genotyping of *Hp* strains was made using by rt-PCR TaqMan, methods

described by Pavlik *et al.* (2007). Specific cellular immune response was detected by LTT-MELISA as described below.

LTT-MELISA for Helicobacter pylori

Modified lymphocyte transformation test, LTT-MELISA is based on evaluation of memory cells proliferation after incubation with antigens. In our test, different *Hp* antigens were used to stimulate lymphocytes isolated from patient's peripheral blood. 30 minutes heat inactivated whole *Helicobacter pylori,* isolated from gastric biopsies of the patient (autologous bacterial *Hp* strain, *aHp*) and from other patients (heterologous *Hp* strain, *hHp*, mixture of cagA+ and cagA- *Hp* strains defined by genotyping) were used in 3 concentrations $1x10^5$, 10^6, 10^7 bacteria/ml (*Hp5, Hp6, Hp7)*. Further, commercial recombinant antigens, *CagA*, *urease*–small subunit (IBT, Germany) in concentration 10 ug/ml, recombinant *Hp protein* (GeneTica, Czech Republic) 5 ug/ml and partially purified *Hp antigen*, *HpAg* (Aalto Bio Reagents, Ireland) 10 ug/ml were applied.

From the blood samples taken to vacuette tubes with natrium citrate (Dialab), peripheral blood mononuclear cells (PBMCs) were isolated on Ficoll-Histopaque (Sigma-Aldrich). After washing, monocyte reduction was made by plastic adherence and dilution of PBMCs to concentration of 1×10^6 cells/ml in medium containing RPMI-1640 with HEPES (Sigma-Aldrich), gentamicin, L-glutamine (Sigma-Aldrich) and 20% pooled, heat-inactivated human AB serum (Biomedica). Lymphocytes were incubated for 5 days at 37°C with 5% CO_2 in 48-well cell culture plate pre-coated with different *Hp* antigens in duplicate or triplicate together with 2 positive controls (lymphocytes in medium and 2 ug/ml pokeweed mitogen, PWM (Sigma-Aldrich)) and 3 negative controls (lymphocytes in medium without antigen, spontaneous proliferation). After 5 days, the cells were pulsed for 4 hours with methyl-^{3}H-thymidine (Lacomed), harvested and the radioactivity was measured in a scintillation counter, expressed as Stimulation Index (SI), defined as a ratio of the cpm (counts per minute) in stimulated culture and the average of non-stimulated cpm values, and also as Δcpm (cpm correlated to non-stimulated control). The lymphoblast transformation after 5 days was confirmed morphologically in stained cytospin preparations (Diff-Quik, Switzerland) – Figure 1.

Statistical evaluation was carried out by Mann-Whitney test and Wilcoxon test.

RESULTS

LTT-MELISA: proliferative response before eradication

In group A of 21 *Hp* infected patients with AT and group B of 13 *Hp* infected patients without AT, immune reactivity before eradication was detected.

In comparison with healthy *Hp* negative controls (group C), immune reactivity to majority of *Hp* antigens (*hHp7, hHp5, aHp7, aHp5*, *HpAg* expressed as SI and *hHp7, hHp6, aHp7, aHp6, aHp5*, *CagA* expressed as Δcpm) was significantly lower in group B before eradication therapy (p<0,05). In group A, lymphocyte proliferative response was low but not significantly in comparison with *Hp* negative controls (p>0.05). There was no significant difference in proliferative response to non-specific mitogen, PWM in group A and B in comparison with healthy controls. The results are shown in Table 1.

Table 1: Immune reactivity (LTT-MELISA) to various *Hp* antigens before eradication - comparison between groups

	group / antigen	A	B	C
SI	***PWM***	69.0	178.0	216.0
	hHp7	12.0	7.0*	27.0*
	hHp6	14.0	4.0	11.0
	hHp5	3.5	2.0*	3.0*
	aHp7	17.5	14.0*	26.0*
	aHp6	12.0	7.0	11.0
	aHp5	4.0	2.0*	7.0*
	CagA	1.0	0.0	1.5
	urease	3.0	1.0	4.0
	Hp protein	3.0	0.0	1.0
	HpAg	14.5	7.0	30.0
Δcpm	***PWM***	25815.0	13224.0	21866.0
	hHp7	2217.0	898.0*	5681.0*
	hHp6	2872.0	445.0*	5168.0*
	hHp5	203.0	165.0	265.0
	aHp7	3574.5	436.0*	4186.0*
	aHp6	654.5	937.0*	4796.0*
	aHp5	122.0	85.5*	1367.0*
	CagA	38.0	-2.0*	170.0*
	urease	379.0	15.5	271.0
	Hp protein	151.0	-9.5	24.0
	HpAg	2991.5	3007.0	16715.0

The values of SI and Δcpm are in medians.

* significant difference (p<0.05)

Comparison of stimulation by autologous and heterologous Hp strain

In two concentrations of autologous *Hp* strain (*aHp7, aHp6* expressed as SI), proliferative response was higher than in heterologous *Hp* strain (*hHp7, hHp6*) in group B ($p<0.05$). In group A, there was higher immune reactivity to autologous than heterologous *Hp* strain but not significant ($p>0.05$).

Proliferative response before eradication in dependence on CagA status

In group of *Hp* infected, CagA positive patients without AT, statistically significantly higher proliferative response was measured to certain *Hp* antigens (*hHp7, aHp7, aHp6* and *CagA* expressed as SI) in comparison with CagA negative patients ($p<0.05$). In group of *Hp* infected patients with AT, significant difference wasn't observed in proliferative response in CagA positive and CagA negative patients.

LTT-MELISA: proliferative response after eradication

In group B, significant increase of immune reactivity was observed in 4 *Hp* antigens (*hHp7, aHp7* and *HpAg* expressed as SI and *aHp7, CagA* expressed as Δcpm) after successful eradication ($p<0.05$). The lymphocyte proliferative response in group A increased but no significantly after eradication ($p>0.05$). There was no significant difference in proliferative response to non-specific mitogen (PWM) in group A and B in comparison before and after successful eradication. The results are demonstrated in Table 2 (relations only after successful eradication).

In the patients with unsuccessful *Hp* eradication, immune reactivity significantly increased in 1 antigen expressed in SI (*hHp6*) in group A.

Table 2: Immune reactivity (LTT-MELISA) to various *Hp* antigens in group A and B - comparison before and after eradication

		Before eradication		*cca 3 months after eradication*		*cca 6 months after eradication*	
	group / antigen	**A**	**B**	**A**	**B**	**A**	**B**
SI	***PWM***	69.0	178.0	147.5	185.0	299.0	237.0
	hHp7	12.0	7.0*	11.5	24.0*	32.0	12.0*
	hHp6	14.0	4.0	8.5	7.0	28.0	9.0
	hHp5	3.5	2.0	3.0	1.0	3.0	2.0
	aHp7	17.5	14.0*	20.5	22.0	29.0	33.0*
	aHp6	12.0	7.0	18.0	16.0	54.0	17.5
	aHp5	4.0	2.0	7.0	2.5	12.0	7.5
	CagA	1.0	0.0	1.0	0.0	2.0	1.0
	urease	3.0	1.0	4.0	0.0	4.0	1.0
	Hp protein	3.0	0.0	1.0	0.5	2.5	0.5
	HpAg	14.5	7.0*	19.0	32.0*	39.0	26.0*
Δ cpm	***PWM***	25815.0	13224.0	34377.0	39114.5	21521.0	5788.0
	hHp7	2217.0	898.0	2431.0	3327.5	3287.0	832.0
	hHp6	2872.0	445.0	3788.0	1070.5	2327.0	27.0
	hHp5	203.0	165.0	783.5	55.0	923.0	332.0
	aHp7	3574.5	436.0*	6474.0	4469.0	5654.0	9312.0*
	aHp6	654.5	937.0	8553.0	1559.0	1721.0	3554.0
	aHp5	122.0	85.5	2323.0	1076.5	1569.0	1766.0
	CagA	38.0	-2.0*	174.0	-16.5	147.0	40.0*
	urease	379.0	15.5	449.0	-18.0	913.0	164.5
	Hp protein	151.0	-9.5	278.0	-25.5	201.5	-15.5
	HpAg	2991.5	3007.0	7477.0	3242.0	6372.5	1399.0

The values of SI and Δcpm are in medians.

* significant difference ($p<0.05$)

Case reports of one patient with *Hp* positive antral gastritis and AT and one patient with *Hp* positive antral gastritis without AT are shown in Figure 2a, 2b. After successful eradication, confirmed by clinical improvement and negative urea breath test, the increase of cellular reactivity to *Hp* antigens was detected in these patients. There was no substantial difference in reactivity of representatives in both groups.

cagA/vacA status of the Hp infection

Anti-CagA antibodies and *Hp* genotyping wasn't performed in all patients because of technical problems.

Serological results: Anti-CagA IgG antibodies were found in 58,3% (7 out of 12) in group A, 40% (4 out of 10) in group B and 0% (0 out of 15) in group C.

Hp genotyping: The similar distribution of cagA and vacA positivity was found in group A and B: cagA+ in group A (36,4%, 4 out of 11), group B (38,9%, 7 out of 18), vacA+ in group A (62,5%, 5 out of 8), group B (66,7%, 12 out of 18).

The presence of anti-CagA antibodies significantly correlated with cagA+ *Hp* genotype.

DISCUSSION

In present study, we have determined specific cellular immune response to *Hp* antigens in *Hp* infected patients with and without autoimmune thyroiditis (AT) using modified lymphocyte transformation test, LTT-MELISA, before and after eradication therapy. Before eradication, in majority *Hp* infected patients, lymphocyte responses to *Hp* antigens were often lower than that of healthy controls. These findings are consistent with previous studies. Several studies have shown inhibitory effects of another important *Hp* virulence factor, vacuolating toxin VacA on the T cell's proliferation (Molinari *et al.* 1998; Boncristiano *et al.* 2003; Gebert *et al.* 2003, 2004; Sundrud *et al.* 2004). *Hp* can induce *Hp*-specific regulatory T cells that actively suppress T-cell response. The elimination of regulatory T cells led to restoration of the proliferative response to *Hp* (Lundgren *et al.* 2003, 2005; Kandulski *et al.* 2008b). Das *et al.* (2006) presented that expression of the co-stimulatory molecule B7-H1 by gastric epithelial cells is higher in *Hp* infected, and this molecule can interact with mucosal T-cells resulting in suppression of T-cell activity. Another studies on *Hp* immunity indicated that infection might induce T cell hyporesponsiveness. Both, peripheral blood lymphocytes and gastric lymphocytes from *Hp* positive patients were shown to respond to *in vitro* stimulation by *Hp* antigens with low cytokine secretion and proliferation relative to *Hp* negative controls (Karttunen *et al.* 1991; Birkholz *et al.* 1993; Knipp *et al*. 1993; Fan *et al*. 1994; Malfitano *et al*. 2006; Windle *et al*. 2005). Chmiela *et al.* (1996a, 1996b) demonstrated that activation or immunosuppression can depend on the concentration of *Hp* and its products. In other study, inhibition of lymphocyte proliferative response to *Hp* by plastic adherent cells was described (Uyub *et al*. 2001).

We observed higher stimulation after autologous than heterologous *Hp* in proliferation response of PBMC. It is in contrast with other study, where proliferative response of PBMC was significantly lower after autologous than after heterologous stimulation. However, there was no significant difference when T cell activation markers were observed (Jakob *et al.* 2001).

After successful eradication of the *Hp* infection, increased cellular reactivity to specific *Hp antigens* was observed but not after non specific stimulation by PWM. In certain *Hp* antigens (autologous and heterologous *Hp* strain, *CagA* and partially purified *HpAg*), increase was statistically significant. Kopitar *et al.* (2007) described a significantly increased response in expression of IFN-gamma and IL-4 molecules by DCs stimulated T cells in subjects that successfully eradicated *Hp* compared with those who failed to eradicate the infection. On the basis of our findings, we can suppose that successful *Helicobacter pylori* eradication restores anti-*Hp* immune response.

LTT used in different modifications, belongs to *in vitro* diagnostic methods in immunology for evaluation of specific cellular immunity in patients with allergy, immunodeficiences, autoimmune, infectious and tumor diseases (Halpern *et al.* 1967; Hybenova and Sterzl 2007). Modified LTT-MELISA has previously been used for the diagnostics of infection in clinically and serologically ambiguous cases of borreliosis (Valentine-Thon *et al.* 2007). This is the reason why we use this newly modified LTT as a possible test for diagnostics and therapy control of *Hp* eradication. In base of our findings (high variability of values, impossibility to determine borders of positive and negative SI values) we think that this test is not suitable for detection of *Hp* infection, but more data are necessary.

For better characterization of immune response to *Hp*, we have also determined cagA/vacA status of *Hp* infection in patients based on serological examination and genotyping of *Hp* strains isolated from patients. CagA protein has been described as a antigen with high pro-inflammatory potential (Crabtree *et al*. 1994; Blaser and Atherton 2004). In cagA positive *Hp* infected patients, we detected higher immune reactivity in comparison with cagA negative patients. CagA protein is main virulence factor of *Hp* so probably strong immune response maybe induced in order to eliminate the invader harming significantly the host.

Although, some studies demonstrated relevance of *Hp* infection in AT (De Luis *et al.* 1998; Bertalot *et al.* 2004) we didn't observed differences between immune cellular reactivity in *Hp* infected patients with or without AT. In agreement with other study (Figura *et al.* 1999), we find higher prevalence of anti-CagA antibodies in patients with AT.

In conclusion, these data indicate that *Helicobacter pylori* might cause inhibition of the specific cellular immune response in *Hp* infected patients with or

without autoimmune diseases such as AT. This immunosupression can be reversed by successful eradication of *Hp*. LTT appears to be a good tool for detection of immune memory cellular response rather than a diagnostic tool of *Hp* infection. Our results give a strong support to eradication therapy of *Hp* in general where eradication could lead to restitution of cell immune response.

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