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INFLUENCE EXERTED BY *HELICOBACTER PYLORI* ON CONCENTRATIONS OF ANTI-INFLAMMATORY T-CELL CYTOKINES AND SUNPOPULATIONS THAT PRODUCE THEM

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Helicobacter pylori is a widely spread pathogenic microorganism. It penetrates the mucous tunic of the stomach and the duodenum and causes diseases in the gastrointestinal tract, including oncologic ones. This agent is able to be chronically persistent in a body and frequently there are no apparent symptoms of it; therefore, it is difficult to detect this pathogen in due time. Risk analysis related to occurrence and development of various pathologies associated with Helicobacter pylori, revealed that their clinical course was to a great extent determined by an immune response that emerged after infection. There are data that Helicobacter pylori is able to influence protective immune reactions making their balance to move to an increase in immune-suppressive components, for example, increased concentrations of T-regulatory cells and cytokines produced by them. However, some data can be found on Helicobacter pylori ability to induce anti-inflammatory responses which include those associated with T-helpers of the 1st and 17th types. Our research goal was to reveal peculiarities of effects produced by this pathogen on γ-interferon as one of basic products by 1st type T-helpers and on contents of the 17th type T-helpers determined as cells belonging to $CD4^+CD161^+$ and $CD4^+IL17^+$ phenotypes under direct contacts between bacteria and lymphocytes. Our research objects were clinical isolates of Helicobacter pylori and blood samples taken from people without helicobacter infection in their case history. We extracted lymphocytes with immunomagnetic separation out of mononuclear blood cells obtained via functioning in density gradient. Their concentrations were assessed with cytofluorometry; cytokines products, with enzyme-linked immunosorbent assay. We showed that $CD4^+CD161^+$ and $CD4^+IL17^+$ cells content didn't change when they were cultivated together for 18 hours under influence exerted by Helicobacter pylori, while products of γ-interferon increased considerably. It can probably be related to activation of the 1st type T-helpers under effects produced by direct contact with bacteria. However, we didn't detect any activation of the 17th type T-helpers. Therefore, we can assume that effects produced by Helicobacter pylori on T-helpers under direct contact cause a response in a form of the 1st type T-helpers activation.

Key words: *Helicobacter pylori, lymphocytes, T-helpers, differentiation, co-stimulation, антитела, flow cytofluorometry, cell cultures.*

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Contemporary healthcare faces variable vital tasks, and one of them is to analyze risk factors that cause occurrence and development of diseases in the gastrointestinal tract. All the age groups are susceptible to risks of damage to the digestive organs, including employable population, elderly people, children, and teenagers. High costs related to necessary treatment and rehabilitation of patients which can be rather expensive make prevention and anti- recurrent treatment of such pathologies not only medical, but also a social problem.

Helicobacter pylori (*H. pylori*) is a widely spread pathogenic microorganism associated with gastrointestinal tract diseases. *H. pylori* is well proven to selectively colonize the mucous tunic of the stomach and duodenum and is considered to be an etiological agent that causes acute and chronic gastritis, ulcer, and other diseases in the gastrointestinal tract [1, 2]. *H. pylori* has a distinctive peculiarity which is its ability to persist in a body for a long time, and this persistence quite often has no symptoms thus making the detection and eradication of the pathogen rather complicated. Such an effect is produced via impacts exerted by the pathogen on the immune system of its host that result in activation of its immune-suppressing component [3, 4]. This hypothesis is confirmed by data found in literature that in some cases helicobacter infection makes for less apparent clinical course of autoimmune and allergic diseases [5, 6].

Besides, *H. pylori* was proven to promote an authentic increase in contents of FoxP3-positive T-regulatory cells (T-reg) and cytokines produced by them; such results were obtained during laboratory tests on model animals [7, 8]. There is also a discussion in the literature on *H. pylori* ability to influence immune cells directly in the stomach thus stimulating changes both in their activity and in levels of cytokines produced by them [9–11]. *H. pylori* ability to stimulate T-reg generation under a direct contact between bacteria and human responder lymphocytes in vitro was described in our previous paper [12]. But at the same time, when attempts were made to reproduce the effect both in vivo and in vitro, there was an increase in anti-inflammatory cytokines such as gamma-interferon INF-γ (INF-γ) and interleukin-17A (IL-17A), and also, apart from T-reg, there was an induction of the 1st and 17th type T-helpers (Th1 и Th17) [13–15].

INF-γ and IL-17A play quite a variable role in development of gastroenterological pathologies. They participate in eliminating infectious agents by neutrophils and macrophages. It is a violent response by the immune system, that is considered to be the most probable cause for development of acute pathologies in the gastrointestinal tract in case of infections with *H. pylori* [16, 17]. However, apart from it, at present IL-17 and Th17 that produce them are viewed as the most probable basic mediators of *H. pylori*-associated autoimmune gastritis [18]. Data on participation of Th1 and cytokines produced by them in autoimmune gastritis development are rarely found but the role they play in development of other autoimmune pathologies has been studied and outlined quite profoundly [19, 20]. So, induction of INF-γ and IL-17A and subpopulations that produce them detected under interaction with *H. pylori* can lead to occurrence of both inflammatory pathologies in the stomach and duodenum and a wide range of extra-gastroduodenal diseases that is confirmed by data taken from literature [21].

Given all the above said, there is a vital scientific and practical task to assess anti-inflammatory effects by *H. pylori* and mechanisms that determine proneness of the agent to both regulatory and anti-inflammatory action. Besides, a preliminarily revealed ability of *H. pylori* directly, without any participating antigen-presenting cells (APC), influence at least some human T-cells subpopulations is also of great interest.

Our research goal was to assess *H. pylori* ability to stimulate occurrence of INF-γ, IL-17A and Th17 under direct contact between bacteria and T-cells, without APC participation.

Data and methods. Our research objects were samples of whole peripheral blood taken from people without *H. pylori*-infection in their case history, its absence confirmed by data obtained via objective research techniques, (n=8), and *H. pylori* isolates taken during diagnostic EGD from people suffering from chronic gastritis (n=6). Blood was only once taken in volume equal to 8-9 ml into vacuum tubes with sodium heparin (Vacuette, Germany). Samples were treated not later than 2 hours after they had been

taken. Mononuclear cells of peripheral blood (MCPB) were extracted out of blood samples via centrifuging (for 45 minutes at 1,500 turns per minute) at "Diakoll-1077" density gradient ("DiaM" Russia). After it, we extracted only $CD4^+$ cells out of obtained MCPB via immune-magnetic separation with Human naïve CD4+ T-cell enrichment Kit (Stemcell technologies, the USA). *H. pylori* was extracted out of biopsy materials taken during diagnostic EGD out of the antral section and body of the stomach; biopsy materials were taken from people with positive CLO-test. The materials were ground mechanically and then sowed on Columbian agar (Becton Dickinson, the USA) with added 10-% defibrinated donor blood as well as with antibiotics for suppressing growth of extraneous microflora and fungi (10 μg/l of Vancomycin, 5 mg/l of Trimethoprim, and 2 mg/l of Nystatin. all produced by Teva, Israel). Cultivating was performed for 7 days under microaerophilic conditions, the temperature being 37 °C. *H. pylori* was identified on the basis of cultural and morphological features.

To assess influence exerted by *H. pylori* on lymphocytes differentiation, we performed joint cultivation of lymphocytes with various concentrations of bacteria (we applied the following ratios of lymphocytes to *H. pylori* one by one: 1:10, 1:20, 1:50) for 18 hours under the following conditions 5% CO2, 37 °C, RPMI-1640 medium (Gibco, the USA) with added 10%-fetal bovine serum and 0.3 g/l of L-glutamine ("Paneko", Russia). A part of lymphocytes was cultivated with bacteria when additional stimulators were present; these stimulators were monoclonal antibodies to CD3 molecule (1 μg/ml, eBioscience, the USA) that imitated influence on T-cell receptor, or a mixture of antibodies to CD3 and CD28 (1 μg/ml, eBioscience, the USA, and 3 μg/ml, Beckman Coulter, France), that imitated influence exerted by APC on T-cells. We had the following cultures in our experiment: lymphocytes with added *H. pylori*, but without any stimulating antibodies; lymphocytes with added antibodies to CD3 and without bacteria; lymphocytes with added antibodies to CD3 and with *H. pylori*; lymphocytes with added antibodies to CD3 and CD28, and without *H. pylori*; and lymphocytes with added antibodies to CD3 and CD28 and with *H. pylori*. The last culture was included into the experiment in order to assess influence exerted by direct presence of the agent on the nature of stimulation. Experiments for all the ratios of lymphocytes and bacteria were performed separately. Lymphocytes without *H. pylori* and stimulating antibodies were negative controls for all the cultures.

After 18 hours 18 we applied cytofluorometry to estimate Th17 as cells of $CD4^+CD161^+$ и $CD4^+IL\text{-}17A^+$ phenotype in all the cultures. To paint the above mentioned markers, we applied antibodies to CD4 tagged with FITC; antibodies to CD161 tagged with PE; antibodies to IL-17A tagged with PE; all produced by eBioscience, the USA. We performed permeabilization of membranes necessary to tag IL-17A with Fix/Perm Concentrate reagents kit and Perm Buffer reagents kit (eBioscience, the USA) according to manufacturer's instructions. Analysis was conducted with FacsCalibur cytofluorimeter (Beckton Dickinson, the USA). Th1 activity was determined by measuring INF-γ concentrations in supernatants of the cultures with ELISA technique (Vector-Best, Russia). we applied Newman-Keuls test to statistically process the obtained data.

Results and discussion. An increase in INF-γ level is a significant part in immune response realization. This cytokine is a macrophages activator and it is involved into a direct response to infection attacks; besides, it enhances effects produced by α and β interferon, promotes an immune response as per Th1 type, and is able to stimulate activities of antigen-representing cells [22].

As we can see from Figure 1, when *H. pylori* was added to a suspension of extracted lymphocytes, both without additional stimulators and together with antibodies to CD3 or CD3/CD28, it led to statistically authentic increase in INF-γ production.

This production in the control culture was equal to only 10±4.08 pg/ml; when bacteria were added in a ratio 1:10, it went up to 835±351.4 pg/ml; in a ratio 1:20, 745±164.1 pg/ml; in a ratio 1:50, 135±121.8 pg/ml. INF-γ production in cultures which were additionally stimulated with antibodies had no statistical discrepancies from a variant with only bacterial stimulations; thus, INF-γ concentration in a culture consisting of T-lymphocytes with *H. pylori*

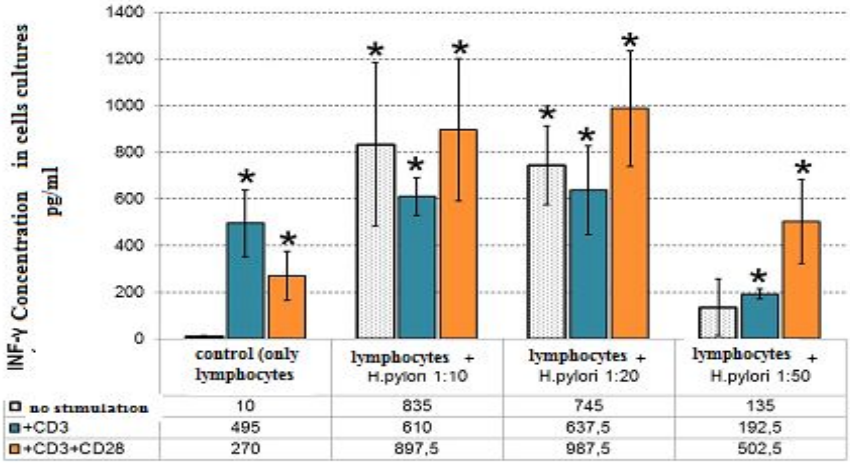

Figure 1. Influence exerted by *H. pylori* on INF-γ production. Variants of stimulation are given below the graph. Control is a culture with lymphocytes only, without added bacteria or antibodies, * means there are authentic discrepancies from the control ($p<0.05$).

and added antibodies to CD3 amounted to 610±081.3 pg/ml at a ratio being 1:10; 637.5±189.7 pg/ml at a ratio being 1:20; and 192.5±21.3 pg/ml at a ration being 1:50. INF-γ concentration in samples with T-lymphocytes with added *H. pylori* and additional stimulation with an admixture of antibodies to CD3 and CD28 amounted to 897.5±300.1 pg/ml at a ration being 1:20; 987.5±249.1 pg/ml at a ratio being 1:20; 502.5±180.01 pg/ml at a ratio being 1:50. All the obtained results coincide with data taken from literature on clinical course of *H. pylori*-associated gastritis accompanied with Th1 accumulation and increased INF-γ in the stomach mucous tunic [13].

Figures 2 and 3 show data on influence exerted by *H. pylori* on differentiation of lymphocytes towards Th17 and them acquiring $CD4^+CD161^+$ phenotype and without any dendritic cells in the cultures.

As we can see, joint cultivation of *H. pylori* and T-cells for 18 hours didn't lead to an increase in $CD4^+CD161^+$ cells number. Their average number in cultures without bacterial stimulation amounted to 20.065±0.72% from all the $CD4^+$ cells; when *H. pylori* was added to responder lymphocytes in a ratio 10:1, it amounted to 22.15±1.49 %. And concentrations of such cells in cultures with ratios 1:20 and 1:50 also had practically no differences from control concentrations of non-stimulated lymphocytes (the number amounted to 24.2±3.41% for a ratio 1:20, and to 23.15±2.73% for a ratio 1:50).

To test significance of co-stimulation, we performed additional experiments adding stimulating antibodies to CD3 and CD3+CD28 molecules; these antibodies sent a signal to T-cells which was similar to that sent by antigen-presenting cells in the process of stimulation. $CD4^+CD161^+$ cells concentration amounted to 23.745±7.3% in a culture of T-lymphocytes without *H. pylori* but with added antibodies to CD3. But at the same time, when both *H. pylori* and antibodies to CD3 were added to a culture, $CD4^+CD161^+$ cells concentration amounted to 23.83±3.30% for a ratio 1:10; 25.4±2.42% for a ratio 1:20; and 25.4±1.75% for a ratio 1:50.

CD161 molecule under its co-expression with CD4 molecule is widely used in world scientific practice as a Th17 population marker. However, occurrence of membrane phenotypic markers doesn't guarantee that responder cells are functionally able to produce IL-17A or, in other words, to fulfill basic Th17 function. Also, some authors state, that it is more reliable to assess Th17 concentration as per occurrence of intracellular IL-17A or its products than to apply CD161 in the process. Given all the above stated, we assessed concentrations of intracellular IL-17A in cultures of lymphocytes that responded to *H. pylori*. To do that, we performed permeabilization of responder cells membranes and

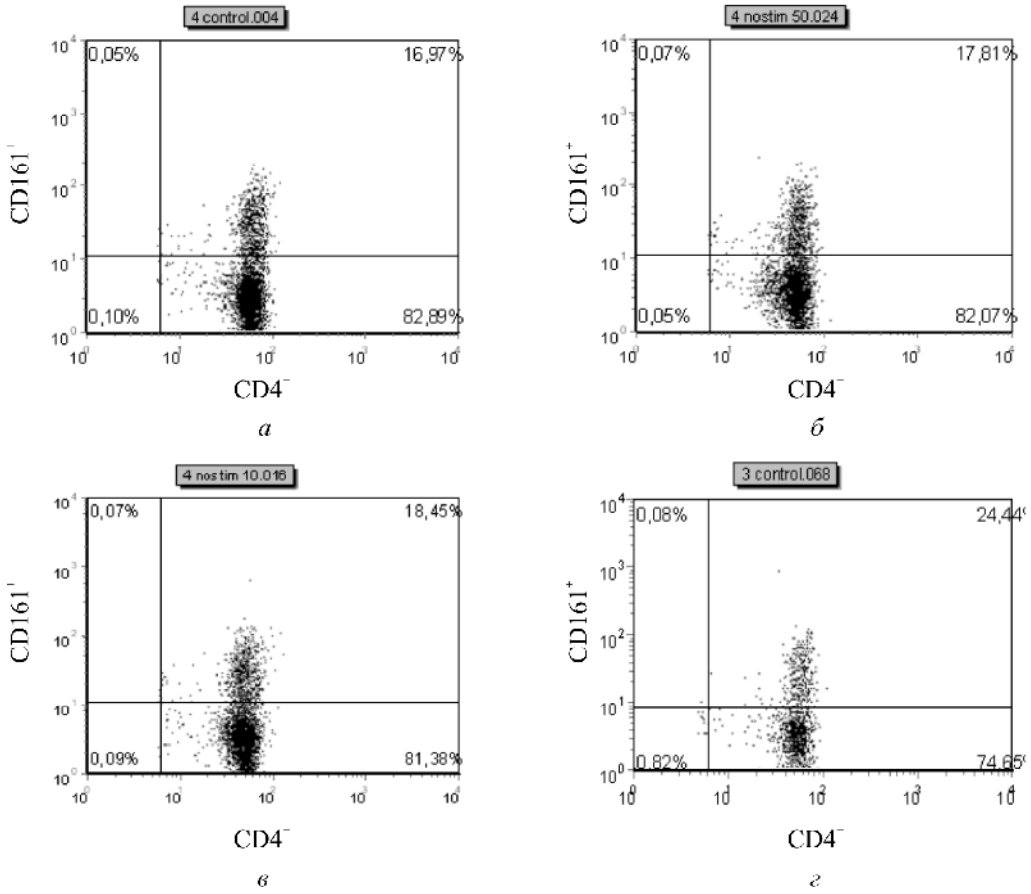

Figure 2. $CD4^+CD161^+$ cells concentrations in T-lymphocytes cultures under joint cultivation with *H. pylori* without additional stimulation, in % of the overall CD4+ cells number (data obtained via a representative experiment): A is a control suspension of lymphocytes without *H. pylori*; B is joint cultivation with *H. pylori* in a ratio 1:10, C and D is joint cultivation with *H. pylori* in ratios 1:20 and 1:50 respectively. A per cent content of cells is given in the angles of the quadrants.

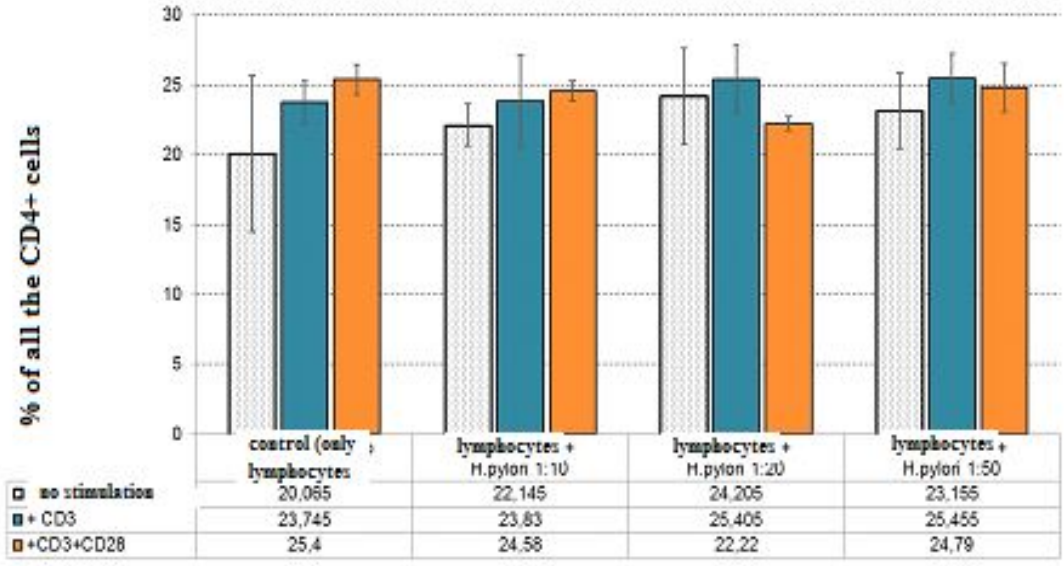

Figure 3. Influence exerted by *H. pylori* on $CD4^+CD161^+$ cells concentration. variants of stimulation are given below the graph. Control is a culture with lymphocytes only, without added bacteria or antibodies

Table

IL-17A$^+$CD4$^+$ cells concentrations under joint cultivation with *H. pylori* and/or additional stimulation with antibodies to CD3 and CD28

IL-17A$^+$ cells concentration (% from all CD4$^+$ cells)	Lymphocytes without *H. pylori* (control)	Lymphocytes without *H. pylori*, CD3	Lymphocytes without *H. pylori*, CD3 and CD28	Lymphocytes + *H. pylori* (1:10)	Lymphocytes + *H. pylori* (1:10), CD3	Lymphocytes + *H. pylori* (1:10), CD3 and CD28
	0,27 ± 0,08	0,21 ± 0,02	0,23 ± 0,033	0,43 ± 0,07	0,26 ± 0,03	0,14 ± 0,04

painted them with monoclonal antibodies to IL-17A. We detected that most cells don't stimulate greater IL-17A expression under cultivation conditions applied in our experiments (Table).

Thus, IL-17A$^+$ cells concentration didn't exceed 0.5% of all the CD4$^+$ cells in any of the applied variants of stimulation and ratios of responder cells and bacteria; such concentration doesn't have any discrepancies from standard IL-17$^+$ cells concentration in human blood [23–25].

Conclusions.

Direct joint cultivation of extracted T-lymphocytes with *H. pylori* promotes a drastic increase in INF-γ production, and it, under these experimental conditions, can most probably mean that Th1 activation occurs. However, Th17 concentrations in % (determined both as CD4$^+$CD161$^+$, and as CD4$^+$IL17A$^+$) changed only slightly under such conditions. We can assume that a response from T-helpers to a direct contact with *H. pylori*, given its anti-inflammatory effects, develops as per Th1 type, without Th17 being significantly involved in the process. A mechanism that determines *H. pylori* ability to stimulate activity of both Th1 and T-reg (which was shown by us earlier) requires further investigation. Overall, the authors think that mechanisms and acting agents that help a pathogen to exert direct influence on a prevailing type of an immune response are of great fundamental and practical interest; they potentially can be applied in development of medications that guide an immune response to a right direction. They are also important for assessing risks of hyper-stimulated immune response development in patients infected with *H. pylori.*

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