MEDICAL AND BIOLOGICAL ASPECTS OF THE ASSESSMENT OF THE RISK FACTORS

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INFLUENCE EXERTED BY HELICOBACTER PYLORI ON REGULATORY T-CELLS DIFFERENTIATION

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Estimating risks of infections induced by gram-negative Helicobacter pylori, is a vital problem for healthcare due to wide spread of the agent and wide range of induced pathologies which include malignant neoplasms in gastrointestinal tract. The agent is prone to long-term chronic persistence despite its "fragility" and its being greatly demanding to culturing conditions. The persistence issue is of special interest here as it is related to data on Helicobacter pylori capability to change immune response in infected people inducing suppressive regulatory immune reactions which are more favorable for the agent, both in stomach and in a whole body. Our research goal was to estimate Helicobacter pylori capability to induce differentiation of regulatory CD4+CD25+FoxP3+ human T-cells as basic mediators of immune response regulation under direct contact

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between bacteria and T-cells without any participation of most professional antigen-presenting cells. Our re-

search objects were clinical isolates of Helicobacter pylori and T-lymphocytes samples taken from people who didn't have Helicobacter pylori-infection in their case history; isolates and samples were jointly cultivated in vitro. We applied cytofluorometry to estimate changes in regulatory T-cells content. We detected that if T-lymphocytes and Helicobacter pylori were jointly cultivated during 18 hours in ratios from 1:10 to 1:50, regulatory T-cells content in cultures increased 2.12 times on average. This effects doesn't require any dendritic cells in a culture and obviously affects T-lymphocytes which are originally committed to regulatory T-cells in their development. Also, in our opinion, influence exerted on regulatory T-cells differentiation is a specific feature of Helicobacter pylori.

Key words: *Helicobacter pylori, lymphocytes, regulatory T-cells, differentiation, co-stimulation, antibodies, flow cytofluorometry, cell cultures.*

Helicobacter pylori (*H. pylori*) is a gram-negative bent bacillus which selectively forms colonies on mucous coat of human stomach and duodenum. At the moment *H. pylori* is considered to be an etiological agent causing acute and chronic gastritis, as well as etiologic pathogenic factor causing stomach and duodenum ulcer, stomach carcinoma and MALT-lymphoma [1, 10]. To succeed in colonies formation on mucous coat of stomach and duodenum, *H. pylori* has to overcome numerous mechanisms of inborn and adaptive immune system of a host which include acute neutrophilic and lymphocytic infiltration of a damaged area, as well as production of bacteria-specific IgM и IgA [2]. However, in spite of immune response evolvement, *H. pylori* can successfully persist in a stomach for decades [8].

There are some mechanisms which favor long persistence of *H. pylori* in a host; increased CD4+CD25+FoxP3+ T-regulatory cells content which can at least be observed at later contamination stages is one of them [9]. T-regulatory cells (T-reg) are a specialized subpopulation of CD4+ T-lymphocytes which is able to suppress activity of other lymphoid cells and either prevent or decrease intensity of inborn and adaptive immune response. T-reg basic physiologic function is to maintain peripheral tolerance [12]; T-reg absence or deficiency lead to evolvement of grave pathologies with autoimmune etiology [11]. At the same time, T-reg excessive activity is known to favor both infection process and tumors growth [13, 16].

Mechanism which helps *H. pylori* to induce increase in T-reg quantity has not been clearly defined yet, although immune-regulatory effects of T-reg induced by helicobacteriosis have been described. Thus, in particular, it is known that experimental autoimmune diseases proceed milder in experimental animals contaminated with *H. pylori*, [5, 15]. An attempt to generate T-regulatory cells via most profoundly described model of immune responses induction (T-lymphocytes activation by dendritic cells stimulated by helicobacter) gave controversial and multidirectional results. It led to induction of both T-reg and T-helpers of the 1st and 17th types which were active immune response stimulators [14]. Moreover, if during an experiment immune response shifts were detected to inhibiting T-reg as well as stimulating T-helpers of the 1st and 17th type, then mixed immune response without prevalence of any part was mentioned in both basic research and contemporary works [7, 8]. In our opinion, the existing data prove there are additional mechanisms which manage T-reg differentiation. They can be either stomach microenvironment conditions or direct effect exerted on lymphocytes by the agent able to contact them in mucous coat. Hypothesis of direct contact is most easily tested and has limited experimental confirmation [3, 4, 6].

Our research goal was to detect whether *H. pylori* exerted any influence on T-reg differentiation under conditions of direct contact between bacteria and T-cells and to assess the degree of such influence, as well as without participation of dendritic cells as most active antigen-presenting cells of immune system.

Data and methods. Our research objects were samples of whole peripheral blood taken from people with gastroenterological diseases (n = 6), who didn't have *H. pylori* in their case history and it was also proved by

objective research data, and also *H. pylori* clinic isolates obtained during diagnostic fibergastroscopy. 8-9 ml of blood were taken only once, into vacuum vials with sodium heparin (Vacuette, Germany). Work with samples started not later than 2 hours after sampling. Mononuclear cells of peripheral blood (MCPB) were extracted from blood samples via centrifuging (45 minutes, 1500 turns per minute) on «Diakoll-1077» density gradient («DiaEm», Russia). The obtained MCPB were divided into adhesive monocytic fraction and non-adhesive lymphocytic one via adhesion on plastic (2 hours). Only lymphocytic fraction was used in further research. *H. pylori* was extracted out of bioptic material obtained during diagnostic fibergastroscopy from antral section and body of stomach performed on patients with positive CLO-test. The obtained material was ground mechanically and sowed on Colombian agar (Becton Dickinson, USA) and 10%-defibrinated donor blood was added to it as well as antibiotics which were needed to suppress growth of extraneous microflora and fungi (10 μg/l of vancomycin, 5 μg/l of Trimethoprimum, 2 μg/l of nystatin, all produced by Teva, Israel). Cultivation was performed under microaerophilic conditions at 37 °C, during 7 days. Helicobacter was identified on the basis of cultural and morphological features. To assess influence exerted by *H. pylori* on lymphocytes differentiation to T-regulatory cells direction, we accomplished joint cultivation of lymphocytes with various bacteria concentrations (we used 1:10, 1:20, and 1:50 lymphocytes to *H. pylori* ratios) for 18 hours under the following conditions: 5 % CO_2, 37 °C, RPMI-1640 medium (Gibco, USA) adding 10% embryonic calf serum and 0.3 g/l L-glutamine ("Paneko", Russia). A part of lymphocytes was jointly cultivated with bacteria with additional stimulators, such as CD3 molecule monoclonal antibodies (1 μg/ml, eBioscience, USA), or CD3 and CD28 antibodies mixture (1 μg/ml, eBioscience, USA and 3 μg/ml, Beckman Coulter, France). Lymphocytes without *H. pylori* supplement were negative control for all cultures. Lymphocytes cultivated with *E. coli* in 1:50 ratio, both with and without antibodies, were additional control. After 18 hours we applied cytofluorometry to assess T-regulatory lymphocytes content in cultures as cells of CD4+CD25+FoxP3+ phenotype. To paint the said markers, we used FITC-tagged CD4 antibodies, APC-tagged CD25 antibodies, PE-tagged FoxP3 antibodies, all produced by eBioscience, USA. Membrane permeabilization necessary for FoxP3 tagging was conducted with the use of «Foxp3 / Transcription Factor Staining Buffer Set» (eBioscience, США) as per manufacturer's instructions. We performed the analysis with the use of FacsCalibur cytofluorimeter (Beckton Dickinson, USA). To statistically process the obtained data, we used Newman-Keuls criterion.

Results and discussion. Figures 1 and 2 present the data on influence exerted by *H. pylori* on lymphocytes differentiation to T-reg direction when dendritic cells were absent in cultures. As we can see, adding *H. pylori* to T-cells culture led to authentic increase in T-regulatory CD4+CD25+FoxP3+ cells after 18 hours of joint cultivating (average T-reg content in cultures without bacterial stimulation amounted to 6.01 ± 0.72 % of all CD4+ cells, but when *H. pylori* was added in 1:10 ratio, T-reg content grew up to 15.04 ± 1.97 %, $p<0.01$).

Cells content in cultures with lymphocytes-bacteria ratio being 1:20 and 1:50 was also authentically higher than control values for non-stimulated lymphocytes (12.7 ± 1.53 % for 1:20 and 10.42 ± 1.97 % for 1:50, discrepancy from control is authentic, $p<0.01$).

At the same time, as we can see, there was a trend of inverse dose relation in a number of ratios. 1:10 ratio had the greatest growth in T-reg quantity which went down as bacteria number in cultures grew. However, this trend remained statistically not authentic. So, the obtained data reveal that *H. pylori* has some influence on responding T-lymphocytes regardless of dendritic cells presence in a culture (as most efficient antigen-presenting cells); in our opinion, this influence was mediated by direct contact between bacteria and responding T-lymphocytes).

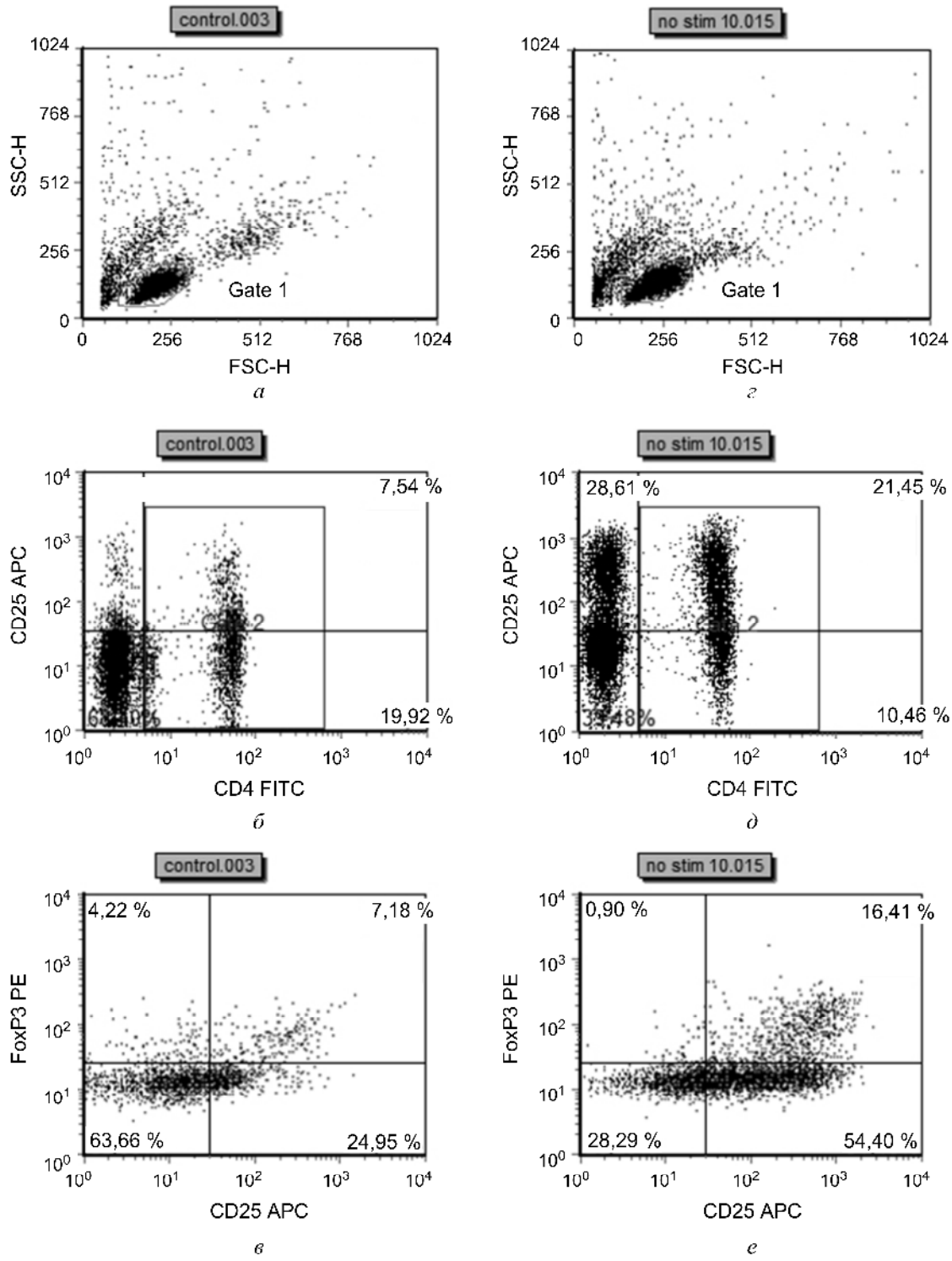

Figure 1. *H. pylori* increases T-regulatory cells content in joint cultivating with lymphocytes: *а – в* are the data obtained in representative experiment, T-regulatory cells content in cultures without *H. pylori* (*а* is cells distribution as per light scattering, *б* is CD4+CD25+ cells extraction, *в* is FoxP3+ T-regulatory cells extraction, the estimated population is in the upper right quadrant); *г – е* the data obtained in representative experiment, T-regulatory cells content in cultures, which grew with *H. pylori* (*г* is cells distribution as per light scattering, *д* is CD4+CD25+ cells extraction, *е* is FoxP3+ T-regulatory cells extraction, the estimated population is in the upper right quadrant). estimated markers and registered parameters are given at axes, cells percentage is given in angles of quadrants

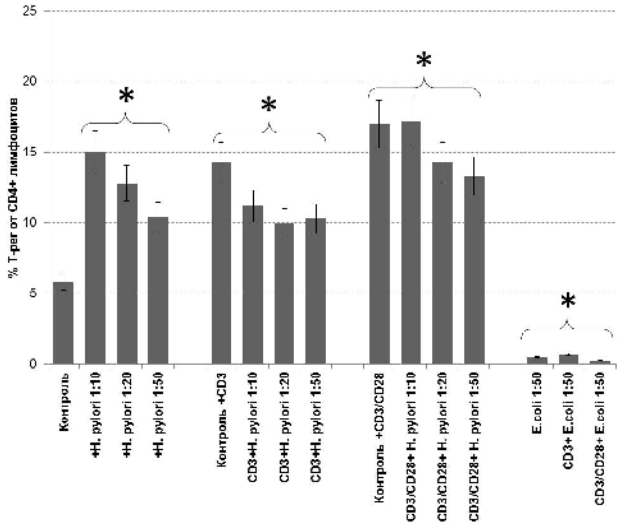

Figure 2. *H. pylory* influence on T-regulatory cells differentiation. Stimulation variants are given below the diagram. Control is lymphocytes without bacteria and antibodies; * discrepancies with control culture are authentic, $p<0.01$

(x-axis: control; y-axis: % of T-reg in CD4+ lymphocytes)

At the same time, there can be some impacts caused by B-lymphocytes present in cultures under these experimental conditions as such cells also have antigen-presenting properties. To exclude their impacts in future, we plan to perform B-cells depletion.

To check co-stimulation significance, we had additional culture types where not only bacteria but also CD3 and CD28 stimulating antibodies were applied; such antibodies gave T-cells a signal similar to antigen-presenting cells stimulation.

Figure 2 presents the results of analyzing cultures with antibodies. As we can see, stimulation with antibodies instantly led to significantly high levels of T-reg content in cultures (14.0 ± 1.29 % of T-reg for cultures where only CD3 without *H. pylori* were added, 16.54 ± 2.13 % for cultures where CD3 and CD28 mixture was added, a model of more comprehensive stimulation, but still without *H. pylori*). *H. pylori* introduction into cultures didn't cause increase in T-reg content (for cultures with CD3 T-reg content amounted to 11.25 ± 1.06 % for 1:10 ratio, 10.04 ± 1.14 for 1:20 ratio, 10.28 ± 1.54 % for 1:50 ratio, there were no authentic discrepancies with culture with CD3 without *H. pylori*; T-reg content amounted to 16.93 ± 3.74 % for cultures with CD3 and CD28 mixture for 1:10 ratio, 13.95 ± 2.13 % for 1:20 ratio, 12..82 ± 1.28 % for 1:50 ratio, there were no authentic discrepancies with culture with CD3+CD28 mixture without *H. pylori*). Just like in case with cultures stimulated by *H. pylori* only, we observed a trend to inverse dose relation between microbe dose in a culture and T-reg content.

All values of T-reg content in cultures with CD3 and CD3+CD28 were authentically different from T-reg content in cultures of lymphocytes without antibodies and *H. pylori* ($p<0.01$) and they had no discrepancies with cultures stimulated by *H. pylori* without antibodies. So, *H. pylori* stimulates lymphocytes differentiation to T-reg at the level of activation by antibodies. Lack of increase in T-reg content in cultures stimulated simultaneously by *H. pylori* and antibodies can probably be explained by polyclonal character of antibodies' activating effect which resulted in differentiation of all CD4+ T-cells initially committed to development into T-reg. To check specificity of *H. pylori* effects, we used lymphocytes cultures stimulated by another microbe which inhabited human gastrointestinal tract, namely *Escherichia coli (E. coli).*

Lymphocytes and *E. coli* cultures which we used grew both with and without CD3 and CD28 antibodies. Lymphocytes and *E. coli* ratio was 1:50. As we can see on Figure 1, E. coli didn't cause T-reg growth in cultures and prevented antibodies' stimulating effects. Thus, the data we obtained show that effects which stimulate T-reg differentiation are caused exactly by *H. pylori* and apparently they are not mediated by pathogens patterns (which are similar in *H. pylori* and *E. coli,* both being active gram-negative microbes).

Conclusions. Assessing whether microbes can modulate immune responses is a vital fundamental and practical task, in particular, if such modulation makes for microbes survival in a host. A better insight

into such mechanisms undoubtedly can help both to predict possible risks caused by a microbe and to raise the efficiency of therapeutic approaches to pathologies caused by such microbes. In our work we showed *H. pylori* ability to exert differentiating effects on T-lymphocytes directing them to T-regulatory cells. If there were no dendritic cells in cultures (immunocytes population), the process as per contemporary data had most apparent ability to manage immune response. Within the examined group, such effects were observed when microbes and T-cells ration in cultures was from 1:10 to 1:50, and they didn't require antigen-presenting cells or stimulating antibodies' presence in cultures. On average, growth in percentage of T-regulatory CD4+CD25+FoxP3+ cells among CD4+ lymphocytes amounted to 2.12 times. Apparently, under this experiment conditions, *H. pylori* influence affected cells initially committed to development into T-reg, as polyclonal stimulation with CD28 antibodies and/or CD3 antibodies "absorbed" the effect from bacteria introduction; cultures stimulated only with antibodies, as well as antibodies together with *H. pylori*, had no statistic discrepancies in T-reg content. Apparently, differentiating effect was a specific feature of *H. pylori*, as lymphocytes stimulation with another microbe able to survive in human gastrointestinal tract for a long time (*E. coli*) didn't lead to increase in T-regulatory cells content. So, we essentially showed that *H. pylori* was able to exert influence on T-regulatory cells differentiation; this effect wasn't mediated by presentation of microbe antigens with dendritic cells, but evidently it depended on direct influence exerted by the microbe on T-cells. Insight into this mechanism' nature may enable determining its active principle which induces T-reg development and hence is potentially a natural tolerogenic factor which can be used as a medication.

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