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The Role of the Reduction of Extracellular Calcium Ion Concentrations in Activation of Human Peripheral Blood T cells

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Abstract—Calcium plays a fundamental role in many essential functions of a living organism. It is well known that many infections can reduce extracellular calcium concentrations ($[Ca^{2+}]_o$) (hypocalcaemia) in human blood. The importance of Ca^{2+} for the T lymphocyte activation has been known for a long time, but the optimal range of $[Ca^{2+}]_o$ levels has not been found yet. The main goal of this work was the study of functional activities of $CD4^+$ cells in human peripheral blood (HPB) in vitro in the conditions of hypocalcemia. Polyclonal activation was induced in whole blood of healthy donors by immobilized monoclonal antibodies (mAbs) toward CD3 molecules (epsilon chain of the T cell receptor) and dissolved mAbs toward CD28 (the major molecule of T cell costimulation). Dependencies of T-cell activation parameters on varied $[Ca^{2+}]_o$ in HPB in vitro were studied. Production of interleukins-2 (IL-2), IL-4, IL-17A, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) as well as $CD4^+CD69^+$, $CD4^+CD25^+$, and $CD4^+$(CYTOKINE)$^+$ T cells were used as activation parameters of the T cell polyclonal activation in HPB. EGTA was used as a tool for the reduction of $[Ca^{2+}]_o$ levels. The results demonstrated that the EGTA-stimulated binding of extracellular calcium in HPB caused a nonlinear dependence of T cell activation on $[Ca^{2+}]_o$. No EGTA influence on resting T cells was found. The dependence of the number of $CD4^+CD69^+$ T cells on $[Ca^{2+}]_o$ was represented as a nonlinear curve with two maxima and a local minimum in between. One of the maxima was in the area of the HPB lower $[Ca^{2+}]_o$ normal level, whereas the other one was even lower, which is characteristic for pathological states. The T cell-induced production of IL-2, IL-4, IL-17A, IFN-γ, and TNF-α as well as the populations of $CD4^+CD25^+$ and $CD4^+$(CYTOKINE)$^+$ T cells were analyzed in HPB in vitro under the same activation conditions. All the dependencies obtained were similar in shape to that of the $CD4^+CD69^+$ T cell population versus EGTA concentration in HPB in vitro and was observed as curves with two maxima. The dependencies of this type were described first. It is likely that the presence of two maxima and a local minimum in between for the dependence of HPB T cell activation parameters on $[Ca^{2+}]_o$ concentrations is of great physiological importance because hypocalcemia has been associated with many pathological states.

Keywords: $CD4^+$ T cell activation, cytokines, EGTA, ELISA, extracellular calcium concentration, FCM analysis, human peripheral blood

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INTRODUCTION

Calcium ion concentration ($[Ca^{2+}]_o$) in human peripheral blood has been considered as an important physiological parameter [1]. Its upper and lower normal levels in HPB (1.38 and 0.93 mM respectively) were found and a long term deviation from these values is regarded as pathology [2, 3]. General sepsis [4–6], AIDS [7], tuberculosis [8], malaria [9], and other infections often cause noticeable blood hypocalcemia [10].

The goal of this work was the analysis of the effect of $[Ca^{2+}]_o$ reduction on the polyclonal activation of HPB $CD4^+$ T cells in vitro. The choice of the whole blood for testing is not accidental. The in vivo T cell activation occurs in the microenvironment, which can be hardly modeled in experiments. If even the presence of erythrocytes is known to change the intensity of T lymphocyte response toward anti-CD3 mAbs [11], the impact of cells of other types for this response can be hardly assessed.

Extracellular calcium concentrations vary even in blood of healthy donors. Therefore, all the experiments were performed according to the following protocol. After $[Ca^{2+}]_o$ value was determined for each donor it was adjusted by the addition of concentrated $CaCl_2$ or EGTA to the value maximally close to 1 mM. A decrease in the $[Ca^{2+}]_o$ was provided by the EGTA addition. The blood preparations were activated with

Abbreviations: DMSO, dimethylsulfoxide; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IL, interleukin; IFN, interferon; Th1, Th2, and Th17, $CD4^+$ T helpers types 1, 2, and 17; TNF-α, tumor necrosis factor-alpha); mAbs, monoclonal antibodies; HPB, human peripheral blood.

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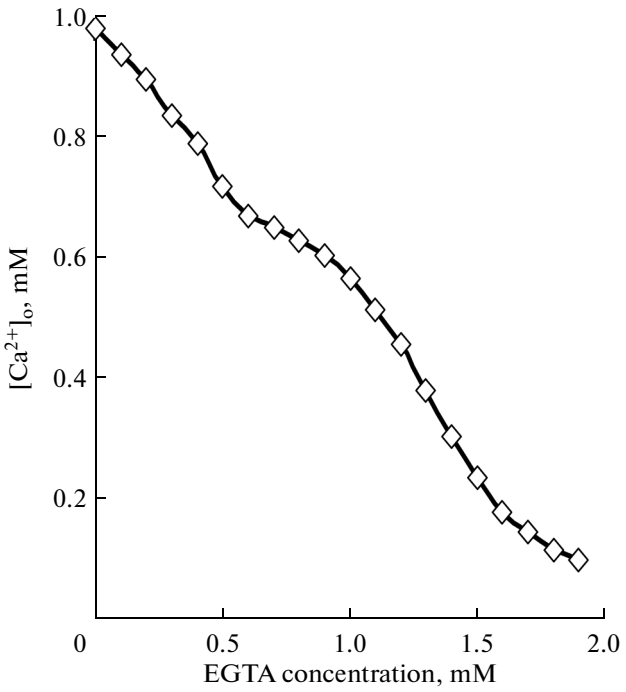

Fig. 1. A typical titration curve of heparinized HPB with increasing EGTA concentrations.

anti-CD3 and anti-CD28 mAbs, and activation parameters of $CD4^+$ T cells were analyzed. The parameters of $CD4^+$ T cell activation obtained at varied EGTA concentrations were normalized relative to the counterparts obtained for the whole blood of the same donor at $[Ca^{2+}]$ close to 1.0 mM. Such an approach provided a considerably lower spread of values even for healthy donors.

RESULTS AND DISCUSSION

Extracellular Calcium Ion Concentration in Healthy Donor Blood

Values of $[Ca^{2+}]_o$ in heparinized blood of healthy donors varied in the range of 1.05 to 0.89 mM and were always lower than those in blood serum [12, 13]. This can be accounted for by the heparin binding to Ca^{2+} [14–16].

In this work, a EGTA concentration rather than absolute $[Ca^{2+}]_o$ values were used as a measure of hypocalcemia. Below, we give the argument in favor of this choice.

First, IFCC (International Federation of Clinical Chemistry) [17] developed tight guidelines for the correct calculation of $[Ca^{2+}]_o$ in HPB long ago [17]. According to them, $[Ca^{2+}]_o$ values are measured under controlled conditions: anaerobiosis or the most severe hypoxia, constant HCO_3^- concentration in blood plasma or constant partial CO_2 pressure in the sample, constant pH (the best if 7.0 to 7.5) and constant sample temperature. At the same time, the $[Ca^{2+}]_o$ values measured at room temperature half an hour after blood sampling are accepted as relevant. We measured $[Ca^{2+}]_o$at varied EGTA concentrations using this time frame. Hence, within this time range there was good correlation between $[Ca^{2+}]_o$ and EGTA values.

Second, the IFCC guidelines have additional restrictions if the samples need to be stored for several hours, which are mainly involved with anaerobiosis, constant pH values, and reduced temperature [17]. These parameters of blood sample storage dramatically differ from the common optimal conditions of human cell cultivation, which are wet 5% CO_2 atmosphere, oxygen, and 37°C. In addition, a long term in vitro incubation of even resting cells under optimal cultivation conditions always causes changes in pH of blood plasma, HCO_3^- concentration, and composition of cell metabolites including those capable of binding to calcium ions [18–20]. Thus, $[Ca^{2+}]_o$ measured in healthy donor blood in the beginning of the experiment (in vivo) under optimal cultivation conditions (in vitro) and in the absence of any activation stimuli do not remain constant even for several hours. Therefore, measurements of $[Ca^{2+}]_o$ values on the basis of IFCC guidelines are hardly possible.

Third, in the course of polyclonal activation of T cells in HPB in vitro the intensity and pathways of numerous biochemical reactions in cells vary and result in considerable changes of pH, HCO_3^- concentration, and cell metabolite composition. All these factors can effect HPB $[Ca^{2+}]_o$ values in vitro. Deviations of $[Ca^{2+}]_o$ values from those found for resting cells of the same donor are all the more ambiguous. Unfortunately, we failed to find in vitro any experimental data on changes in $[Ca^{2+}]_o$ values in the course of polyclonal activation of HPB T cells of healthy donors. Most probably, this problem has not attracted attention of researchers so far. At the same time, EGTA concentrations added to the blood in vitro prior to T cell stimulation cannot vary in the process of activation and/or incubation of these cells. Therefore, EGTA concentrations in HPB in vitro can be reproduced more strictly in any experiment.

The Dependence of Calcium Ion Concentrations in Fresh Heparinized Blood of Healthy Donors on EGTA Added in vitro (Titration Curve)

The starting point for the analysis of all the results obtained was a shape of the titration curve of fresh heparinized HPB with increasing EGTA concentrations (Fig. 1). The curve for HPB heparinized blood was obtained first. Its shape was similar to those of well-known EGTA titration curves of complex solutions containing calcium ions [21, 22]. All the EGTA

concentration dependencies on the tested parameters of polyclonal activation of $CD4^+$ T cells in HPB in vitro were compared with the shape of this very curve. The shape conformity of these curves was the most predictable result before the initiation of our work, but none of the curves obtained describing the dependencies of polyclonal activation of $CD4^+$ T cells in HPB in vitro repeated its shape.

The Criteria of HPB $CD4^+$ T Cell Activation in vitro

The de novo appearance of CD69 molecules on the surface of $CD4^+$ T cells is accepted as a phenotypical criterion of the earliest stages of $CD4^+$ T cell activation [23–25]. The next stage of their activation is characterized by the expression de novo on their surface of CD25 molecules, which are α-chain of the IL-2 receptor [26]. A functional criterion of activation of all $CD4^+$ T cells was a sharp increase in the production of IL-2, IL-4, IL-5, IL-10, IL-12, IL-17, IFN-γ, TNF-α, and others if compared with their initial levels in the blood of the same donor. This functional characteristic of cell activation was also estimated by a population of $CD4^+(CYTOKINE)^+$ T cells relative to the initial levels in the blood of the same donor. Both criteria, phenotypical and functional, were used for the analysis of the effect of EGTA concentration on the activation capacity of $CD4^+$ T cells in HPB in vitro in the presence of anti-CD3 and -CD28 mAbs. If it is not stated otherwise, activation of $CD4^+$ T cells in HPB T cells will mean "polyclonal activation of $CD4^+$ T cells in heparinized peripheral blood of healthy donors in the presence of anti-CD3 and -CD28 mAbs."

Unequal Sensitivity of Early Stages of Activation of HPB $CD4^+$ T Cells toward $[Ca^{2+}]_o$ Variations in vitro

In blood of healthy donors only single $CD4^+CD69^+$ T cells were detected, and this observation agreed well with the published data [23–25]. (Thereafter, "a population of $CD4^+(X)^+$ T cells" will imply "a population of $CD4^+(X)^+$ T cells among all $CD4^+$ T cells in HPB in vitro.") The $CD4^+CD69^+$ T cells values in blood of each donor prior to stimulation were taken as basal levels. An addition of 1.5 mM EGTA did not change the percentage of $CD4^+CD69^+$ T cell populations in initial blood samples. After the 18 h stimulation with a mAbs mixture a sharp increase to 52.3 ± 5.9% in the $CD4^+CD69^+$ T cell population was observed. Absolute values of this parameter differed among the donors, but were always higher than 42%. Similar results were obtained for 12 donors of middle age and agreed well with the data for healthy people [23–25].

The dependence of the number of $CD4^+CD69^+$ T cells on EGTA concentrations varying from 0 to 1.5 mM was represented as a curve with two maxima of different width and positions and a local minimum in between (Fig. 2). The first maximum was relatively wide and was always in the region of the $[Ca^{2+}]_o$ lower normal level. The second, which was narrower, was in the area of even lower $[Ca^{2+}]_o$ values. When concentrations of $[Ca^{2+}]_o$ decreased to 0.1 mM or lower, the number of $CD4^+CD69^+$ T cells fell sharply but not to zero. Intensities of the maxima and the depth of the minimum varied among the donors. Hence, $CD4^+$ T cells capable to activation in vitro at various $[Ca^{2+}]_o$ were present even in blood of healthy people. Such dependence was found first.

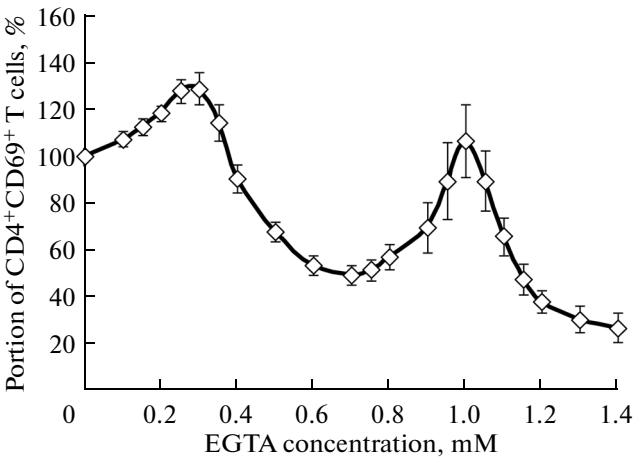

Fig. 2. The dependence of $CD4^+CD69^+$ T cell portion activated with a mixture of mAbs toward CD3 and CD28 in vitro on EGTA concentrations in HPB of healthy donors. Mean values of 12 independent measurements are shown. Experimental spread along the Y axis was corresponded to SEM values.

Unequal Sensitivity of IL-2-Dependent Activation Processes of HPB $CD4^+$ T cells to $Ca^{2+}]_o$ in vitro

In healthy donor blood IL-2 molecules were found in minimal quantities and the number of $CD4^+(IL\text{-}2)^+$ T cells rarely exceeded 0.2%. These values agreed well with the published data [26, 27]. The addition of 1.5 mM EGTA to HPB in vitro did not cause any changes in the population of $CD4^+(IL\text{-}2)^+$ T cells or IL-2 in the initial blood even after the 8 h incubation at 37°C in a wet 5% CO_2 atmosphere. These values of $CD4^+(IL\text{-}2)^+$ T cells and IL-2 molecules in the initial blood of each donor prior to stimulation were taken as a basal level. (Since similar data were also obtained for other cytokines and $CD4^+(CYTOKINE)^+$ T cells, these data are regarded as a basal level.)

The dependencies of the populations of activated $CD4^+(IL\text{-}2)^+$ T cells and produced IL-2 on EGTA concentrations were described on the "EGTA scale" by curves with two maxima located lower than $[Ca^{2+}]_o$ minimal normal values in peripheral blood of healthy donors (Figs. 3a and 3b). At the same time, these curves significantly differed in shape from the $CD4^+CD69^+$ T cell–EGTA dependence shown in

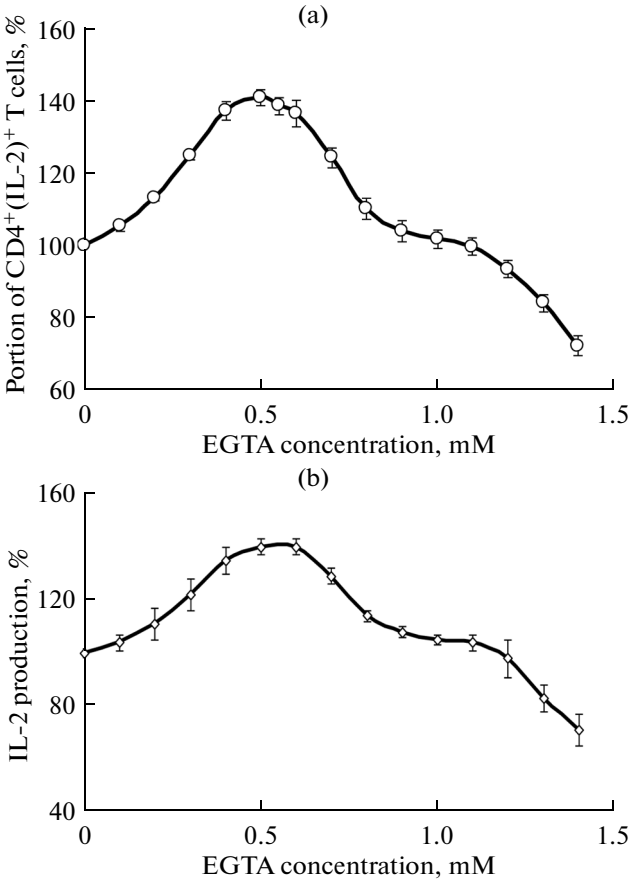

Fig. 3. The dependence of $CD4^+(IL\text{-}2)^+$ T cell portion activated with a mixture of mAbs toward CD3 and CD28 (a) in vitro and (b) production of IL-2 on EGTA concentrations in HPB of healthy donors. Mean values of five independent measurements are shown. Experimental spread along the Y axis was corresponded to SEM values.

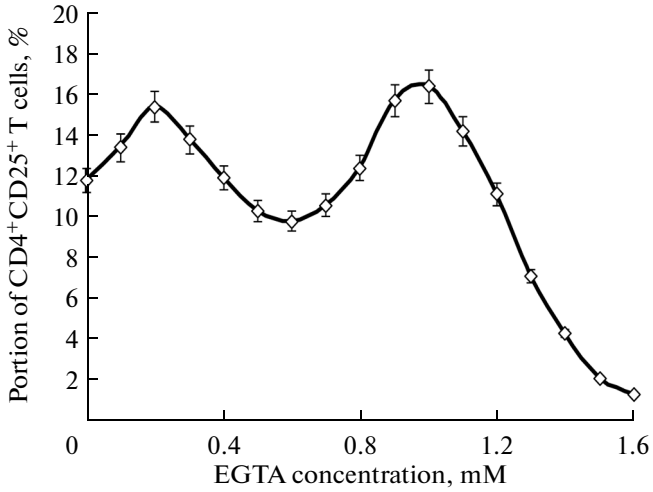

Fig. 4. The dependence of $CD4^+CD25^+$ T cell portion activated with a mixture of mAbs toward CD3 and CD28 in vitro on EGTA concentrations in HPB of healthy donors. Mean values of four independent measurements are shown. Experimental spread along the Y axis was corresponded to SEM values.

Fig. 2. As a rule, the maximum, which was close to normal $[Ca^{2+}]_o$ values, was always higher than the second one. Both of these maxima were very wide and considerably overlapped. Therefore, the local minimum was weakly marked. Similar results were obtained for four donors. Hence, after the in vitro activation $CD4^+$ T cells in HPB could produce IL-2 even at low $[Ca^{2+}]_o$ values. Such dependencies were found first.

The expression de novo of CD25 molecules on the surface of activated $CD4^+$ T cells is accepted to be a sign of their intensive proliferation [27, 28]. For healthy donors, a portion of $CD4^+CD25^+$ T cells was always less than 6% (upper normal level [24]) and the most common values were in the range of 1.5 to 3.5%. Portions of $CD4^+CD25^+$ T cells in the initial blood of any donor were taken as basal levels. These values were not nearly affected by the in vitro addition of 2.0 mM EGTA even after the 8 h incubation of the blood at 37°C in a 5% CO_2 atmosphere. Polyclonal activation in vitro always resulted in a sharp growth of $CD4^+CD25^+$ T cell population, five to fifteen times depending on the donor, and this fact agreed well with the published data [28].

The $CD4^+CD25^+$ T cell percentage in activated blood was influenced by EGTA (Fig. 4) and corresponded to a curve with two maxima and a local minimum in between. Although unexpectedly, this curve in its shape and maximum positions was closer to the curve shown in Fig. 2 rather than to the EGTA concentration dependence of $CD4^+(IL\text{-}2)^+$ T cells or production of IL-2 (Figs. 3a and 3b). Such dependencies were found first. Any experimental data supporting them have not been described so far. The reasons for the differences in the curve shapes in Figs. 2–4 remain unclear and obviously will the further studied.

One of the key stages of the T cell activation involves IL-2 molecules. The EGTA concentration dependencies of $CD4^+CD25^+$ and $CD4^+(IL\text{-}2)^+$ activated T cells in HPB as well as IL-2 production in response to polyclonal activation differed in shape (Figs. 3 and 4). The influence of the calcium ion removal from the medium using EGTA on the T cell-induced expression of CD25 molecules in vitro, which was strongly dependent on the time of chelator addition, was described recently [29]. As a rule, the earlier were calcium ions removed from the incubation medium, the more it influenced on the expression of CD25 molecules [30–32]. Likely, calcium ions are absolutely necessary for the induction of synthesis of CD25 molecules de novo [29, 33].The expression of CD25 molecules on activated T cells, which had passed the induction stage, was far less affected by EGTA concentrations [33–35].

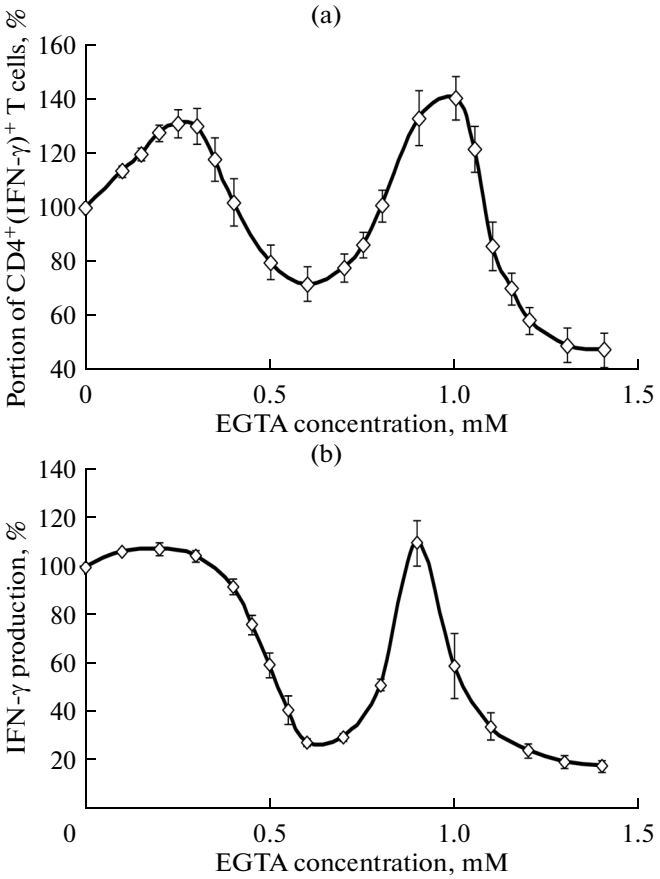

Fig. 5. The dependence of $CD4^+(IFN\text{-}\gamma)^+$ T cell portion activated with a mixture of mAbs toward CD3 and CD28 (a) in vitro and (b) production of IFN-γ on EGTA concentrations in HPB of healthy donors. Mean values of five independent measurements are shown. Experimental spread along the Y axis was corresponded to SEM values.

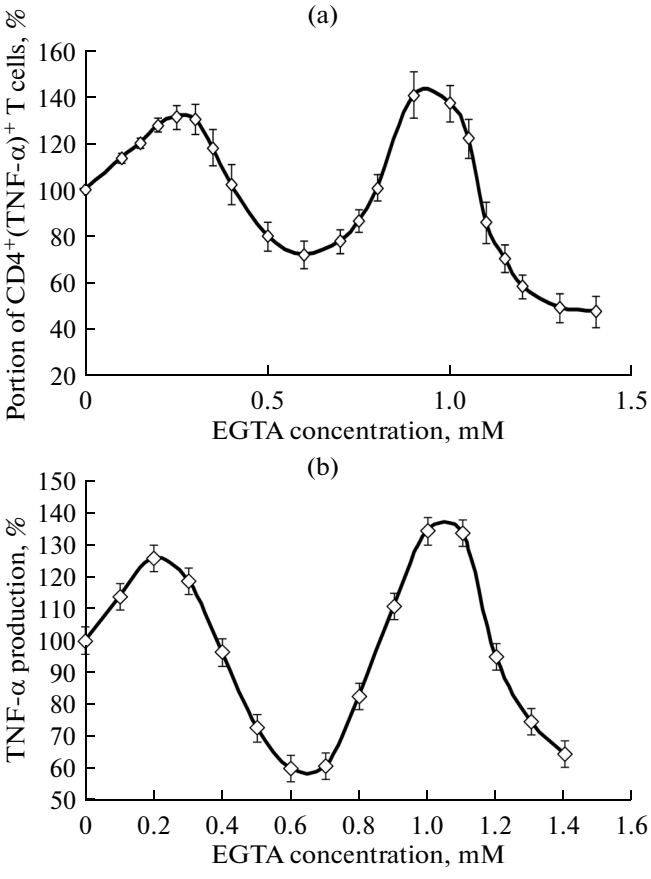

Fig. 6. The dependence of $CD4^+(TNF\text{-}\alpha)^+$ T cell portion activated with a mixture of mAbs toward CD3 and CD28 (a) in vitro and (b) production of TNF-α on EGTA concentrations in HPB of healthy donors. Mean values of six independent measurements are shown. Experimental spread along the Y axis was corresponded to SEM values.

In later activation stages, the effect of EGTA on $CD25^+$ T cells or IL-2 binding to its receptor was not observed [32–35]. Probably, this result can explain the differences in the EGTA concentration dependencies of $CD4^+CD25^+$ and $CD4^+(IL\text{-}2)^+$ T cells in HPB in vitro as well as IL-2 production by blood cells in vitro in response to the polyclonal activation (Figs. 3 and 4).

The Influence of $[Ca^{2+}]_o$ on Activated Th1 Cells in HPB in vitro

According to the current data, a generic feature of all activated Th-1 cells is the IFN-γ production [36]. In addition, they produce TNF-α [37]. The production of IFN-γ and TNF-α increased greatly after HPB T cell activation. However, although all Th-1 cells belong to the same helper type, they produce different quantities of IFN-γ and TNF-α. As a rule, T cells of healthy donors under optimal activation conditions in vitro produce 6.0 to 11 ng/mL IFN-γ and only 40 ± 15 pg/mL TNF-α. The reasons for this difference remain unclear.

These results agreed well with the published data [38, 39]. The percentage of $CD4^+(IFN\text{-}\gamma)^+$ and $CD4^+(TNF\text{-}\alpha)^+$ T cells also grew sharply in response to the polyclonal activation in vitro. In the same blood sample the portion of $CD4^+(IFN\text{-}\gamma)^+$ activated T cells was higher than that of $CD4^+(TNF\text{-}\alpha)^+$ T cells, although they belong to the same type of Th1 cells. There are no reliable data unambiguously explaining the regularities obtained. Production of IFN-γ and TNF-α by activated T cells as well as $CD4^+(IFN\text{-}\gamma)^+$ and $CD4^+(TNF\text{-}\alpha)^+$ T cells depended on the EGTA concentrations in a similar mode (Figs. 5a and 5b and 6a and 6b). Positions of maxima on the "EGTA scale" agreed well enough. Excluding small quantitative differences, their shape was identical to the shape of the $CD4^+CD69^+$ T cell–EGTA dependence shown in Fig. 2. Hence, the EGTA concentration dependencies of the production of IFN-γ and TNF-α by T helpers type 1 in HPB in vitro were analogous. Similar results were obtained for seven donors. Such dependencies of the production of IFN-γ and TNF-α by T helpers type 1

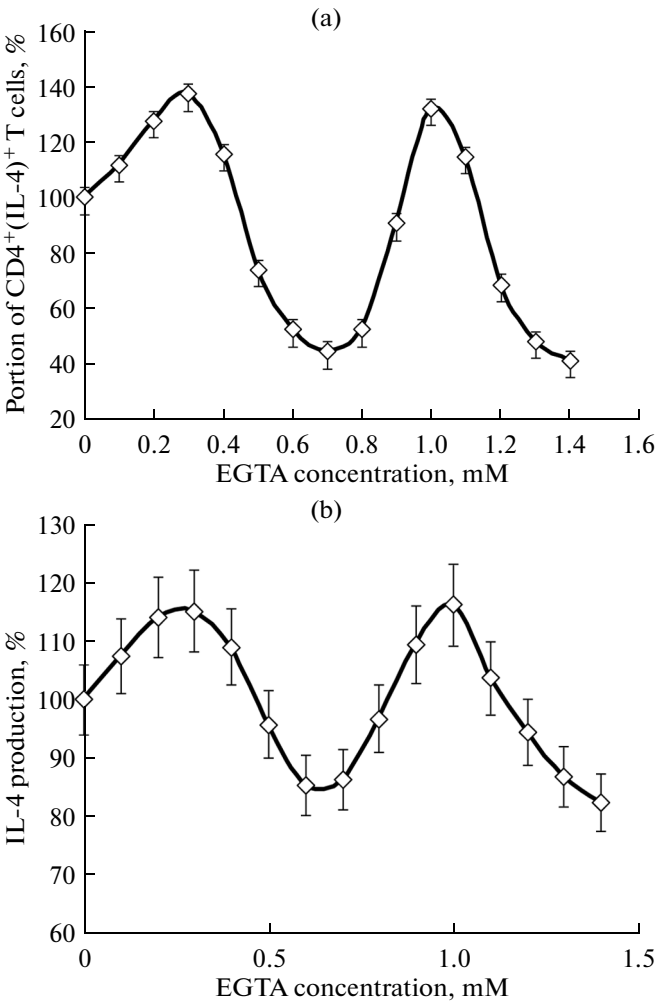

Fig. 7. The dependence of $CD4^+(IL\text{-}4)^+$ T cell portion activated with a mixture of mAbs toward CD3 and CD28 (a) in vitro and (b) production of IL-4 on EGTA concentrations in HPB of healthy donors. Mean values of five independent measurements are shown. Experimental spread along the Y axis was corresponded to SEM values.

on the EGTA concentrations in HPB in vitro were found first.

The Influence of $[Ca^{2+}]_o$ Reduction on Activated Th2 Cells in HPB in vitro

A characteristic property of all Th2 cells is their capacity to intensify greatly the IL-4 production in response to the activation stimulus. As a rule, $CD4^+(IL\text{-}4)^+$+ T cells and IL-4 numbers are minimal in the initial blood. These values were taken as basal levels for each donor and agreed well with the published data [36–38]. The percentage of $CD4^+(IL\text{-}4)^+$ Th2 cells in the blood of healthy donors was always lower than that of $CD4^+(IFN\text{-}\gamma)^+$ Th1 cells and also agreed well with the published data [38]. The response of T cells from healthy donor blood to polyclonal activation in vitro was always accompanied by a sharp increase in the IL-4 production and a simultaneous growth of $CD4^+(IL\text{-}4)^+$ T cell population in it. This fact correlated well with the published data [39, 40].

The IL-4 production by activated T cells and the portion of activated $CD4^+(IL\text{-}4)^+$ T cells depended on the presence of EGTA (Figs. 7a and 7b). Both dependencies (Figs. 7a and 7b) only insignificantly differed in shape and can be described as curves on the "EGTA scale" with two maxima and a local minimum in between located in the region of hypocalcemia for healthy donors. The shape of these curves agreed well with the $CD4^+CD69^+$ T cell–EGTA dependence shown in Fig. 2. Similar results were obtained for five donors. Hence, the IL-4 production by activated $CD4^+$ T helpers type 2 in HPB in vitro depended on $[Ca^{2+}]_o$ values in a like way. Such dependencies were found first.

The Influence of $[Ca^{2+}]_o$ Reduction on Activated Th17 Cells in HPB in vitro

The Th17 cells got their name from IL-17, which they produce in response to activation [41]. The percentage of these cells rarely exceeded 0.1–0.2% and most often only traces of IL-17A could be found. These results are confirmed by the previous data [42]. The Th-17 cell activation was always accompanied by a sharp growth of IL-17 production and a simultaneous multiple increase in $CD4^+(IL\text{-}17A)^+$ T cells in HPB in vitro. This result correlated well with the published data [31]. The IL-17 production by activated $CD4^+$ T cells and the portion of activated $CD4^+(IL\text{-}17A)^+$ T cells depended on the presence of EGTA. Both dependencies (Figs. 8a and 8b) only insignificantly differed in shape and can be described on the "EGTA scale" as curves with two maxima located in the region of hypocalcemia for healthy donors and a local minimum in between. The positions of maxima of the activated T cell-induced IL-17A production and a $CD4^+(IL\text{-}17A)^+$ T cell portion in blood (Figs. 8a and 8b) coincided well enough with each other. In addition, the shape of these curves agreed well with the dependence shown in Fig. 2, which described the function of in vitro activated $CD4^+CD69^+$ T cell versus EGTA concentrations. Hence, the dependence of IL-17A production and a $CD4^+(IL\text{-}17A)^+$ T cell portion in HPB on the $[Ca^{2+}]_o$ values after the in vitro activation was the same. Such dependence was found first.

Thus, all the T helpers tested responded nearly equally to the polyclonal activation in healthy donor blood in the presence of the varied EGTA concentrations.

The Analysis of Relationship between the Cytokine Production and CD4⁺(CYTOKINE)⁺ T Cell Population after the HPB Activation in vitro

In this work we used the following parameters as functional characteristics of the T cell capacity to polyclonal activation in HPB: production of IL-2, IL-4, IL-17A, TNF-α, and IFN-γ, percentage of $CD4^+CD69^+$, $CD4^+CD25^+$, and $CD4^+(CYTOKINE)^+$ T cells. The three latter activation parameters were directly measured in HPB on $CD4^+$ T cells and their dependencies at $[Ca^{2+}]_o$ reduced with EGTA could be easily compared. A comparison of activated T cell-induced IL-2, IL-4, IL-17A, TNF-α, and IFN-γ production with portions of the corresponding $CD4^+(CYTOKINE)^+$ T cells seemed to be more ambiguous, because these cytokines can also be produced by some populations of cytotoxic $CD3^+CD8^+$ T cells.

However, with the exception of some details, the curves on Figs. 2, 5–8 agreed well enough in shape. It can be accounted for by several reasons. First, $CD4^+$ T helper cells are principal producers of cytokines and they are responsible for the synthesis of most of IL-2, IL-4, IL-17A, TNF-α, and IFN-γ. Second, they constitute more than a half of all $CD3^+$ T cells in HPB. Third, the capacity of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in HPB to be activated by polyclonal stimuli may be similar. All these three factors can serve the reason for the identity of curve shapes in Figs. 5–8.

Indirect Influence of $[Ca^{2+}]_o$ on the T Cell Activation in HPB in vitro

The HPB titration curve with EGTA increasing concentrations (Fig. 1) never coincided in shape with the dependencies of T cell polyclonal activation parameters in the conditions of $[Ca^{2+}]_o$ reduction using EGTA (Figs. 2–8). This fact only implies that calcium ions in the concentration range tested did not directly affect the sensitivity of the T cell polyclonal activation parameters in HPB. Otherwise the dependencies of these parameters would be completely identical in shape to the curve in Fig. 1. Most probably, some mediated mechanisms of $[Ca^{2+}]_o$ influence on the T cell capacity to polyclonal activation are implemented in $CD4^+$ T cells. We failed to find any experimental data on the $[Ca^{2+}]_o$ effects within the concentrations studied on the T cell capacity to polyclonal activation in HPB.

Physiological Importance of Non-Monotonic Influence of $[Ca^{2+}]_o$ on Functioning of Activated $CD4^+$ T Cells in HPB

The presence of two maxima on the dependence of the T cell capacity to polyclonal activation in the conditions of EGTA-induced reduction of $[Ca^{2+}]_o$ values (Figs. 2–8) was found first. The position of the first maximum corresponded to [Ca2+]o of 0.82 to 0.85 mM (within 0.25 to 0.35 mM EGTA) and it was located in the region of the lower normal level of calcium ions of healthy donor blood. Likely, a considerably increased capacity of $CD4^+$ T cells to activation in HPB, when the calcium concentrations are on the border of healthy and pathological states, is necessary for the prevention of disease development. Unfortunately, the data on the influence of $[Ca^{2+}]_o$ values within this range on the $CD4^+$ T cell capacity to polyclonal activation in HPB are absent. Further decrease in $[Ca^{2+}]_o$ in blood was accompanied by an unexpected and sharp reduction of the capacity of $CD4^+$ T cells to activation in HPB. This fall was observed within the calcium concentrations characteristic for numerous pathological states [3–10]. The region of $[Ca^{2+}]_o$ 0.85 to 0.6 mM (within 0.35 to 0.8 mM EGTA) seems a vulnerable zone, within which the ability of the immune system to fight infections is essentially limited. The appearance of the second maximum at $[Ca^{2+}]_o$ lower than 0.6 mM (at EGTA concentrations higher than 0.8 mM) was a great surprise and was

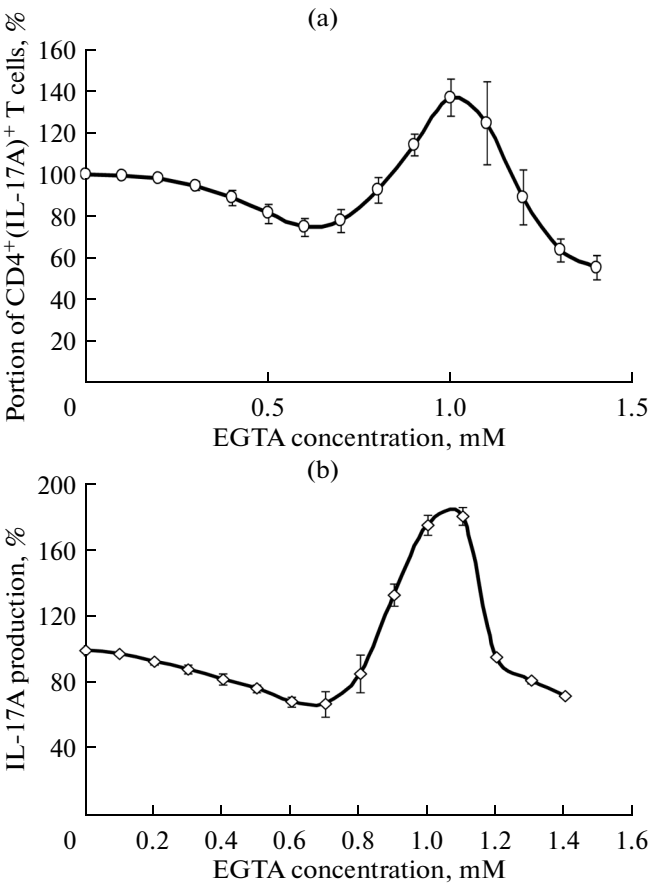

Fig. 8. The dependence of $CD4^+(IL\text{-}17A)^+$ T cell portion activated with a mixture of mAbs toward CD3 and CD28 (a) in vitro and (b) production of IL-17A on EGTA concentrations in HPB of healthy donors. Mean values of six independent measurements are shown. Experimental spread along the Y axis was corresponded to SEM values.

found first. We failed to find any hints evidencing why $CD4^+$ T cells can be activated at such low $[Ca^{2+}]_o$ concentrations. In this case, we cannot exclude the existence of some $CD4^+$ T cell populations, one of which operates in the normal conditions and the other is specialized in functioning at pathological $[Ca^{2+}]_o$. However, the results of this work do not allow us either to confirm or to reject this assumption. Such a strange type of the dependence of the capacity of $CD4^+$ T cells to activation on $[Ca^{2+}]_o$ values will obviously be a subject of further studies.

EXPERIMENTAL

Reagents. In this work we used DMSO, EGTA, HEPES, Brefeldin A, and paraformaldehyde purchased from Sigma (United States). For all pharmacologically active compounds, their optimal concentrations, at which the maximal effect was displayed at minimal toxicity during the total experimental time, were preliminarily assessed. All plastics was from NUNC (Denmark). All mAbs, Optilyse C, and IntraPrep kit were from Immunotech (France). Kits for cytokine analysis by ELISA were from Bender MedSystems (United States), BIOSOURCE (United States), and INVITROGEN (United States).

Donors and sample preparation. Blood cell counts and cell immune status determined using the mAbs affinities toward CD3/CD19, CD3/CD4, CD3/CD8, CD45/CD14, (CD16 + CD56)/CD3, CD3/CD25, and CD3/HLA-DR were within the norm for healthy subjects [43]. The $[Ca^{2+}]_o$ values conformed to those of healthy people. Blood cell counts were assayed on an ACT 5 DIFF Beckman-Coulter hematological analyzer (United States). Titration of blood EGTA was performed in microtubes with the given volume of the solution containing 0.5 M EGTA and 0.35 M HEPES, pH 7.45. Fresh heparinized blood was added to the tubes, the suspension was stirred, incubated for 2–3 min, and $[Ca^{2+}]_o$ values were measured on a 9180 ionometer Electrolyte analyzer (Roch, Switzerland).

Flow cytofluorometry. The cells were analyzed on an EPICS XL MCL flow Coulter cytofluorimeter (United States). For the correct exclusion from the analysis of the particles inconsistent in the size and granularity to live lymphocytes, adequate logic limitations were introduced in the histograms of particle distribution using low angle and 90-angle light scattering detection. The limits of the "lymphocyte window" were additionally monitored by cell staining with mAbs-CD45 and -CD14 combinations. The data were treated using the WinMDI program (United States). More than 10^4 cells were analyzed in each sample. mAbs corresponding to the mAbs type and class were used for isotype controls.

T cell activation with mAbs toward α-CD3 immobilized on the plastic surface (500 ng/mL) overnight and mAbs toward α-CD28 (2.5 μg/mL) was performed in total heparinized blood (0.1 mL) for 18 h. The compounds added constituted less than 2% of the total sample volume. The control samples contained the proper volumes of phosphate buffer and DMSO. Prior to stimulation, the cells were incubated with the proper EGTA concentrations for 15 min in a 5% CO_2 atmosphere at 37°C.

After 18 h incubation in a 5% CO_2 atmosphere at 37°C, the tubes were placed on an ice bath to terminate the reactions and then centrifuged. The supernatant was taken out and a mixture of proteinase inhibitors was immediately added. The mixture was stirred and filtered via membranes with a pore diameter of 0.1 μm in order to remove the cells and their debris. The solutions were analyzed ex tempera or they were frozen and kept at –70°C. Cytokines were detected according to the supplier's recommendations in duplicate. The cytokine number before activation was considered as a basal level, which was subtracted from all the values obtained for the activated cells. The cytokine number produced by the activated cells without EGTA was taken as 100%. All the values obtained in the presence of EGTA were normalized to this value.

Percentage of $CD4^+(CYTOKINE)^+$ T cells was assessed using the same protocol. Twelve hours after the cell activation or incubation without stimuli (control) Brefeldin A was added to the concentration of 100 μM. After 6 h of incubation the cells were transferred to an ice bath and stained with mAbs toward CD4 according to the supplier's recommendations. Staining with the proper mAbs toward cytokines was carried out using an IntraPrep kit according to the supplier's recommendations. The number of $CD4^+(CYTOKINE)^+$ T cells without activation was considered as a basal level, which was subtracted from all the values obtained for the activated cells. The number of $CD4^+(CYTOKINE)^+$ T cells without EGTA was taken as 100%. All the values obtained in the presence of EGTA were normalized to this value.

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