MINISTRY OF HEALTH OF THE REPUBLIC OF UZBEKISTAN
SAMARKAND STATE MEDICAL UNIVERSITY

STIMULATION IMMUNITY IN A MODEL OF CUTANEOUS LEISCHMANIASIS

Abdullaev Davlat Mukumovich, Ashirov Zokhid Fayazdanovich
Abdullaev Hasan Davlatovich, Akhmedova Makhbuba Makhmudovna

MONOGRAPH

Samarkand 2024

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The process of cutaneous leishmaniasis is characterized by a sluggish, long-term course due to weak local anti-infective immunity. Therefore, we tried to stimulate local immunity using the official gramicidin paste. To assess the contribution of the antibiotic action of gramicidin S, monomycin ointment was used in one of the control groups as the most effective anti-leishmania drug among the antibiotic agents used to treat cutaneous leishmaniasis.

The significantly more effective therapeutic effect of gramicidin in the main group compared to monomycin indicates that the effect of gramicidin is determined not only by its antibiotic activity. There is enough reason to assume that gramicidin S has a local immunostimulating effect, which, in combination with the antibiotic effect, gives a very good therapeutic effect for cutaneous leishmaniasis.

Authors:

Abdullaev Davlat Mukumovich - Associate Professor of the Department of Dermatovenerology and Samarkand State Medical Institute

Abdullaev Hasan Davlatovich - assistant at the Department of Dermatovenerology and Samarkand State Medical Institute

Akhmedova Mahbuba Makhmudovna - Associate Professor, Department of Pediatrics FPDO Samarkand State Medical Institute

Reviewers:

Ziyaduliaev Sh. Kh. Doctor of Medical Sciences, Professor, Deputy Director for Researchof the Institute of Immunology and Human Genomics of the Academy of Sciences of the Republic of Uzbekistan

Samieva G. U. Doctor of Medical Sciences, Professor, Head of Department Patological fiziology SSMU.

CONTENT

LIST OF ABBREVIATIONS

ATPh-adenosine triphosphate

IEA – immunoenzyme analysis

AFC - antibody-forming cells

PAA - polyacrylic acid

WSA - water-soluble antigen

PREFACE

Data on the immunostimulating effect of official membrane-active substances: gramicidin S have independent scientific and practical significance. They allow us to recommend the use of these substances as immunoadjuvants in the induction of antibody genesis against antigens from pathogens of various infections. At the same time, the work presents detailed data on the dose dependencies of the immunoadjuvant effect of gramicidin S, on the dynamics of the production of IgM and IgG antibodies, on the possibility of enhancing both the primary synthesis of antibodies and immune memory, as well as secondary production of antibodies, on the dependence of the severity of the immunoadjuvant effect on the level of the modified reaction (on the dose of the immunogen) and on the structural features of the antigens being studied. All this is information that can be useful for a more targeted use of gramicidin S and its analogues in the treatment of leishmaniasis. The results obtained in this work should be developed in subsequent studies. This is also confirmed by successful attempts to use gramicidin S to stimulate the immune response against antigens of salmonella and the causative agent of anthrax, together with the significant therapeutic effect of gramicidin S in cutaneous leishmaniasis, which is characterized by a protracted sluggish course due to weak local immunity.

This monograph is relevant for dermatologists, infectious disease specialists and therapists. The materials of the monograph can be used in the educational process when teaching such disciplines as “Dermatology”, “Infection”, “Therapy”, to students of medical universities, as well as to master’s and clinical residents.

INTRODUCTION

The introduction of water-soluble polyacids or polybases into the body can lead to activation of the processes of migration, interaction, reproduction, maturation and functioning of both lymphoid cells and their precursors, starting with hematopoietic stem cells. The discovered phenomenon is used successfully to effectively enhance immune responses. In this case, the result of stimulation is much more significant if the polyelectrolyte is introduced not in a mixture with the antigen, but in the form of a covalent antigen-polymer complex.

The mechanisms of the immunostimulating effect of polyelectrolytes are being actively studied. In particular, significant advances have been made in analyzing the participation of subpopulations of immune cells in polymer-enhanced immune responses.

This monograph indicates a set of works on the study of the molecular mechanisms of activation of the response of an immunocompetent cell by polyelectrolyte. We had to determine the degree of participation of the ion transport system of the cell membrane in the activation of immunocompetent cells by poly ions during the treatment of leishmaniasis. And also to investigate the possibility of activation of the immune system by inducers of ion permeability of a non-polymer nature in leishmaniasis.

CHAPTER 1. LITERATURE REVIEW

Before moving on to a description of the experiments we conducted, we should consider in more detail the facts established before us. First, what is known about the immunostimulating effect of polyelectrolytes. Secondly, about the role of membrane ion transport in the mechanism of activation of lymphoid cells by lectins.

1. Stimulation of immunogenesis with polyelectrolytes.

Polyelectrolytes are soluble polymers of different structures that have multiple charges. It has been shown that by introducing these substances into the body of animals, it is possible to intensify the processes of hematopoiesis and immunopoiesis. Co-administration of a polyelectrolyte with an antigen leads to the production of antibodies 3-5 times higher than with the introduction of the antigen itself (11, 32, 46). Stimulation of the final result of the immune system reaction is a consequence of the activation of specific units of immunopoiesis by the polymer. For example, in vivo administration of a polyelectrolyte significantly activates the relocation of hematopoietic stem cells from the bone marrow to the spleen (7). In addition, under the influence of polymers (polyacrylic acid, poly-4-vinylpyridine), the growth of hematopoietic colonies is intensified (13).

Such important links in immunopoiesis as the migration of T cells from the thymus to the spleen, as well as B cells from the bone marrow to the lymphoid organs, are also enhanced after the introduction of a polyelectrolyte into the mouse body (16). These migration processes are a necessary condition for the bringing together of T and B cells within the same tissue, which allows these cells to interact. Moreover, exposure to polyelectrolyte intensifies the process of cooperation between T and B cells during the induction of antibody synthesis (16). This was established in model experiments when T and B cells were introduced into a syngeneic organism deprived of its own lymphocytes due to total gamma irradiation.

Interestingly, in these same experiments, polyelectrolytes stimulated B cell responses even in the absence of T cells (21). There was an assumption about the direct activating effect of polyeletrolyte on B-lymphocytes. The data obtained were regarded

as evidence of the possibility of polyelectrolyte replacement of T-helper cells during antibody induction.

In in vivo experiments, any effects of polymers could be interpreted as a direct activating effect on hematopoietic or lymphoid cells only with certain reservations, since influences mediated through the hormonal, nervous and other systems of the body could not be excluded. Therefore, experiments in in vitro cell cultures can be considered strict proof of the direct activating effect of polyelectrolytes on lymphocytes and macrophages. It was found that polyanions and polycations induce activation of the early phases of the cell division cycle. In resting lymphocytes, under the influence of the polymer, RNA synthesis was activated (GI phase), and in the presence of macrophages, DNA synthesis was activated (S-phase) (4a). The proliferative response of lymphocytes to polyion differs significantly from the response of cells to mitogenic lectins (34). The latter enhance DNA synthesis 20-30 times and induce a series of successive mitoses lasting several days. On the contrary, under the influence of a polyion, DNA synthesis is activated no more than 3-7 times. Cells, if they do, do only one division cycle. Rather, the polymer induces only the initial phases of the division cycle, without creating the conditions for its full completion.The reasons for the inferiority of the mitogenic signal of polyelectrolytes seem to be that these substances, unlike lectins, do not induce the secretion of growth factors (3). In general, polyelectrolytes can be classified as weak mitogens.

Apparently, the lack of the ability to induce a series of full divisions and secretion of differentiation factors is also associated with the weak polyclonal effect of polyelectrolytes on the maturation of B cells into Ig-secreting cells (33, a). At the same time, the effect of polyion on lymphocytes strongly activates the process of antigen-dependent differentiation of B cells, their transformation into specific antibody producers. When inducing antibody synthesis in cell culture in vitro, the polyelectrolyte increased the number of antibody-secreting cells 4-5 times (23).In vivo production of antibodies upon joint administration of antigens with polyelectrolytes can be 3-7 times higher than the level of response to “pure” antigens (16).Consequently, the polyelectrolyte, acting directly on lymphoid cells, induces a powerful adjuvant

signal and a weak mitogenic one. In the following presentation, when speaking about the activation of the response of lymphoid cells by polyelectrolyte, we will mean the activation of antigen-dependent differentiation of lymphocytes and the release of resting lymphocytes into the initial phases of the cell division cycle.

2. Study of molecular mechanisms of activation cells by polyions

For a number of years, R.I.Ataullakhanov and co-authors have been studying the earliest molecular changes in lymphoid cells that occur when they are activated by polyions. First, a theoretical analysis was carried out, which made it possible to focus primarily on the processes in the outer membrane of the cell. Moreover, the authors named several key systems through which a signal about the effect of an exogenous polyelectrolyte on the cell could be transmitted into the cell. The experiment showed that the polyelectrolyte does not affect such an important signaling mechanism as the system of membrane enzymes that regulate the level of cyclic nucleotides (2). The polymer did not cause rapid significant changes in the lipid matrix system of the membrane. This was demonstrated using fluorescent probes for the viscosity of membrane lipids and the surface area of the membrane lipid matrix (5). In the first minutes after treatment of lymphocytes and macrophages with the polycation, a significant change in the morphology of the cell plasma membrane was detected (44). The surface of the cells was smoothed: membrane protrusions, villi, and ridges were shortened. At the same time, the elementary “grain” of the membrane matrix noticeably enlarged over a significant part of the cell surface. There was an assumption about the formation of numerous complexes of exogenous polymer molecules with molecules of the cell membrane. By cryopreservation of membranes and electron microscopy of fractures, it was possible to detect microaggregates of membrane proteins, consisting of 5 - 50 or more intramembrane protein particles (5a). Clustering of membrane proteins occurred within the first minutes after exposure of cells to polyion. At the same time, a change in the permeability of the cell membrane occurred. This change was first detected by fluxes of labeled nucleosides, and then by fluxes of Ca^{2+} and K^{+} (4). The authors proposed a hypothesis that linked changes in

permeability to aggregation of membrane proteins and flattening of membrane configuration (44). According to the hypothesis, polyelectrolyte molecules form many electrostatic complexes with complementarily charged groups of membrane proteins. Cross-linking of protein particles with polymer filaments leads to the formation of protein clusters. Protein clusters themselves contain cracks and pores, which increase the permeability of the membrane. Moreover, the pores are heterogeneous and non-selective; ions, water, and small molecules up to nucleosides penetrate through them.

The change in permeability is associated with a slight increase in cell volume and smoothing of membrane protrusions.

It was important to determine whether any of the detected changes (protein clustering, membrane flattening, changes in membrane permeability) in the properties of the cell membrane were essential for triggering the cell's response to the polymer.

In an experiment, we had to study the significance of one of the detected changes, an increase in the permeability of the membrane for ions. Therefore, it is necessary to consider in detail the literature on the role of ion transport in activating the response of lymphoid cells, although not by polyelectrolytes, but by lectins.

3. The role of membrane ion transport in the mechanism lymphocyte activation

The contents of eukaryotic cells differ significantly from the extracellular environment in the concentration of many ions. Particularly important for the life of a cell are the differences in Na+, K+, Ca2+ and H+ ions. Special enzyme systems function in the cell membrane, ensuring the creation and maintenance of the ionic identity of cells at a certain level. In particular, membrane enzymes (Na+, K+) - and Ca2+ -transporting ATPhases are those unique structures that penetrate the outer cell membrane and ensure the creation of concentration gradients of these cations.

The ionic identity of the cell in relation to the pericellular environment and the differences in ion content between cell compartments are primarily critical for many enzymes and, therefore, for the activity of many metabolic systems within the cell. In addition, it is believed that the ionic heterogeneity of the cell in relation to the extracellular environment is the most important acquisition of evolution, which makes

it possible to easily and quickly “signal” about the slightest damage to the outer cell membrane.

Numerous studies in various fields of biology have proven the key role of ion “signaling” in triggering a cell’s response to external stimuli. For example, for an egg, a signal for activating cleavage divisions can be the fact of damage to the membrane by the sperm. For a muscle cell, the signal to contract is a change in the permeability of the outer membrane induced by a neurotransmitter (for example, acetylcholine or norepinephrine). For a secretory cell, a signal inducing the secretion of granules and the production of new portions of secretion can be an increase in the permeability of the outer membrane for ions, in particular Ca2+. This usually occurs due to the landing of a hormone, releasing factor or kinin on the corresponding membrane receptors of the secretory cell.

Apparently, cells of the lymphoid system use the same signaling system when responding to many external factors. The nature of these factors is determined by the specificity and diversity of the receptor apparatus of the lymphoid cell. The nature of the lymphocyte's reaction is predetermined by its structural and functional specialization that occurred during the previous stages of differentiation. As a rule, a lymphocyte can respond to an external stimulus by activating divisions or transforming into a secretory cell, or a combination of these two types of reactions.

Modern literature has accumulated a lot of data indicating the importance of ion transport in triggering the response of lymphoid cells to external signals.

Changes in ion transport parameters upon exposure
on lymphoid cells with mitogens and immunostimulants.

a) **Passive ion flows.** Allwood et al. [36] were among the first to show that when human lymphocytes are exposed to PHA, there is an increase in Ca2+ entry into the cells. Similar observations have been described in many other studies when lymphocytes from the thymus, spleen or peripheral blood were exposed to various mitogens [19, 25, 33, 43, 55]. It turned out that an increase in permeability to Ca2+ occurs already in the first minutes after exposure of the cell to the mitogen.

The increase in Ca2+ entry induced by mitogens depends on the Ca2+ concentration in the medium. With an increase in the concentration of extracellular calcium in the range of 0.1-3 mM, an increase in Ca2+ entry is observed, then the curve reaches a plateau [52, 57]. The Ca2+ entry also increases with increasing lectin concentration, and this increase is not limited to the region of low concentrations at which the lectin has mitogenic properties, but continues with a further increase in lectin concentration, up to its toxic doses. This result was obtained in a number of studies in which Ca2+ entry was studied over a wide range of changes in mitogen concentrations [60, 43, 26, 17]. Some authors [52, 59], when studying Ca entry, observed a local maximum in the region of mitogenic lectin concentrations; however, with a further increase in the mitogen concentration, Ca2+ entry increased even more [33, 37]. It should be noted that some authors did not find a significant change in passive Ca2+ fluxes when using mitogenic concentrations of the lectins concanovalin A and PHA [52, 42, 27]. In these studies, a noticeable increase in Ca2+ entry was recorded when using concentrations exceeding the optimal immunogenic dose.

Similar to changes in calcium permeability, a number of studies have found an increase in passive K+ transport [38, 39, 60, 32]. The passive flow of K+ is directed from the cell to the extracellular environment in accordance with the transmembrane gradient of this cation.

Changes in Na+ transport under the influence of mitogens have been little studied. There are no direct results on measuring Na+ fluxes from the extracellular environment into the cell (along the concentration gradient) in the literature. Indirectly, an increase in the Na+ flux can be judged by a slight increase in the Na+ concentration inside cells and by an increase in the RB+ isotope flux [38, 39, 56].

b) **Active transport of K+, Na+, Ca2+ cations.**

Many authors have noted an increase in the activity of (Na+, K+) - and Ca2+ - ATPhases in the membrane of activated lymphocytes [37, 60, 31].

In some studies, the activation of (Na+, K+)-ATPhase was judged by an increase in the K+ flux against the concentration gradient. The increase in K+ transport can be quite significant: the rate of K+ entry into lymphocytes sometimes doubles [33, 45].

The issue of changes in Ca2+ -ATPhase activity in the membrane of lymphocytes when exposed to mitogens is still far from a final solution. Some studies note a significant increase in the activity of the calcium pump [58]. In other studies, it was not possible to register significant changes in the active transport of Ca2+ compared to the corresponding control. However, it should be noted that in most studies, Ca2+ ATPhase activity was measured in membrane preparations obtained after cell destruction. At the same time, it is well known that disruption of the membrane integrity leads to a rapid increase in the Ca2+ concentration near the cytoplasmic segments of the Ca2+ ATPhase and, consequently, to the rapid activation of this enzyme to the maximum possible level.

Apparently, in some cases, the extreme activation of the enzyme in control preparations can mask the true activation of the enzyme in preparations of cell membranes stimulated with mitogen.

Obviously, in order to judge relatively small physiological changes in the activity of ion transporting ATPhases, other methodological approaches are needed to measure their activity in a living, fully functioning cell, and not in fragments of a destroyed cell, as was done in the vast majority of the experiments mentioned above.

c) **Concentration of ions inside the cell.**

When lymphocytes are exposed to mitogens, as noted above, the fluxes of cations along their concentration gradient increase significantly. At the same time, the activity of transport ATPhases increases, pumping the same cations in the opposite direction. It is still not completely clear what these processes lead to in relation to the concentration of these cations inside the cell. In other words, how does the concentration of the intracellular pool of K+, Na+, Ca2+ change against the background of significantly activated counter transmembrane flows.

In the literature one can find the most contradictory experimental results on this issue. Some studies found an increase in the Ca2+ concentration inside the cell, in others a decrease, and in still others, the intracellular Ca2+ content did not change [58].

The situation is the same with the results on the K+ content in cells activated by mitogen.

Early studies [42, 33] reported an increase in intracellular K+ concentration. Subsequently, more accurate methods were used to show that the K+ concentration does not change significantly [60, 34], or even decreases slightly [38, 39, 42].

A physiologically active dose of an immunostimulant causes a mild increase in membrane permeability, a slight increase in passive cation fluxes, which almost instantly turns on compensatory activation of transport ATPhase s. In this case, active ATP-dependent ion transport can either partially or completely compensate for passive flows. In the first case, the concentration of K+ in the cytosol will decrease, and the concentration of Ca2+ will increase. In the second case, the concentration of both cations will remain unchanged.

It is possible that the enhanced active fluxes will for some time exceed the value of the passive fluxes, which will lead, accordingly, to a small and temporary increase in the K+ concentration or a decrease in the Ca2+ concentration inside the cell. The most likely, according to the authors, are small fluctuations in the intensity of two counter flows, which are accompanied by relatively weak fluctuations in the concentration of ions inside the cell near the initial values. Apparently, such processes may be the reason for the receipt of contradictory data by different experimenters.

Despite the above-mentioned contradictions in the literature data, it should be noted that the issue of the kinetics of Ca2+ levels in the cytosol after activation of lymphocytes by lectin has been significantly clarified by work in recent years [48, 54-59, 54, 52]. It has been established that ligands that activate lymphocytes cause a faster increase in the concentration of Ca2+ in the cytosol, followed by a gradual decrease in the level of Ca2+. It turned out that at the initial moment, Ca2+ comes from the pericellular environment, and then the effect is enhanced by Ca2+ released from intracellular stores. Moreover, the latter effect is regulated through the activation of the membrane enzyme phospholipase C and the production of a secondary messenger - inositol triphosphate.

The mitogenic response of lymphoid cells significantly depends on the concentration of ions in the medium and the functional state of ion-transporting ATPhase s.

Very often, researchers use two groups of facts as an argument in favor of the key role of ion transport in lymphocyte activation. The first group of data is represented by experiments on the removal of various ions from the nutrient medium. The second group of facts includes work on the study of the mitogenic response of lymphocytes in the presence of inhibitors of ion transporting ATPhase s.

A decrease in Na+ concentration in the medium by 30-35% led to a significant inhibition of the response of lymphoid cells to Con A [43]. On the contrary, a decrease in the concentration of K+ in the medium by 5-6 times (to the level of 1 mM) had almost no effect on the response of lymphocytes to mitogens [61]. Complete removal of K+ from the medium led to inhibition of lymphocyte proliferation.

Extracellular Ca2+ is necessary for the development of the lymphocyte response to mitogen [49, 37]. A number of studies suggest that extracellular Ca2+ is necessary starting from the 10th hour of activation by mitogen. At early stages, activation does not depend on the presence of Ca2+ in the extracellular environment [47, 21]. These data can be questioned due to the presence of the cell's own Ca2+ reserves (for example, the membrane-bound Ca2+ pool, mitochondrial Ca2+, etc.), which can be released into the cytoplasm at the early stages of cell activation. It is unlikely that the exclusion of any component from the culture medium, and in particular, the removal of ions from the medium, can serve as reliable evidence in favor of the importance or, conversely, the insignificance of the transmembrane flows of these ions in triggering the cell's response to external influences. Removal of an important component of the environment can disrupt any metabolic processes in the cell, prohibiting its physiological reaction, without affecting the initial moment of the launch of this reaction.

The situation is similar with the "proof" of the key role of membrane ATPhase s in triggering the reaction of lymphoid cells. As noted above, ATPhase s, which transport ions across the membrane, are a critical organ of the cell. Without the normal functioning of ATPhase s, the full functioning of a cell is impossible, and even more so the activation of its metabolism in response to external influences is impossible. Therefore, numerous experiments in which inhibition of ion-transporting ATPhase s

(for example, inhibition of (Na+, K+) - ATPhase by ouabain) is accompanied by a violation of the response of lymphocytes to mitogens, in our opinion, cannot unambiguously indicate the key role of (Na+, K+) - ATPhase s in the mechanism of triggering the lymphocyte response.

Effect of ionophores on functional activity lymphocytes.

To test the possible role of K+, Na+, Ca2+ flows in the mechanism of triggering the physiological response of lymphocytes, a number of studies used substances that facilitate the diffusion of these cations through the membrane - ionophores.

Valinomycin, cyclic depsipeptide, highly specific K+ ionophore. The addition of valinomycin to the in vitro culture led to a significant inhibition of the proliferative response of lymphocytes to the mitogen. Another K+ ionophore, nigericin, also had a similar effect. The ionophore monensin, which transfers Na+ in exchange for H+, also inhibited lymphocyte proliferation [42, 43].

It should be noted here that all the carriers listed above, penetrating into the cell, transport the corresponding cations through the membranes of intracellular organelles. In particular, valinomycin, due to this effect on mitochondria, uncouples oxidative phosphorylation. Consequently, the intracellular effects of monovalent cation transporters can block important metabolic systems of the cell, making it impossible for it to respond to the mitogen. However, it is impossible to judge the significance of the effects of these same ionophores on the outer cell membrane in the initiation of the cellular response.

Ca2+ ionophore A23187, an antibiotic that transports Ca2+ ions across the membrane [18], has a weak mitogenic effect in in vitro culture of lymphocytes. The mitogenicity of the ionophore A23187 was considered as one of the main evidence of the functional role of Ca2+ entry in cell activation, therefore a number of studies were devoted to comparing changes in Ca2+ transport caused by A23187 and mitogenic lectins [52, 48, 57, 60]. There is also a report that another Ca2+ ionophore, ionomycin,

has mitogenic properties [16]. The third Ca2+ ionophore studied, X537A, had a mitogenic effect on cells [40].

Just like other mitogens, the ionophore A23187 interacts with the cell membrane of lymphocytes and ultimately leads to DNA synthesis and cell division. According to some characteristics, the ionophore A23187 differs from lectins. It increases the intensity of DNA synthesis to a lesser extent than PHA and Con A [54], but to the same extent as other mitogens, it stimulates the synthesis of RNA and protein [32]. Ionophore A23187 also induces early biochemical changes characteristic of the onset of cell division: increased metabolism of phosphoinositides, entry of glucose and amino acids into the cell [50]. Moreover, in the first hours of activation, it does not matter whether the mitogen is Con A or the ionophore A23187.

Replacing Con A with an ionophore for a short time - the first 3 hours of mitogenesis (followed by washing the cells from the ionophore and introducing Con A) - does not reduce the intensity of DNA synthesis. If Con A is replaced with an ionophore 15 hours after the onset of mitogenesis, then the intensity of DNA synthesis will decrease [37]. Compared to other ionophores, A23187 is more sensitive to the concentration of Ca2+ in the medium and at low concentrations of extracellular calcium (10-4 M) loses its mitogenic properties [43].

In general, the presented literature data indicate the undoubted importance of ion transport as one of the mechanisms for regulating the response of lymphoid cells to a mitogenic ligand. Together with the data described in the review on the absence of effects of polyelectrolytes on the cyclase system and the lipid matrix of the cell membrane and in connection with the discovery of R.V. Petrov and co-authors changed the permeability of the cell membrane during activation of lymphocytes by polyions, it was necessary to investigate the significance of modification of ion transport for the activation of not only and not so much the response of lymphocytes in vitro, but also the immune response in vivo.

It was impossible to do this on the basis of literary data alone. Firstly, the mechanisms of cell activation by polyions remained undiscovered. Secondly, data on the effects of ionophores in vitro were contradictory. It was necessary to investigate to

what extent the induction of ion permeability at the level of the outer cell membrane could determine the stimulation of the immune response upon the introduction of a polyion in vivo. It was for this purpose that we began experimental work in which we compared the ionophore-like effect of polyanions with their mitogenic effect and studied the immunoadjuvant properties of membrane-active compounds with different structures.

Chapter 2. MATERIALS AND METHODS OF RESEARCH

1. Antigens. Model of induction of antibody genesis in vivo.

Heterologous erythrocytes obtained from the peripheral blood of a donor sheep, as well as killed Salmonella microbial cells (S. typhimurium, strain 415), were used as an immunogen to induce the primary or secondary synthesis of specific antibodies. Before use, heparinized red blood cells were washed at least three times by centrifugation in a 20-fold volume of Hanks' balanced salt solution (without NaHCO3, pH 7.2) at 1500 rpm for 10 minutes.

During immunization, experimental mice were injected intraperitoneally with either a suspension of salmonella killed by formaldehyde from 1 to 100 μg (in terms of dry microbial sediment), or a suspension of sheep erythrocytosis (BE) in doses from 106 to 5 x 108. Suspensions were prepared using Hanks' solution. Each mouse was injected with no more than 0.5 ml of cell suspension.

In a series of experiments, CBA mice were immunized with the water-soluble protein antigen p90 from the anthrax pathogen. We used chromatographically homogeneous antigen p90 from the diagnosticum of the Stavropol Scientific Research Institute of Humanities. The p90 antigen was dissolved in physiological NaCl solution and administered intraperitoneally to mice at a dose of 3 μg (per mouse). Repeated immunization with the same dose of p90 was carried out after 1 month.

During the primary and secondary immune response, blood from the orbital venous sinus was collected weekly from experimental mice. Blood from 5-7 mice within one experimental group was pooled, serum was obtained, which was poured into 3 identical microtubes and frozen at - 20°C. Sera obtained 7, 14, 21, 28 days after primary immunization at the same time after secondary immunization were

accumulated. At the end of the experiment, the level of antibodies to p90 was tested simultaneously in all accumulated sera.

Registration of immune response.

4-5 days after immunization of mice with sheep erythrocytes, the content of antibody-forming cells (AFC) was determined in the spleens of immune mice, and after 7-8 days, the level of specific antibodies to BE in the blood serum was determined.

Spleen cell suspensions were prepared using a glass homogenizer. Each spleen was homogenized in 10 ml of Hanks' solution, then the suspension was filtered through a 4-layer nylon filter. According to the method, 20-200 μl of a suspension of spleen cells was mixed with 2.5 ml of 0.65% agarose sol, heated to 48ºC. A 20% suspension of BE was first added to the agarose sol at the rate of 27 μl per 1 ml of agarose. The agarose sol containing spleen cells and EB was carefully mixed and poured into Petri dishes (diameter 100 mm) placed on a horizontal plane.

After the gel hardened, the dishes were incubated for 1.5 hours at 37°C, and then diluted (1:10) guinea pig serum was applied as a source of complement. They were incubated for another 1 hour at 37°C, after which the dishes were analyzed or fixed with a 4% solution of farmaldehyde and stored until counting. In obliquely transmitted light, hemolysis zones were counted, which made it possible to determine the number of AFCs secreting hemolytic IgM antibodies that are specifically adsorbed on BE surface antigens. To determine IgG antibodies, before applying complement, rabbit antiserum specific to mouse immunoglobulins was added to the dishes. In the presence of anti-Ig serum (1:60 dilution), the dishes were incubated for 30 minutes, after which they were carefully washed with Hanks' solution and complement was applied.

To record cells secreting antibodies to Salmonella antigens, the above-described gel hemolysis method was used. But in this case, BE was added to the agarose, previously sensitized (“coated”) with 0 - antigen from the same Salmonella. Sensitization of erythrocytes with 0-antigen was carried out according to the method of V.V. Solovyov and co-authors. 0 - the antigen was previously “activated” in an aqueous solution (5 mg/ml) at 100º C for 2 hours. Then the “activated” 0-antigen was added to the BE suspension, constantly stirring with a magnetic stirrer. After the

adsorption of 0-antigen on the surface of the BE, the latter were washed several times to remove unbound antigen by centrifugation in a 20-fold volume of Hanks' solution.

After immunization of mice with the p90 protein antigen (from the causative agent of anthrax), the intensity of the immune reaction was assessed by the accumulation of p90-specific antibodies in the blood serum. For this purpose, enzyme-linked immunosorbent assay (IEA) was used. Optimal conditions for IEA with p90 antigen were selectedana (formerly) an employee of the Institute of Immunology A.I. Pereverzev.

When performing IEA, we carried out the following operations step by step. 100 μl of a solution of p90 in 0.1 M carbonate-bicarbonate buffer pH 9.6 was added to a well of special 96-well panels “Linbro” (Flow Lab., UK) (the final concentration of p90 was 3 μg/ml). Antigen panels were left overnight at 4°C. Then the wells of the panels were washed three times with a special washing solution (0.15 M NaCl solution, buffered with 0.01 M phosphate buffer pH 7.2-7.4 with 0.05% Tween-20). A Titertek Microplate Washer 120 (Flow Lab., UK) was used to wash the panels.

After washing off the antigen not bound to the plastic, 150 μl of a 1% solution of bovine serum albumin in physiological NaCl solution buffered with phosphate buffer was added to the well of the panels. The panels were incubated for 1 hour at 37°C and then washed three times.

Serial dilutions of the analyzed sera, starting from a starting dilution of 1:20 with a step of 2, were added to the well of the panels pre-treated with the antigen. Sera were diluted in an incubation solution (0.15 M NaCl, in 0.01 M phosphate buffer pH 7.2-7.4 with 0.1% Tween - 20). To add dilutions of serums, as well as to add any solutions into the wells of micropanels, an automatic dispenser Titertek Autodrop (Flow Lab., UK) was used. After adding serum dilutions, the panels were incubated for 1 hour at 37°C.

Upon completion of incubation, the panels were washed three times and 100 μl of a solution of rabbit antibodies specific to the γ-chain of mouse immunoglobulins or rabbit antibodies specific to mouse IgM (Miles Scientific, USA) was added to the wells. Primary antibodies were added at a final dilution of 1:500 in incubation solution. The

panels were incubated for 1 hour at 37°C. Primary antibodies were washed three times, and then 100 μl of a solution of secondary antibodies conjugated with peroxidase (Miles Scientific, USA) was applied.The panels were incubated at 37°C for 1 hour, washed three times, after which 100 μl of a substrate solution was added to the wells - 0.6 mg/ml orthophenylenediamine dihydrochloride (Sigma, USA) in 0.1 M citrate-phosphate buffer pH 5.0 s 0.015% hydrogen peroxide. The panels were incubated for 10 min in the dark. The reaction was stopped by adding 50 μl of 2 M sulfuric acid into the panel well. The optical density of solutions in the wells of micropanels was measured at a wavelength of 492 nm on a special Titertek Multiscan MCC photometer (Flow Lab., UK). The IEA results were analyzed on an IBM computer using a special program written by an employee of the Institute of Immunology A.I. Pereverzev. This program made it possible to calculate titers of specific antibodies in sera and confidence intervals for their determination at P = 0.05.

2. Cultures of lymphoid cells in vitro

Depending on the tasks being solved, we used in our work both short-term incubation of lymphoid cells in vitro for several hours, and longer-term cultivation of them for several days.

A suspension of mouse lymphoid cells was prepared according to standard methods. Mice were killed by cervical spinal dislocation. The spleen and lymph nodes (inguinal, axillary, submandibular, mesenteric) were removed under aseptic conditions. The suspension was prepared using a glass homogenizer. Cells were washed 1-2 times with Hanks solution (without NaHCO3, pH 7.2), buffered with 10-20 mM. The osmotic shock method was used to remove red blood cells. After shock, the lymphocyte suspension was filtered through a layer of sterile cotton wool and further washed by centrifugation in a 20-fold volume of buffered Hanks solution. Cell viability was assessed by microscopy in the presence of 0.1% trypan blue.

Short-term incubation was carried out either in buffered Hanks solution supplemented with 1% fetal bovine serum (FBS) and 50 U gentamicin, or in RPMI-1640 medium supplemented with 2 mM L-glutamine, 5x10-5 mercaptoethanol, 1% FBS and 50 U gentamicin and 20 mM HEPES buffer.

Cultivation of lymphoid cells for 3 days was carried out in RPMI-1640 medium with the same additives, but the medium contained 5% FBS. The cultures were kept in a special incubator in an atmosphere of 5% CO2 in the air.

In some experiments, a suspension of lymphocytes was separated into two fractions - enriched in T cells and enriched in B lymphocytes. Separation was carried out according to the method of M. Julius coauthors, using specially prepared nylon wool. Leucko-color Leuccosyte Filter (USA) cotton wool was boiled for two days in distilled water, replacing the water several times a day. The cotton wool was then dried and weighed. A plastic column (height 140 mm, cross-sectional diameter 22 mm) was tightly filled with 3 g of dry nylon wool.

The column was washed with a small amount of medium 199 supplemented with 5% FBS and placed in a thermostat at 37°C for 30 minutes. Then, 2 ml of a lymphocyte suspension containing 50x10 living cells in 1 ml was added to the upper part of a vertically installed column. Another 5 ml of medium was added and placed in a thermostat at 37°C. After 45-50 minutes, the column was carefully washed with 80 ml of medium heated to 37°C. The eluate was collected (first portion).

After several cycles of mechanical compaction with nylon wool, the column was washed again with a medium that did not contain ETS. The second portion of the eluate was also collected. Both portions of the eluate contained living cells. The first fraction is cells that do not adhere to cotton wool, the second fraction is cells that adhere to nylon wool. The resulting cells from this separation have been characterized previously. It has been shown that the fraction of non-adherent cells is rich in mature T cells and contains virtually no B cells.

On the contrary, the fraction of cells adhering to cotton wool contains mainly B lymphocytes, and has a small admixture of T cells adhering to nylon.

In our work, to control the quality of separation, we characterized the fractions in a cytotoxic test with immune sera against Thy I, 2 antigen and against in the presence of complement. The nonadherent fraction contained 93-95% Thy I, 2-positive cells and 1-2% cells bearing surface Ig. The adherent fraction contained 6-7% Thy I, 2-positive and 80-84% Ig-positive cells.

To determine the activation of lymphocytes when adding any immunostimulant in vitro, we examined the intensity of DNA synthesis in the first 48-72 hours after exposure. On the second day of incubation, 3H-labeled thymidine was added to the cultures at a concentration of 1 μCurie per 1 ml. After 24 hours, the suspensions were filtered through Sinpor No2 filters. The cells deposited on the filter were washed with Hanks' solution and lysed with 0.5% trichloroacetic acid. The acid-insoluble precipitate was washed with 96° ethyl alcohol. After drying, the filters with radioactive material were placed in scintillation liquid. The level of radioactivity of the filters was determined using a liquid scintillation β-counter.

3. Polymers.

The polyanions used in the work were polyacrylic acid (PAA, molecular weight about 100 kilodaltons) and dextran sulfate (SD, molecular weight more than 500 kilodaltons). PAA was synthesized by T.V., an employee of the Institute of Immunology. Abramenko. SD is a commercial drug produced by Pharmacia (Sweden).

Typically, polymer solutions were prepared on the eve of the experiment. For more complete dissolution, the newly prepared solution was actively stirred for several minutes, then left in a thermostat at 37°C for 1 hour. The mixture was thoroughly mixed again and sterilized by filtration through Millipore filters (USA) with a pore diameter of 0.22 μm. The prepared sterile polymer solutions were stored in the refrigerator at 4°C.Before use, the polymer solutions were left for several hours at room temperature and thoroughly mixed. The polymers were dissolved in a buffered Hanks solution at a concentration 10-100 times higher than intended. This made it possible to add a small volume of solution containing the polymer to the cell culture. As a rule, to create the intended final concentration, 10-100 μl of a polyion matrix solution was added to 1 ml of culture.

4. Membrane-active effectors.

The following membrane-active substances were used in this work. Gramicidin S 2% alcohol solution of domestic production, pharmacopoeial preparation. Levorin, sodium salt is a pharmacopoeial drug of domestic production. Nystatin is a polyene antibiotic, a domestic drug. Gramicidin A is a pentadecapeptide, an antibiotic produced

by Sigma (USA). Saponin is a membrane-active substance produced by **Calbiochem** (USA).

Solutions of membrane-active substances were prepared immediately before use.

5. Measuring cell membrane permeability for ions.

The permeability of the plasma membrane of lymphocytes to ions was determined by changes in the concentration of K+ in the extracellular environment, and Ca2+ in the cytoplasm.

The permeability of the cell membrane to Ca2+ was measured using the radio indicator method. For this, the 45Ca isotope was used. Experiments were carried out with a suspension of lymphocytes (5 – 10 x 106 in 1 ml). Cells were placed in RPMI-1640 medium supplemented with 20 mM HEPES buffer, 2 mM glutamine, 50 U/ml of a mixture of penicillin and streptomycin, and 5% fetal calf serum.45CaCl salt was added to the cell suspension to a final concentration of 0.1 μCurie per 1 ml. After 1 hour of incubation in the presence of 45Ca, samples of 200-1000 μl were taken from the suspensions. The cells contained in the samples were washed from the extracellular isotope by 2-3-fold centrifugation in a large volume of medium 199. Then the dense cell sediment was lysed with 300 μl of 5% perchloric acid to destroy the cells and extract the intracellular contents.Extraction was carried out for 1-2 minutes, constantly shaking the samples. The resulting extracts were centrifuged at 100 rpm for 10 minutes. Then 5-10 μl of a saturated K2CO3 solution was added to 200 μl of extract to neutralize perchloric acid. The extracts were left for several minutes to allow the insoluble potassium perchlorate “flakes” to settle. The light part of the extracts was selected to measure radioactivity on an Intertechnique SL - 40 β spectrometer. A dioxane scintillator was used (900 ml dioxane; 4 g PPO; 0.2 g POPOP; 60 g naphthalene). Extract samples were mixed with scintillation liquid in a ratio of 1/50.

The radioactivity of extracts from cultures activated with the mitogenic polyanion was compared with the radioactivity of extracts from control non-activated lymphocytes. This made it possible to evaluate the effect of the polyanion on the penetration of 45Ca from the culture medium into the cells.

Measurement of cell membrane permeability to K+.

To prepare lymphocyte suspensions, buffered Hanks solution was used. For this purpose, Hanks' solution (NaHCO3) was supplemented with 20 mM HEPES buffer and 50 IU of peniillin sodium. Suspensions of mouse spleen lymphocytes containing 3–5 x 107 living cells in 1 ml of Hanks' buffered solution were used in the experiments. The cells were placed into a special thermostated (37°C) fluoroplastic cell.A valinomycin electrode, which has selective sensitivity to K+ ions, was installed in the cell, as well as a double electrical bridge that connected the measuring cell to the EVL-1MZ reference electrode [4]. The slope of the characteristic of the valinomycin electrode in the potassium concentration range from 10-4 to 1 M was 60 mV per order of magnitude of the K+ concentration.The K+/Na+ selectivity is no less than 104. The setup we used made it possible to continuously record the K+ concentration in the extracellular environment using an N-339 recorder. Any increase in the permeability of the cell membrane should have led to an increase in the flow of K+ from the cytoplasm into the extracellular environment and to an increase in the level of K+ in the culture medium.

6. Study of active ion transport, mediated by (Na+, K+) - and Ca2+ - ATPhase s plasma membrane.

The rate of operation of membrane ATPhase s transporting Na+, K+ or Ca2+ was assessed by inhibitory analysis. Using the method we used [1], it was possible to estimate the proportion of a specific ATPhase in the total ATPhase of cells. In turn, the total rate of ATPh consumption and the equal rate of ATPh production were judged by the rate of oxygen consumption by cells.As is known, the rate of ATPh production in mitochondria is in direct proportion to the rate of O2 consumption by the mitochondrial oxidative phosphorylation system. Activation of ATPh production in mitochondria requires an equal increase in the rate of O2 consumption. In living, functional cells, the rate of ATPh production is equal to the rate of its consumption. Consequently, by changing the rate of O2 consumption by cells, one can judge the change in the rate of ATPh production by mitochondria and the equal rate of ATPh

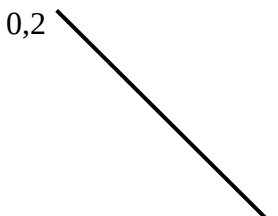
consumption by all cellular reactions taken together that require the energy of ATPh hydrolysis.

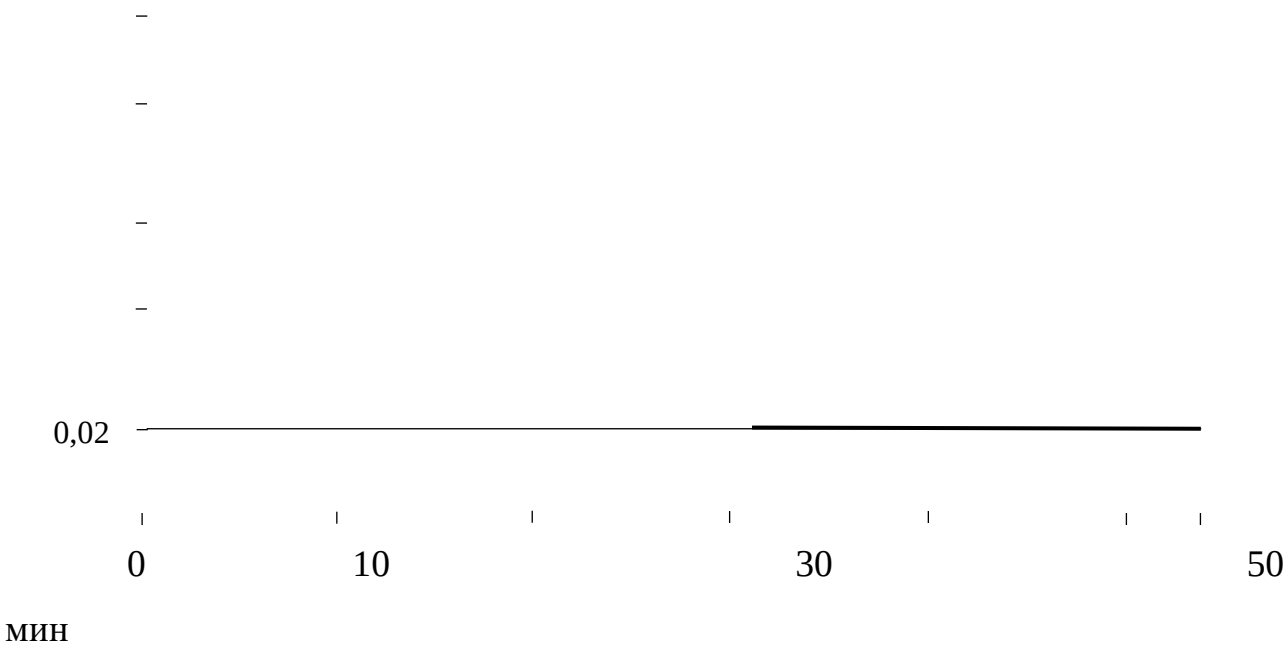
The above reasoning is correct if two conditions are met. First, the rate of ATPh consumption must equal the rate of ATPh production. This condition was fully observed in our experiments. During the entire period of measuring the rate of O2 consumption by lymphocytes, the level of ATPh in the studied cells did not change [1]. The second condition is the relationship between the gross consumption of O2 by cells and the use of O2 in these same cells for the needs of oxidative phosphorylation in mitochondria.The fact is that in some cells a significant proportion of O2 can be consumed to support peroxidation processes or other non-mitochondrial reactions. For example, such cells are phagocytes and hepatocytes. But lymphoid cells in our experiments spent more than 99% of consumed O2 precisely for the needs of oxidative phosphorylation in mitochondria. Turning off the “breathing” of mitochondria using sodium cyanide led to a drop in the rate of O2 consumption to almost zero.

We measured the rate of O2 consumption by lymphocytes using the polarographic method in a special hermetic cell into which a platinum O2 electrode was mounted. 700 µl of a suspension containing 2 – 3 x 107 mouse spleen lymphocytes (calculated per 1 ml of nutrient medium) was placed into the cell.

It should be noted that due to the reduction of O2 on the platinum electrode when the cell was sealed, O2 was absorbed by the platinum electrode itself. The effect of O2 absorption by the electrode was significantly less than the O2 absorption by cells. Despite this, the electrode effect was measured and included in each analysis. To do this, before studying the rate of O2 absorption by the cell suspension, we recorded the O2 absorption by the electrode. Each measurement of cell “respiration” was corrected for the “electrode effect.”

When lymphocytes were incubated in a sealed polarographic cell under thermostatic conditions (37° C), the rate of O2 consumption by cells was constant in the O2 concentration range from 0.02 to 0.2 mM.[O2] mM





Rice. 1. O2 absorption by mouse spleen cells placed in a sealed polarographic cell. Time 0 corresponds to the beginning of incubation of cells in a sealed polarograph cell. An O2 level of 0.2 mM corresponds to the initial content of incubation O2 in the medium at time 0 at a temperature of 37º C. An O2 level of 0.02 mM (marked by a thin horizontal line) is the minimum possible oxygen concentration in the incubation medium placed in a given polarographic cell at a given degree of cell sealing.

When 2 - 3 x 107 lymphocytes were incubated in 1 ml of medium, the cells consumed O2 at a stationary rate, reducing the O2 concentration to 0.02 mM in approximately 45-60 minutes (Fig. 1). This allowed us, during continuous recording of the respiration of cells placed in a sealed cell, to study the effect of an “injection” of a drug on the rate of O2 consumption by cells. That is, we could measure the rate of O2 consumption by cells before exposure and in dynamics after exposure of cells to any substance.

If cells were exposed to an ion transporting ATPhase inhibitor, then a decrease in the rate of O2 consumption in the first minutes after exposure to the inhibitor showed the share of this ATPh-consuming process in the total consumption of O2 by the cell and, consequently, in the total consumption of ATPh by the cell. Hereinafter we will call this part “the share of a given ATPhase in the total cellular ATPhase .”

Thus, the effect of specific inhibitors of ion transporting ATPhase s on the intensity of O2 consumption by intact (non-activated) lymphocytes was analyzed. A selective blocker of (Na+, K+) - and ATPhase s was used - suabain at a final concentration of 10-4 M and a selective inhibitor of Ca2+ - ATPhase - lanthanum (LaCl3) at a final concentration of 10-3 M. Under the influence of lanthanum, such a weak respiratory depression was observed in non-activated lymphocytes that it could not be reliably measured (Fig. 2). On average, lanthanum reduced the rate of O2 consumption by cells by 5% with a 4-5 percent measurement error. This means that during the functioning of Ca2+ - ATPhase , non-activated lymphocytes consume no more than 5% of the total ATPh produced in the cell.

Specific inhibition of (Na+, K+)-transporting ATPhase led to a decrease in the intensity of cellular respiration by an average of 18% (Fig. H). That is, in non-activated lymphocytes, about 18% of the total ATPh produced in the cell was consumed for the work of (Na+, K+)-ATPhase of the plasma membrane.

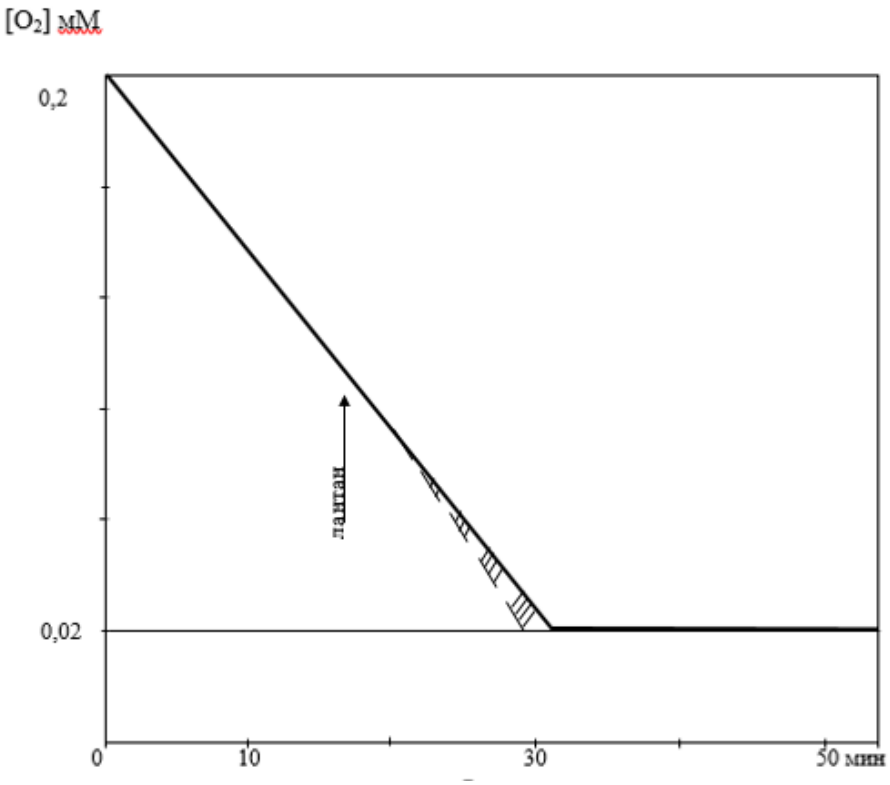


Figure. 2. The effect of lanthanum (10-3 M), a Ca2+ ATPhase inhibitor, on the rate of O2 uptake by lymphoid cells from the mouse spleen placed in a sealed polarographic cell. The abscissa axis is the time (min) of incubation of cells in a sealed cell, the ordinate is the concentration (mM) of O2 in the incubation medium. The initial O2 level in the incubation medium is 0.2 mM; the minimum O2 level in the incubation medium is 0.02 mM. The arrow indicates the moment of "injection" of the inhibitor into the cell suspension.

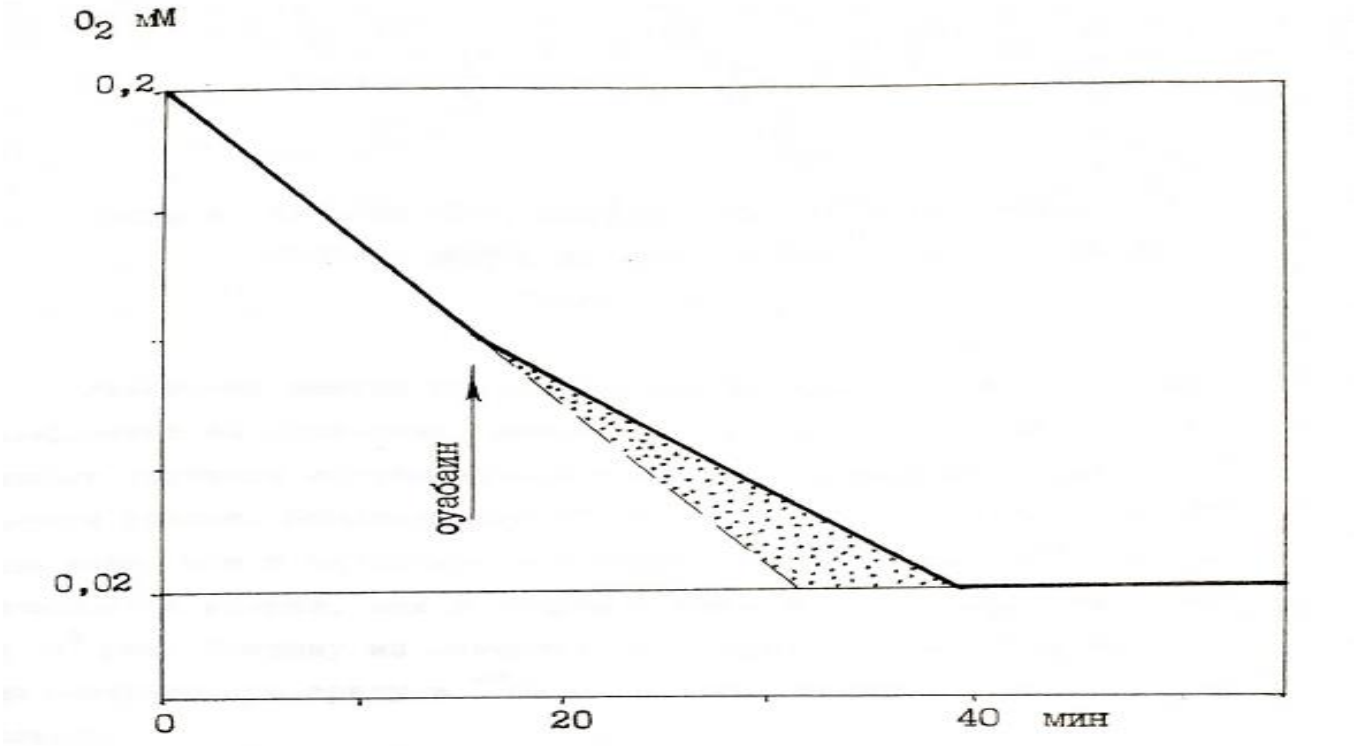


Figure. 3. Effect of ouabain (10-4 M), an inhibitor of (Na+, K+)-ATPhase , on the rate of O2 consumption by lymphoid cells from the mouse spleen placed in a sealed polarographic cell. The abscissa axis is the time (min) of incubation of cells in the cell after its sealing; the ordinate axis is the concentration (mM) of O2 in the incubation medium. The initial O2 level in the incubation medium is 0.2 mM, the minimum O2 level in the incubation medium is 0.02 mM. The arrow indicates the moment of "injection" of the inhibitor into the cell suspension.

RESEARCH RESULTS

Chapter 3. INFLUENCE OF POLYANION IMMUNOSTIMULATORS ON THE PERMEABILITY OF THE LYMPHOCYTE MEMBRANE FOR POTASSIUM AND CALCIUM IONS

We measured the ionic permeability of the plasma membrane and lymphocytes based on the existing differences between the ionic composition of the incubation medium and the ion content inside the cells. The concentration of K+ in the extracellular environment is 30-35 times lower than in the cytosol. On the contrary, the Ca2+ concentration inside lymphocytes is at least 103 times less than in the incubation medium. Therefore, we measured passive fluxes of K+ from the cell into the extracellular medium and 45Ca from the incubation medium into the cytoplasm.

1. Passive K+ flows.

When spleen lymphocytes were incubated in a measuring cell with a K+ electrode at 37°C for 1 hour, we did not observe a significant change in the level of extracellular K+ (Fig. 4). This means that the naturally existing transmembrane K+ flows from the cell to the extracellular environment under physiological conditions are precisely compensated by the active transport of this ion from the extracellular environment - into the cytoplasm. The last one is like.

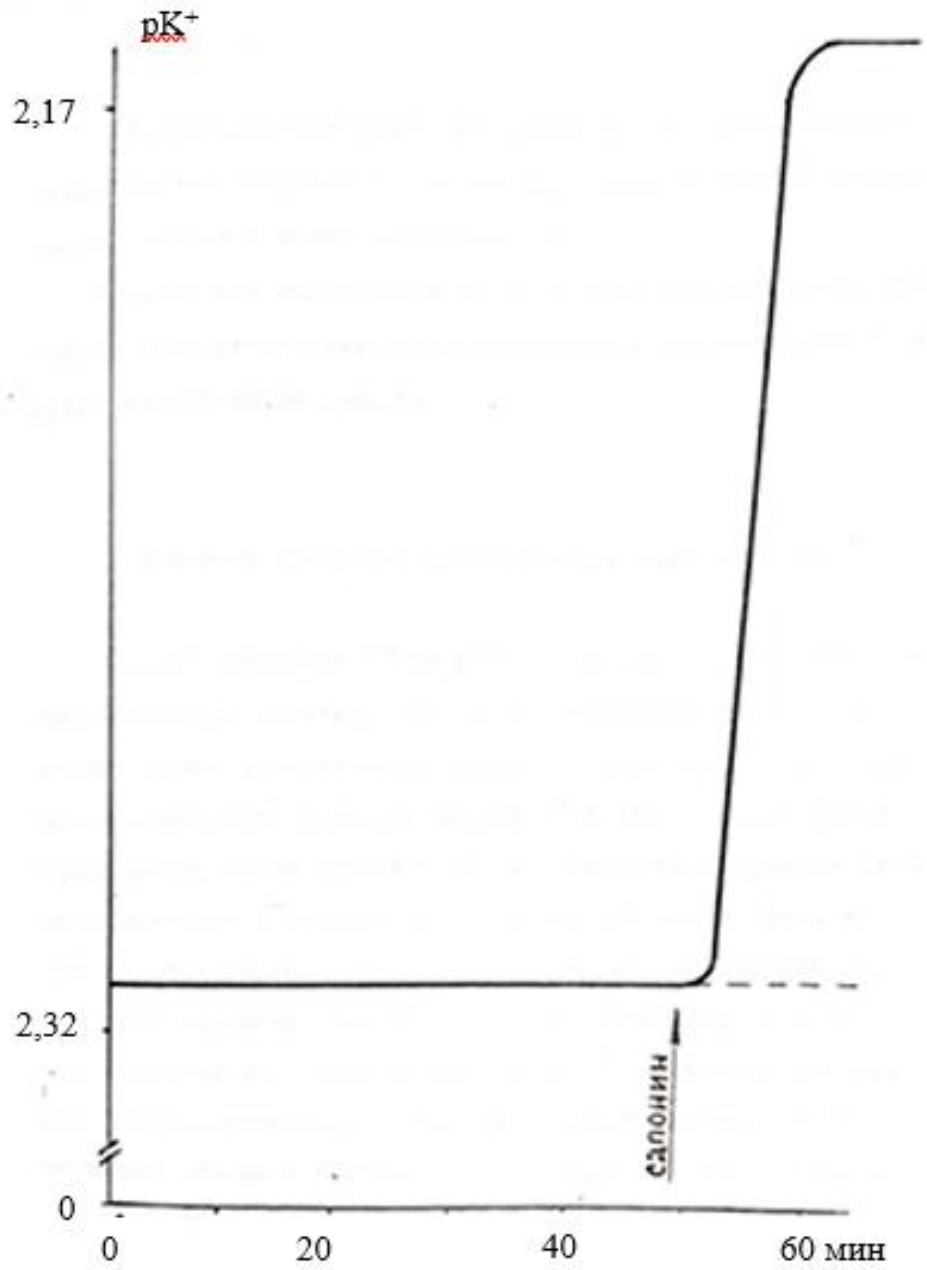


Figure. 4. Stability of the level of extracellular K+ in a suspension of spleen lymphoid cells when they are incubated in a thermostated (37º C) cell with a valinomycin electrode. The abscissa axis is the time of cell incubation in vitro, the ordinate axis is the concentration of K+ in the extracellular environment (pK+ = - Ig [K+]). During 50 min and incubation of cells, the concentration of K+ in the medium does not change. To illustrate the maximum release of K+ from cells, data are presented for complete cell lysis with saponin. The moment of lysis is marked with an arrow.

It is known to be performed by (Na+, K+) - ATPhase . This dynamic equilibrium was not disturbed when cells were incubated in the measuring cell in our experiments.

When exposed to polyanion (PAA) during cell incubation, we found a clear increase in the K+ concentration in the culture medium (Fig. 5).

Kinetics of measuring membrane permeability to K+.

The process of K+ leakage from cells activated by PAA had clearly nonlinear kinetics. In Fig. Figure 5 shows the kinetics of measuring the level of extracellular potassium after adding PAA to a suspension of lymphocytes. Signs of K+ release from cells are visible already in the first minute after exposure to PAA. The highest average rate of K+ leakage was observed in the first 3-5 minutes (phase **a)**.Moreover, in this period, as a rule, it did not acquire a stationary value. On the contrary, in the interval between 5 and 10 minutes (phase b), the rate of K+ leakage, having decreased slightly, stabilized. A steady state of the K+ leakage rate was usually observed over the next 15-20 minutes (phase c). It was during this period that we could accurately measure the steady-state value of the K+ outflow rate. Later, 30-40 minutes after the “injection” of the polyanion into the lymphocyte suspension (phase d), the rate of K+ leakage gradually decreased to almost zero values

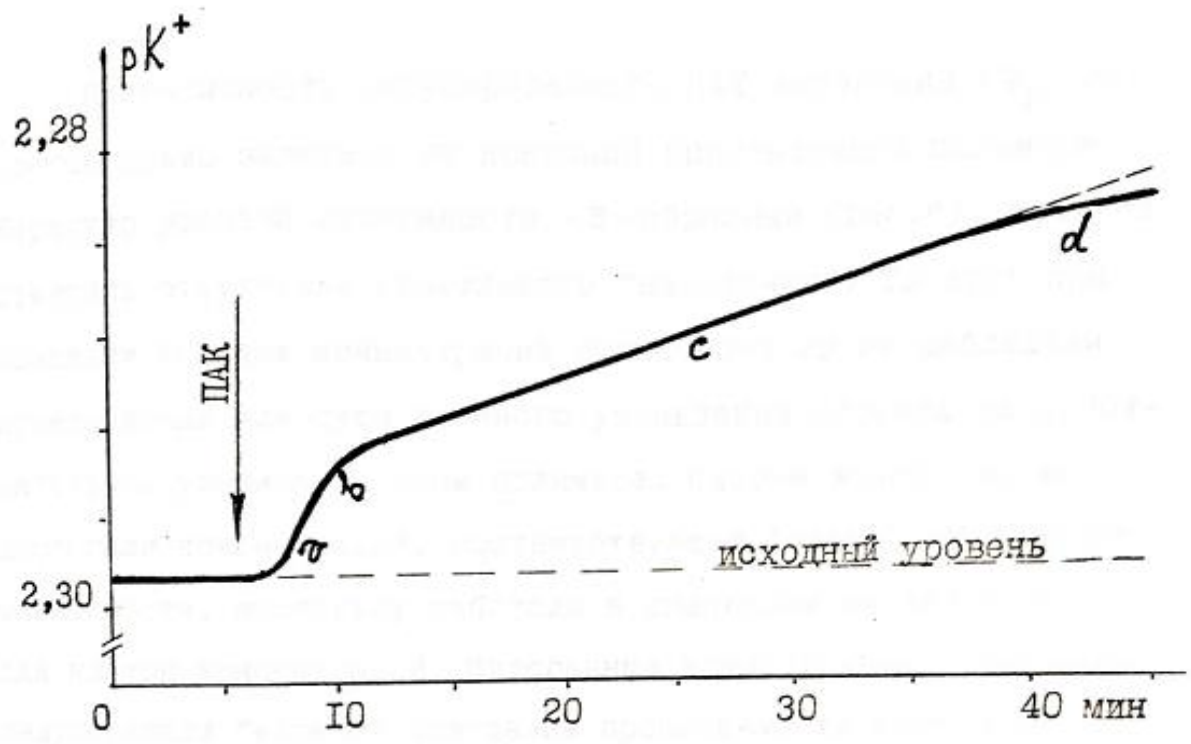

pK+
2,28
2,30
ПАК
a
b
c
d
исходный уровень
0
10
20
30
40 мин

Figure. 5. Kinetics of increasing K+ flux from cells into the extracellular environment under the influence of a polyanion (polyacrylic acid, final concentration 10-6 M). The arrow indicates the moment of “injection” of the polyanion into the suspension of lymphoid cells. The phases of the kinetic wing are designated a, b, c, d (explanations in the text). Along the axes: abscissa - time of incubation of cells in vitro: ordinate - concentration of K+ in the extracellular environment (pK+ = - Ig [K+]).

Dose dependence.

The intensity of PAA-induced efflux (VK) from cells was directly dependent on the final polymer concentration. The nature of the dose dependence is S-shaped (Fig. b). It should be noted that there is no clear “saturation”. That is, when creating high concentrations of the polyanion, we did not observe the disappearance or significant decrease in the effect of an additional increase in the polymer dose. Most likely, we did not reach concentrations corresponding to the “plateau” in the dose dependence, since we worked in the range of concentrations that were not lethal for cells. Mitogenic concentrations of the polyanion induced a “mild” increase in cell membrane permeability.In 1 hour, under the influence of the immunostimulating polymer, 100-1000 times less K+ was released from the cells into the medium than when the same cells were completely lysed by saponin. In addition, as noted above, the effect of K+ leakage induced by the polyanion practically ceased within 35-40 minutes after the start of exposure (see Fig. 5).

Like PAA, another polyanionic mitogen, dextran sulfate, also caused an increase in the permeability of the lymphocyte membrane to K+. The kinetics of K+ leakage was almost the same as when using PAA (Fig. 7). Only the lag phase preceding phase “a” was not several tens of seconds, but 2-3 minutes. The dose dependence when exposed to dextran sulfate was also similar to PAA. She also had an S-shaped appearance without a distinct "saturation". At the same time, it should be noted that an ionophore-like effect of dextran sulfate occurs at molar concentrations an order of magnitude higher than when using PAA (Fig. 8).

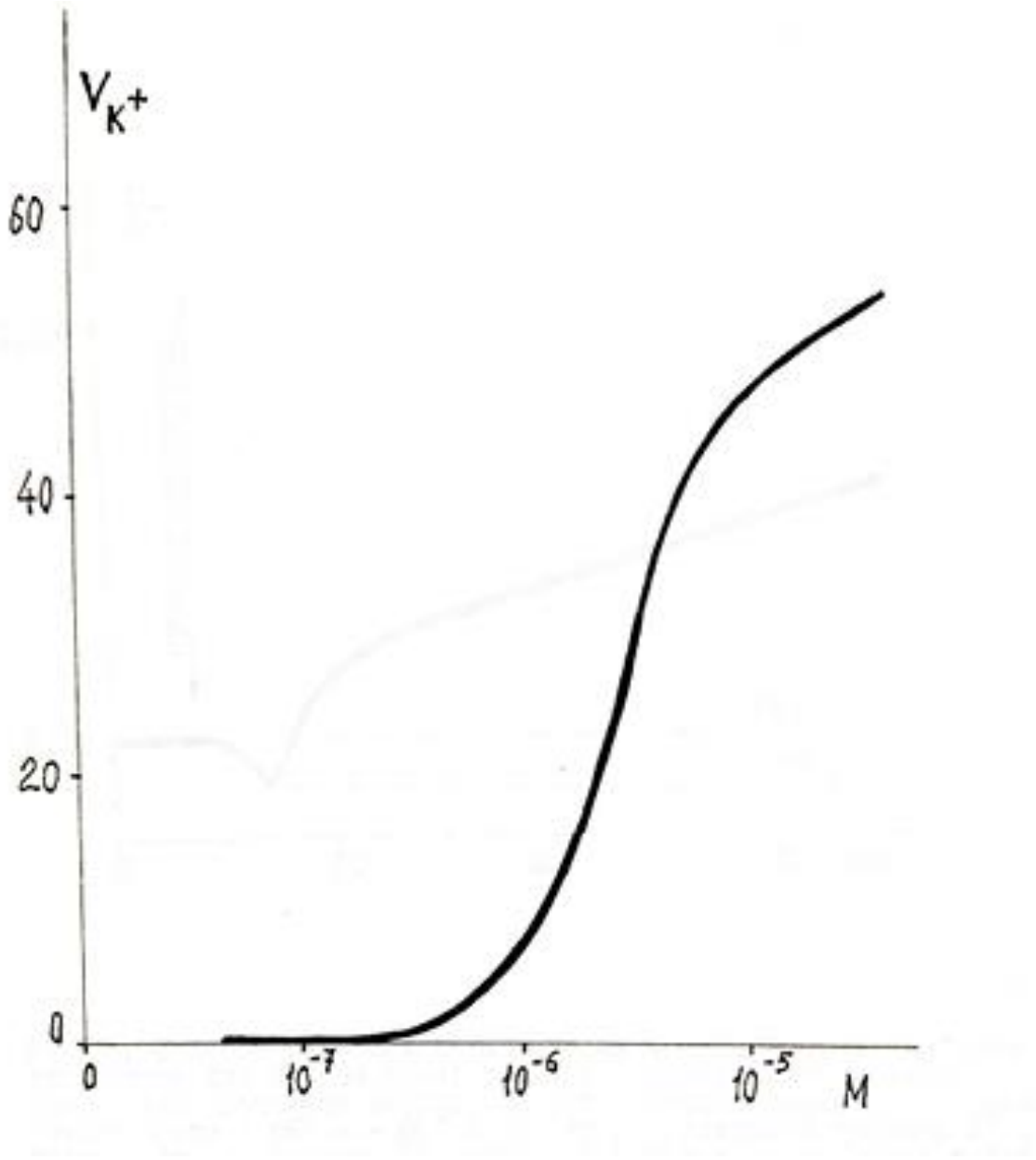


Figure. 6. Dose dependence of the ionophore-like effect of the polyanion on the plasma membrane of lymphocytes. Along the axes: abscissa - final concentration (M) of polyacrylic acid in a suspension of lymphoid cells in vitro; intensity of the ionophore-like action of the polymer (VK+, see text). The dose-response curve was plotted using VK+ values in the stationary portion of the kinetic curves obtained using different polyanion concentrations.

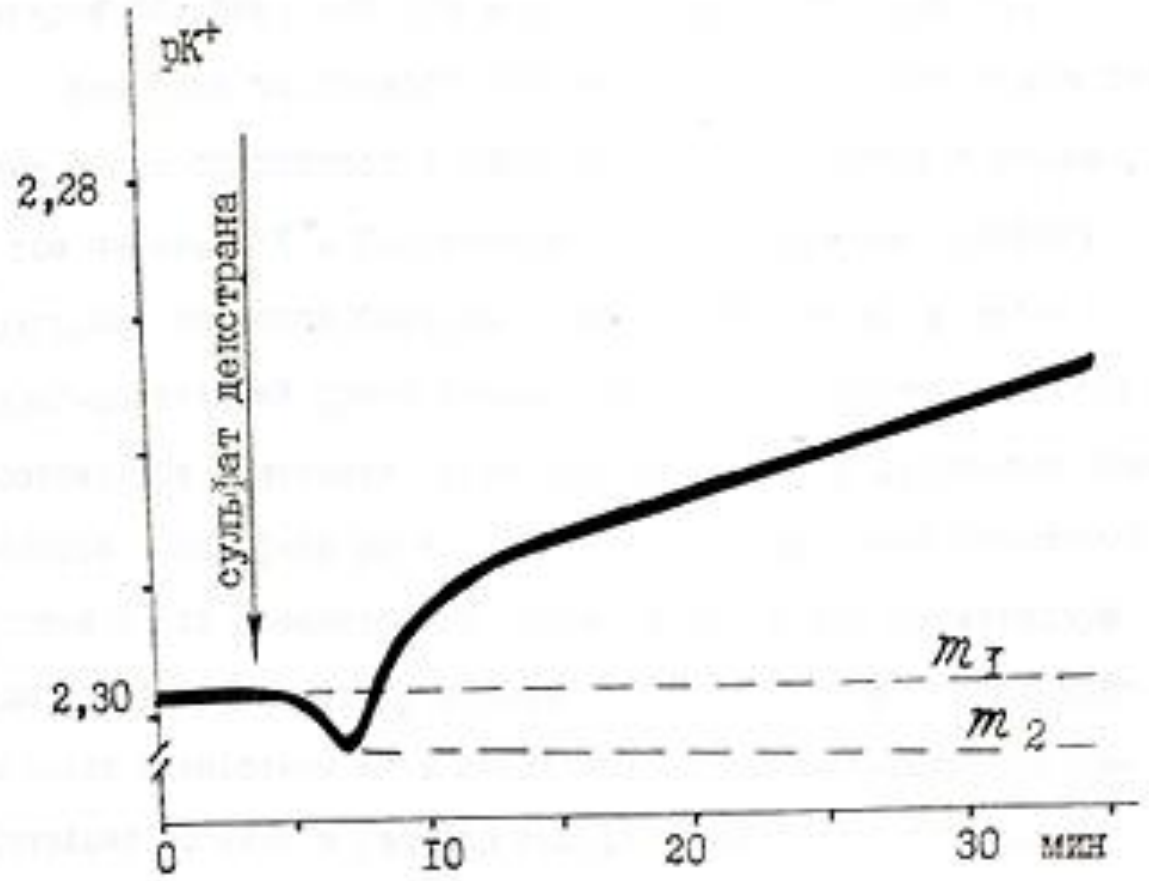


Figure. 7. Effect of dextran sulfate on the permeability of the lymphocyte membrane to K+. Along the axes: abscissa - time of incubation of lymphoid cells, in vitro; ordinate – K+ concentration in the extracellular environment (pK+ = - Ig [K+]). m1 is the initial level of K+ in the medium, m2 is the level of K+ established in the medium after adding dextran sulfate to it (2 x 10-6 M, final concentration). The moment of "injection" of the polyanion into the cell suspension is indicated by the arrow.

Another feature of the effects of dextran sulfate is associated with its ability to form complexes with ions, including K+. The consequence of this property of dextran sulfate is a rapid decrease in the level of K+ in any potassium-containing medium (regardless of the presence of cells). Therefore, when "injecting" dextran sulfate into a suspension of lymphocytes in the lag phase, we could observe a slight decrease in the level of K+ in the extracellular environment, and then the whole picture of K+

leakage from the cells, characteristic of a polyanion. The initial effect of reducing K+ in the medium could be avoided by first adding a small volume of 1 M KS 1 solution to the dextran sulfate solution to “saturate” the dextran sulfate with potassium. This preliminary “saturation” of dextran sulfate with potassium did not affect the ionophore-like activity of the polyanion.

2. Passive Ca2+ flows

As was described in the chapter “Materials and Methods,” using the radioactive isotope 45Ca we could study the rate of penetration of extracellular 45Ca into the cytoplasm, in other words, the permeability of the plasma membrane of lymphocytes to 45Ca. By comparing this indicator in cultures of non-activated cells and in cultures of cells activated by a polyanion, one could judge the effect of the immunostimulating polymer on the permeability of the membrane to Ca2+.

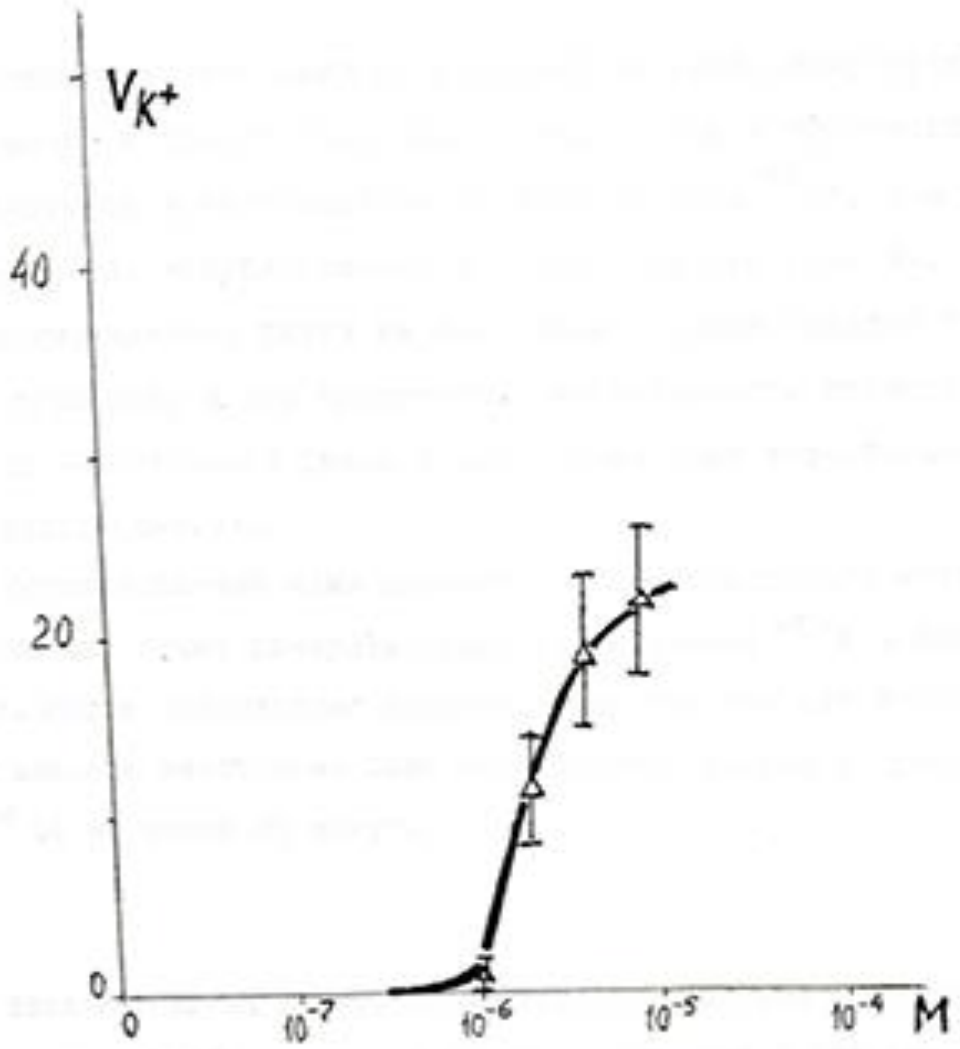


Figure. 8. Dose dependence of the ionophore-like effect of the polyanion, dextran sulfate, on the plasma membrane of lymphocytes. Along the axes: abscissa - final concentration (M) of dextran sulfate in a suspension of lymphoid cells in vitro; ordinate is the intensity of the ionophore-like action of the polymer (VK+). The dose-response curve was plotted using VK+ values in the stationary portion of the kinetic curves recorded using different polyanion concentrations.

It turned out that mouse spleen lymphocytes incubated for 30-60 minutes in the presence of immunostimulating concentrations of PAA include several times more of the 45Ca isotope than the same lymphocytes incubated in the absence of PAA (Fig. 9).

Dextran sulfate had essentially the same effect, since in its presence the intensity of penetration of 45Ca from the extracellular environment into the cytoplasm also increased significantly (Fig. 9).

The version of the radiotracer method we used did not allow us to accurately measure the rate of 45Ca incorporation in the first minutes after exposure to polyanions, since for a reliable analysis it was necessary to incubate the cells in the presence of 45Ca for at least 30 minutes.

3. Effect of PAA on the ionic permeability of the membrane under conditions of complete blocking of the ion-transporting ATPhase .

The increase in passive fluxes of K+ and Ca2+ across the plasma membrane that we discovered could be a consequence of the inhibition of ion transporting ATPhase s under the influence of the polyanion. In an intact cell, ion flows in the direction of their electrochemical gradient (passive flows) are exactly compensated by the active transfer of the same ions in the opposite direction. Ion transport against the electrochemical gradient is carried out by membrane ATPhase s. Inhibition of the work of ATPhase s by any exogenous substance (for example, a polyanion) would lead to an apparent increase in membrane permeability, since the experimenter would observe an apparent increase in passive fluxes. In fact

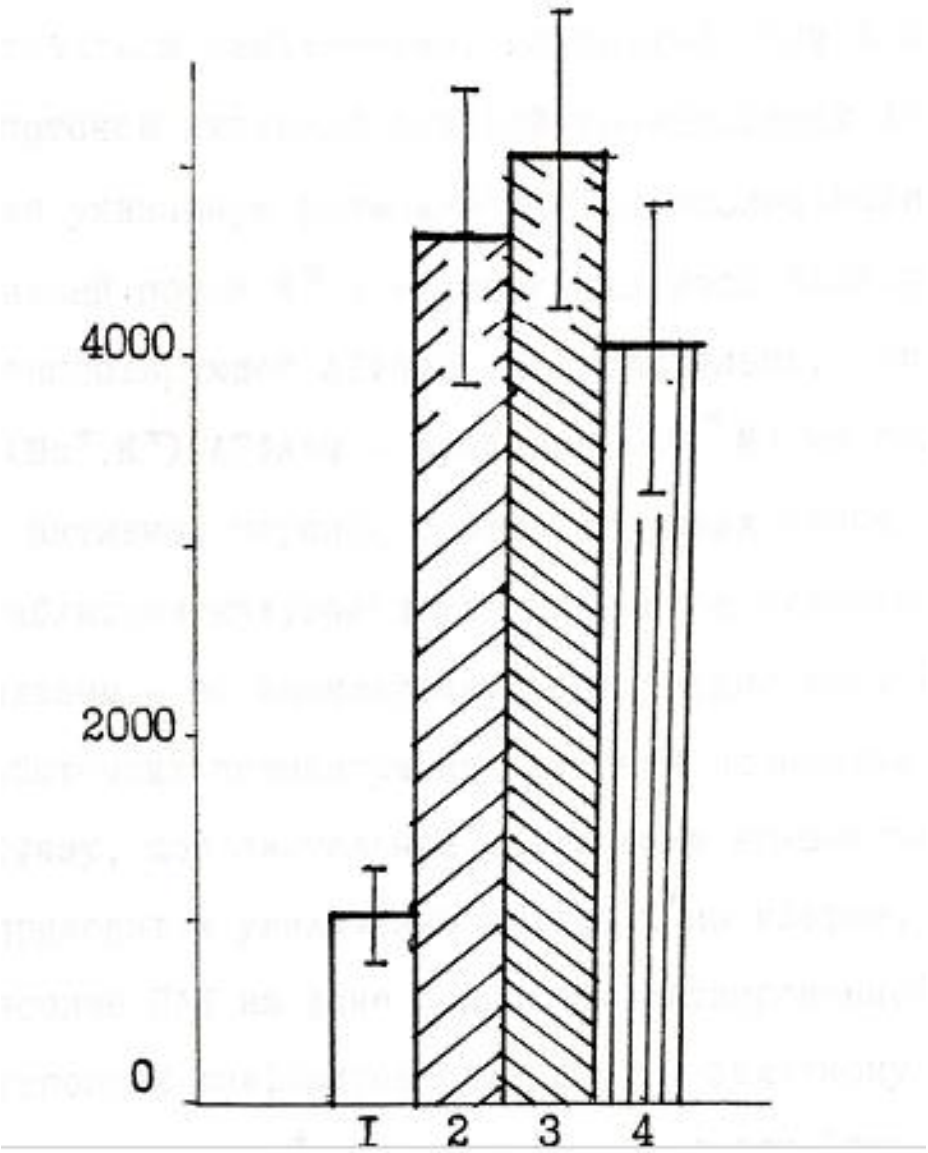


Figure. 9. The influence of polyanions on the incorporation of 45Ca from the medium into the cytosol of lymphoid cells. The ordinate shows the radioactivity of cell extracts (cpm). Extracts were obtained from the following cell variants: (I) control non-activated lymphocyte culture from the mouse spleen; (2) cells activated by PAA (50 μg/ml); (3) cells activated by PAA (200 μg/ml); (4) cells were activated with dextran sulfate (100 μg/ml).

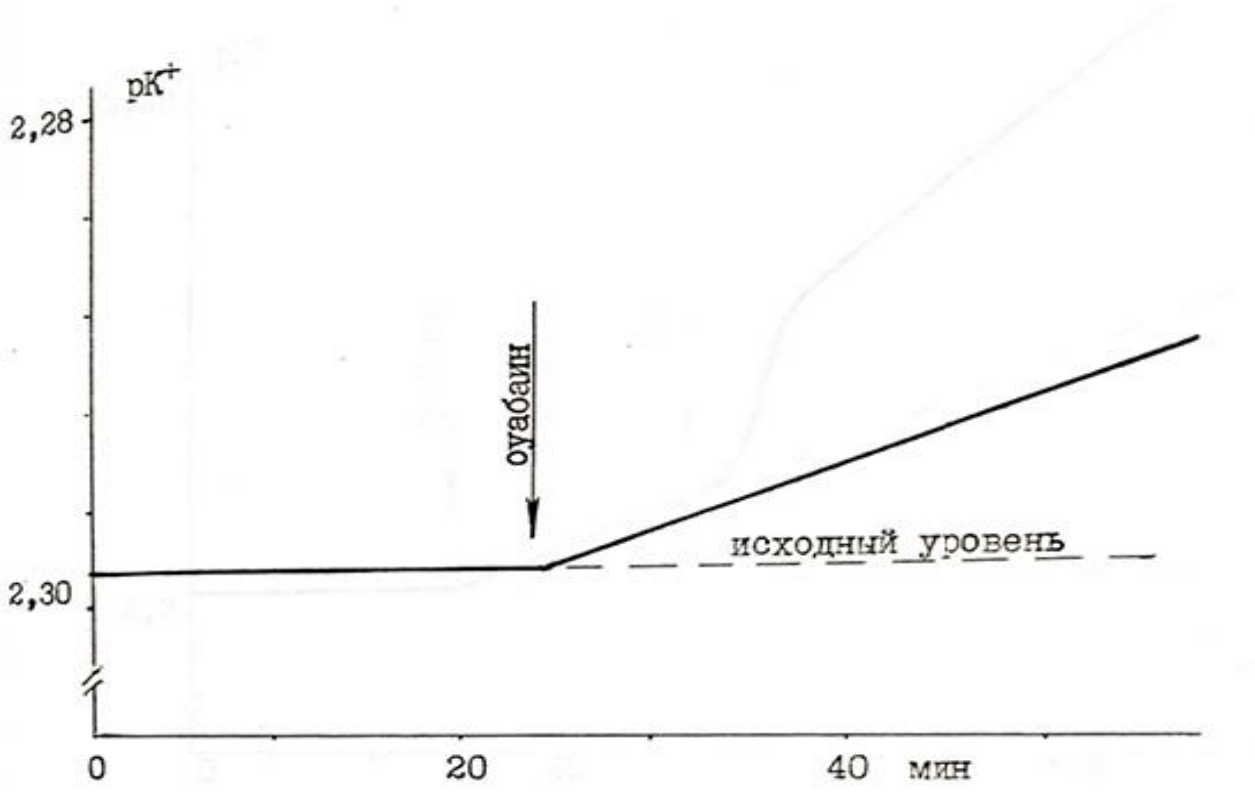
Passive flows could remain unchanged, but compensation of these flows by the active work of transport ATPhase s was blocked.

Taking this possibility into account, we studied the effect of PAA on the passive K+ flow under conditions of complete blocking of the (Na+, K+)-transporting

ATPhase . Indeed, when adding the (Na+, K+)-ATPhase inhibitor - ouabain (10-4 M), we completely “turn off” the active transport of the corresponding ions. As a result, we observe an apparent increase in the passive flow of K+ from the cytoplasm into the extracellular environment (Fig. 10).In the excess concentrations used, ouabain completely blocks (Na+, K+)-ATPhase ; an additional increase in the concentration of the inhibitor does not lead to an increase in the release of K+ from cells. Despite this, the introduction of PAA against the background of completely blocked (Na+, K+)-ATPhase into the lymphocyte suspension led to a noticeable increase in the passive flow of K+ into the extracellular medium (Fig. 11). Moreover, the effects of PAA on intact and ouabain-treated cells were comparable in magnitude.

Consequently, under the influence of the polyanion, a true rather than apparent increase in the ionic permeability of the cell membrane occurs. The effect of increasing passive fluxes is not associated with inhibition of active transport of the same ions by the corresponding membrane ATPhase s.

Despite the absence of signs of inhibition of ion transporting ATPhase s of the cell membrane by polyanion, we conducted special studies of the state of (Na+, K+) - and Ca2+ - ATPhase s using inhibitory analysis. The results of this study are described in the



next chapter.

Figure. 10. Change in the level of extracellular K+ with complete blocking of (Na+, K+) - ATPhase with ouabain (10-4 M). Along the axes: abscissa - time of cell incubation in vitro; ordinate - concentration of K+ (pK+ = - Ig [K+]) in the extracellular environment. The moment of “injection” of ouabain into the cell suspension is indicated by an arrow.

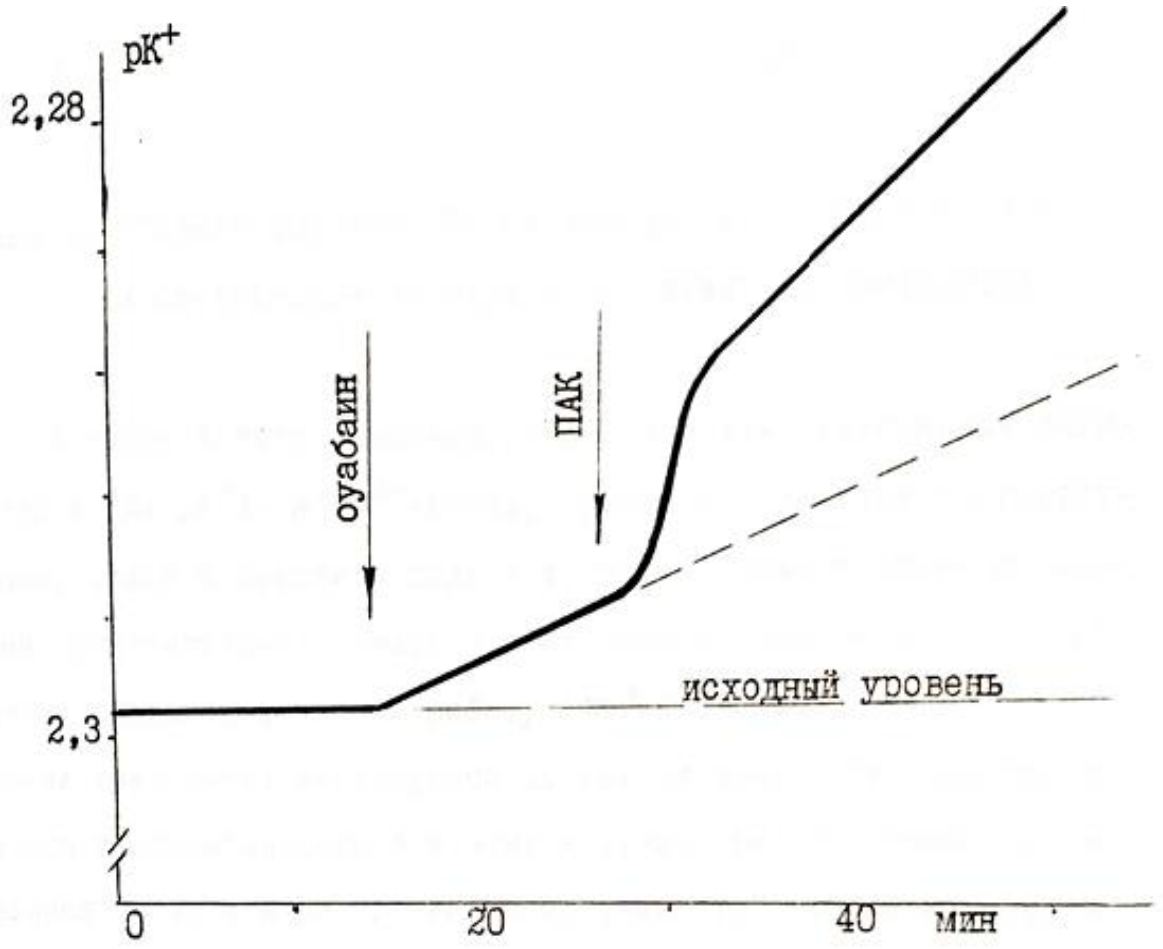

pK+
2,28
2,3
оуабаин
ПАК
исходный уровень
0
20
40
мин

Figure. 11. Effect of PAA polyanion on the permeability of the cell membrane under conditions of complete blocking of (Na+, K+)-ATPhase with ouabain (10-4 M). Along the axes: abscissa - time of incubation of lymphoid cells in vitro; ordinate - concentration of K+ (pK+ = - Ig [K+]) in the extracellular environment. The moments of "injection" of effectors into the cell suspension are indicated by arrows.

Chapter 4. INFLUENCE OF POLYANIONS ON THE FUNCTIONING OF (Na, K) - and Ca - ATPHASE S TRANSPORTING THE LYMPHOCYTE MEMBRANE.

In Chapter 3, it was noted that with the help of selective inhibitors of veno, (Na+, K+) - and Ca2+ - ATPhase s, ouabain and lanthanum, respectively, it is possible to determine the proportion of ATPh consumed by these enzymes relative to the total cellular ATPhase of intact lymphocytes. It turned out that the work of (Na+, K+)-ATPhase in non-activated lymphocytes consumes up to 18% of the total ATPh produced and consumed in these cells (Fig. 3).Such a significant cell-scale "energy intensity" of the sodium-potassium pump of the outer cell membrane apparently indicates the importance of this mechanism in cell viability. At the same time, this fact may be a consequence of the fact that in a non-dividing (resting) lymphocyte, all processes that require significant energy costs (for example, the biosynthesis of DNA, protein, RNA macromolecules) are reduced to a minimum. And therefore, the share of (Na+, K+)-ATPhase in the total consumption of ATPh in resting lymphocytes is quite large.The process of active transport of Ca2+ against its concentration gradient requires significantly less energy expenditure. The Ca2+ ATPhase inhibited by lanthanum in resting lymphocytes consumes so little ATPg that we were unable to measure it accurately. Considering the magnitude of the error in the method used, we can firmly assume that the work of Ca2+ - ATPhase in non-activated lymphocytes consumes no more than 4-5% of the total ATPh produced in these cells (Fig. 2).

The injection of a mitogenic polyanion - PAA - into a suspension of lymphocytes led to a significant activation of the total ATPh-consuming activity of

these cells. The rate of O2 consumption by cells, and therefore the rate of synthesis and consumption of ATPh, increased under the influence of the polyanion by an average of 1.3-1.5 times (Table 1). At the same time, inhibitory analysis showed that in such polyanion-activated cells there is a significant activation of the work of ion-transporting ATPhase s.

The inhibitory effect of lanthanum on lymphocyte respiration increased sharply. The rate of O2 consumption by lymphocytes pre-activated by PAA was reduced by 19-39% under the influence of lanthanum (Table 1, Figs. 12 and 13). Consequently, after exposure to the mitogenic polyanion PAA, a strong activation of Ca2+-transporting ATPhase occurred in the plasma membrane of lymphocytes. The speed of operation of (Na+, K+) - ATPhase after the addition of PAA also increased, but to a lesser extent than Ca2+ - ATPhase . Thus, inhibition of (Na+, K+)-ATPhase in lymphocytes activated by PAA with ouabain led to a decrease in the rate of O2 consumption by cells by 26-27%.

It was of interest to find out whether the entire activation of total cellular ATPhase upon the addition of PAA is ensured only by activation of (Na+, K+) - and Ca2+ -ATPhase s. To do this, we measured the inhibitory effect of simultaneously added lanthanum and ouabain in suspensions of non-activated lymphocytes or cells stimulated with PAA. It turned out that the rate of O2 consumption by cells decreases after simultaneous in vitro injection of both inhibitors to 78-80% of the initial level. In cell suspensions pre-activated with PAA.

Table 1.

The effect of polyacrylic acid on the rate of O2 consumption by lymphocytes

Dynamics of V - O2 during continuous recording*	**No. of series of experiments**				**Note**
	1	2	3	4	
VISH, mm/h	0,130	0,104	0,117	0,125	-
"Injection" PAK in vitro, mg/ml	50	50	200	200	-

VPAK, mM/h	0,166	0,135	0,176	0,183	-
(VPAK - VISKH)/VISKH, %	28	30	50	46	Indicators of total ATPhase activation under the influence of PAK
Vlant, mm/h	0,134	-	0,130	-	-
(VPAK – Vlant)/VISH, %	25	-	39	-	Indicators of Ca2+ dependent ATPhase activationпосле "injections" PAK
(VPAC – Vlant)/VPAK, %	19	-	26	-	
Vlant/VISH, %	-	0,107	-	0,150	
Vstr, mm/h	-	27	-	26	
(VPAK – Vstr)/VISH, %	-	21	-	18	Activation indicators (Na+, K+) of ATPhase after “injection” of PAA
(VPAK – Vstr)/VPAK, %	-	103	-	121	
Vstr/VPACK	-	0,107	-		

* V - O2 – rate of oxygen consumption (mM/h); VISH = V-O2 of intact cells before exposure; VPAK = V-O2 after “injection” of PAA; Vlant = V-O2 after adding lanthanum (10-3 M) to PAA-activated cells; Vstr = V-O2 after adding strophanthin (10-4 M) to cells activated by PAA.

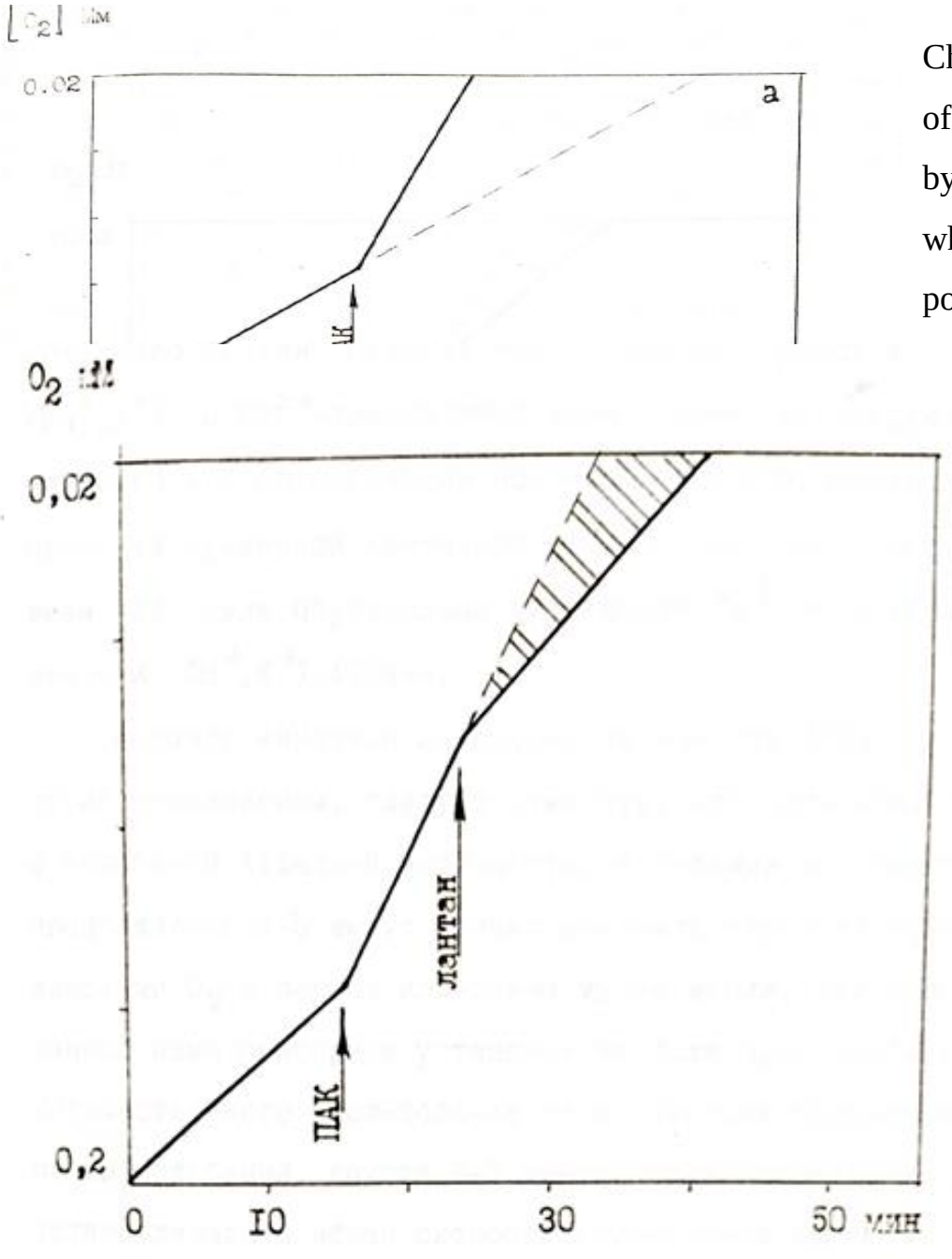


Figure. 12. Change in the rate of O2 consumption by lymphocytes when exposed to: (a) polyacrylic acid (10-6 M) and or (b) successively polyacrylic acid (10-6 M) and then an inhibitor (Na +, K +) - ATPhase , ouabain. Along the axes: abscissa – time of in vitro incubation of cells, ordinate – concentration (mM) of O2 in the incubation medium. The arrows indicate the moments of “injection” of effectors into the test suspension of lymphocytes.

Figure. 13. Change in the rate of O2 consumption by lymphocytes upon sequential exposure to polyacrylic acid (PAA, 10-6 M) and then to the Ca - ATPhase inhibitor, lanthanum (10-3 M). The abscissa axis is the time of in vitro cell incubation, the ordinate axis is the concentration (mM) of O2 in the incubation medium. Arrows indicate the moments of "injection" of effectors into the lymphocyte suspension.

Total cellular ATPhase reached 148% of the initial level. Consequently, after treatment with PAA, in addition to (Na+, K+) - and Ca2+ -transporting ATPhase s, some other ATP-consuming processes are activated. However, the overwhelming majority of the increase in total cellular ATPhase occurring under the influence of PAA was due to activation of Ca2+ ATPhase and, to a lesser extent, (Na+, K+) ATPhase .

Regarding the kinetics of changes in ATPhase activity after exposure to a polyanion, it should be noted that activation began with fluctuations in ATPhase

activity. Fluctuations in ATPhase activity lasted 3-5 minutes. We could not accurately measure the rate of O2 consumption by cells during the period of oscillations, since the instrumentation we used was not suitable for a quantitative study of such rapid processes. At the end of the oscillations, 3-5 minutes after exposure to the polyanion, a new rate of O2 consumption by cells was established, which exceeded the initial one.The magnitude of the excess, that is, the magnitude of the effect of activation of ATPhase s by the polyion, directly depended on the dose of the polymer. When using immunostimulating doses of PAA, as a rule, we did not observe activation of ATPhase s more than 150% of the initial level. A further increase in the polymer concentration did not lead to an additional enhancement of the effect, but could lead to a sharp decrease in it. The reasons for this oppression are not known. It could be a consequence of the toxic effect of the polyanion on cells. In addition, at high concentrations of polyacid, acidification of the environment becomes significant, which may be associated with many unknown effects.

In general, it is clear that nontoxic immunostimulating doses of PAA markedly and rapidly activate the functioning of membrane ion transporting ATPhase s. Another immunostimulating polyanion, dextran sulfate, had essentially the same effect [1]. We assumed [4] that the observed stimulation of active transport of K+, Pa+, Ca2+ ions is a secondary phenomenon, occurring as a result of an increase in passive flows of the same ions.

Apparently, the polyanion, interacting with the cell membrane, induces a rapid increase in its permeability to ions. Changes in transmembrane ion gradients, in turn, lead to rapid activation of the corresponding ATPhase s, which transport the same ions across the membrane in the direction opposite to the passive flow. That is, the activation of ATPhase s is aimed at compensating for increased ion flows and restoring the original ratio of ions between the cell and the environment.

Chapter 5. INCREASE VALUE ANALYSIS CELL MEMBRANE PERMEABILITY TO ACTIVATE LYMPHOCYTE RESPONSE TO POLYANION.

The polyanion induces a rapid increase in the permeability of the cell membrane to ions and a compensatory reaction of ion transporting ATPhase s. What is the significance of this, key (for triggering the response of the lymphoid cell) or concomitant, unrelated to the process of activation of lymphocytes? To analyze this issue in the experiment, we used two approaches. First, we investigated the effect of polyanion on the membrane permeability of subpopulations of lymphocytes that do and do not respond to polyanion proliferation in vitro. Secondly, we studied the immunomodulatory properties of substances of different chemical structures that are not polymers, but are capable of increasing the permeability of the cell membrane to ions.

I. Effect of polyanion on ion transport in the membrane T- and B-lymphoitis.

It is known that polyanions (PAA, dextran sulfate) exhibit the properties of B-cell mitogens and do not activate the response of T lymphocytes [44]. Therefore, it was of interest to study the membrane-active effect of the polyanion on T and B lymphocytes separately.

As described in the Materials and Methods chapter, splenic lymphocytes were separated according to their ability to adhere to nylon wool. At the same time, the fraction adhering to nylon was enriched in B cells and contained a small admixture of T lymphocytes. Most of the T lymphocytes were contained in the fraction of cells that did not adhere to nylon. This fraction of cells that did not adhere to nylon was poor in B lymphocytes. Conventionally, we will call non-adherent and adherent cells “T - fraction” and “B - fraction”, respectively.

When exposed to polyanion (PAA, 50 μg/ml) in vitro, activation of DNA synthesis was induced only in the B-fraction, but not in the T-cell fraction (Fig. 14). This was quite consistent with previously published data [44].

A study of the action of PAA at the level of membrane ion transport also showed the selectivity of the polyanion in relation to B lymphocytes. Thus, activation of membrane ion-transporting ATPhase s, as well as an increase in passive fluxes of K+ and Ca2+, was observed in our experiments only in a suspension of B cells (Fig. 15 a, b, c). Mature T-lymphocytes from the spleen did not respond to the addition of polyanion by activating membrane ion transport systems. In connection with the data obtained, the following working hypothesis was put forward [90].It was based on numerous information about the differences between T - and B - lymphocytes in surface electrical charge. It is well known that mature mouse T lymphocytes have a high density of negative surface charge. This is expressed in the high mobility of T cells in an electric field, in the absence of adhesion of T cells to a similarly charged substrate - plastic, nylon, etc.The working hypothesis that guided our work suggested that the lack of effect of PAA on mature T cells of the spleen is determined by the high density of negatively charged groups on the surface of these cells. It followed from the hypothesis that PAA should act on T cells with a less negative surface charge, in particular, on immature T cells from the mouse thymus and on mature T cells pretreated with neurons.

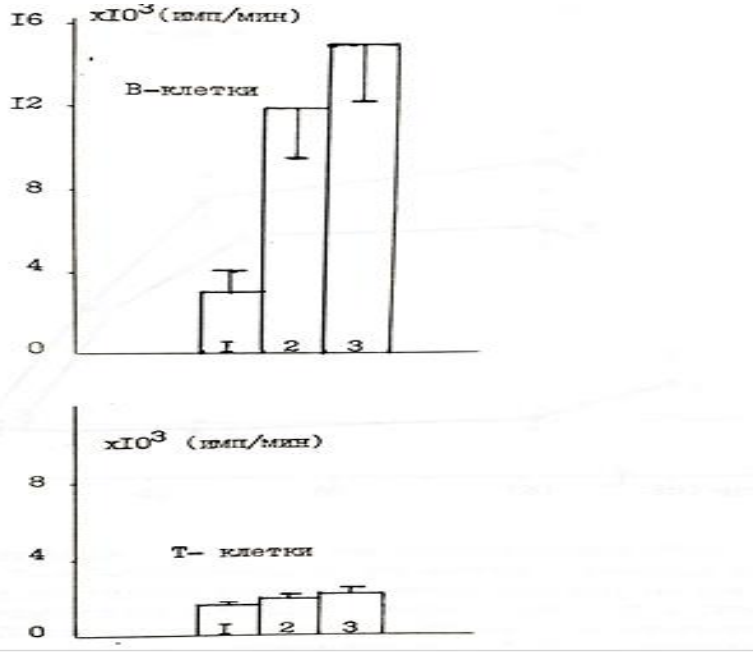


Figure. 14. Mitogenic effect of polyanions on enriched subpopulations of T- and B-lymphocytes in in vitro cell culture.

1 - intact non-activated cells; 2 - cells activated with polyacrylic acid; 3 - cells activated with dextran sulfate. The ordinate axes shows the intensity of 3H-thymidine incorporation by cells (imp/min).

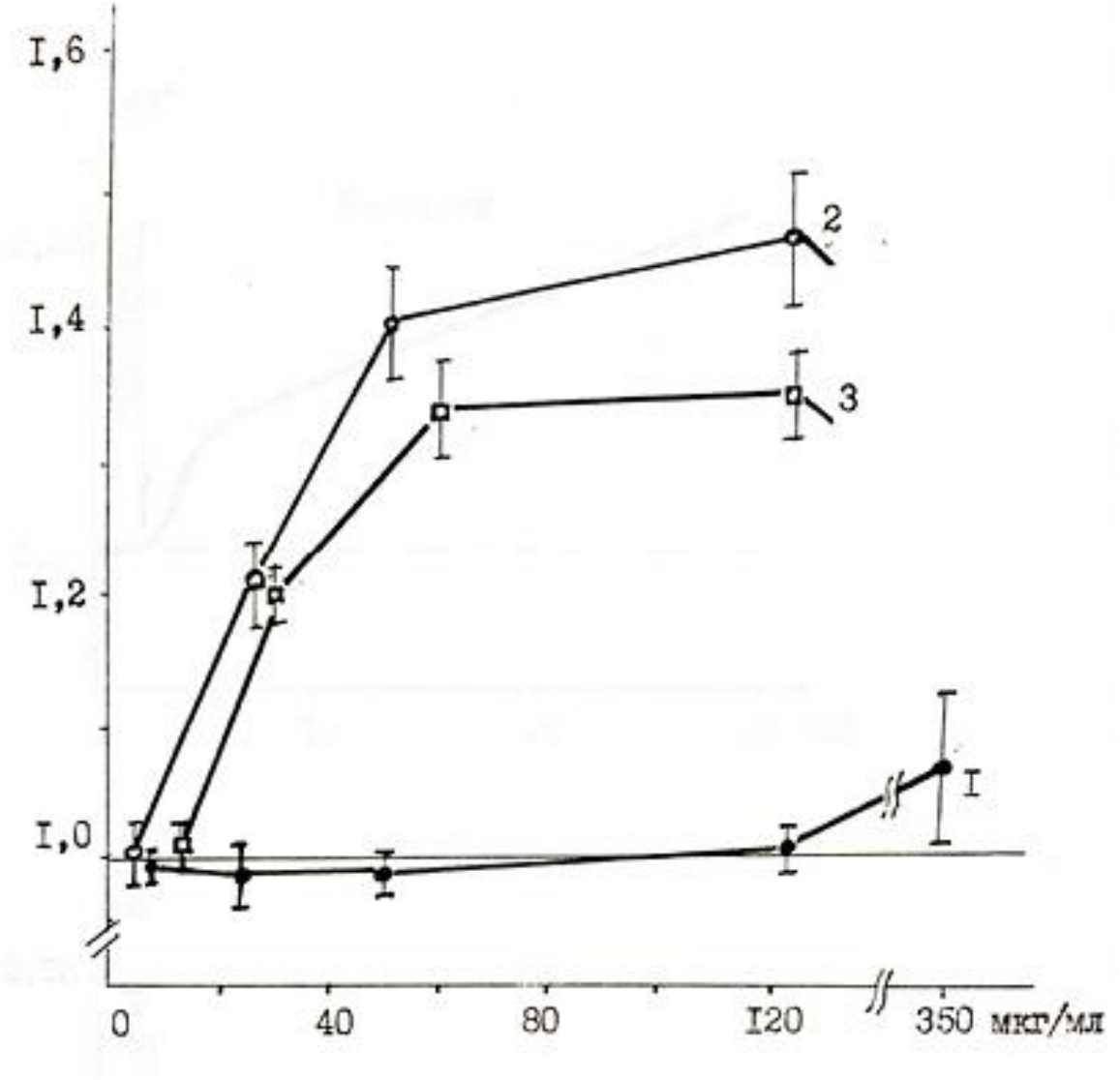


Figure. 15 Activation by polyanion of ion-transporting ATPhase s in the membrane of T - and B - lymphocytes. The x axis is the final concentration of polyacrylic acid in vitro (μg/ml). On the ordinate axis is the activation coefficient of the total cellular ATPhase (the level of ATPh, the consumer activity of cells immediately before their activation by polyion, is taken as 1.0). The data obtained from the activation of a fraction of T-cells of the spleen (1), a fraction of B-cells of the spleen (2), and such T-cells of the spleen pre-treated with neuraminidase (3) are presented.

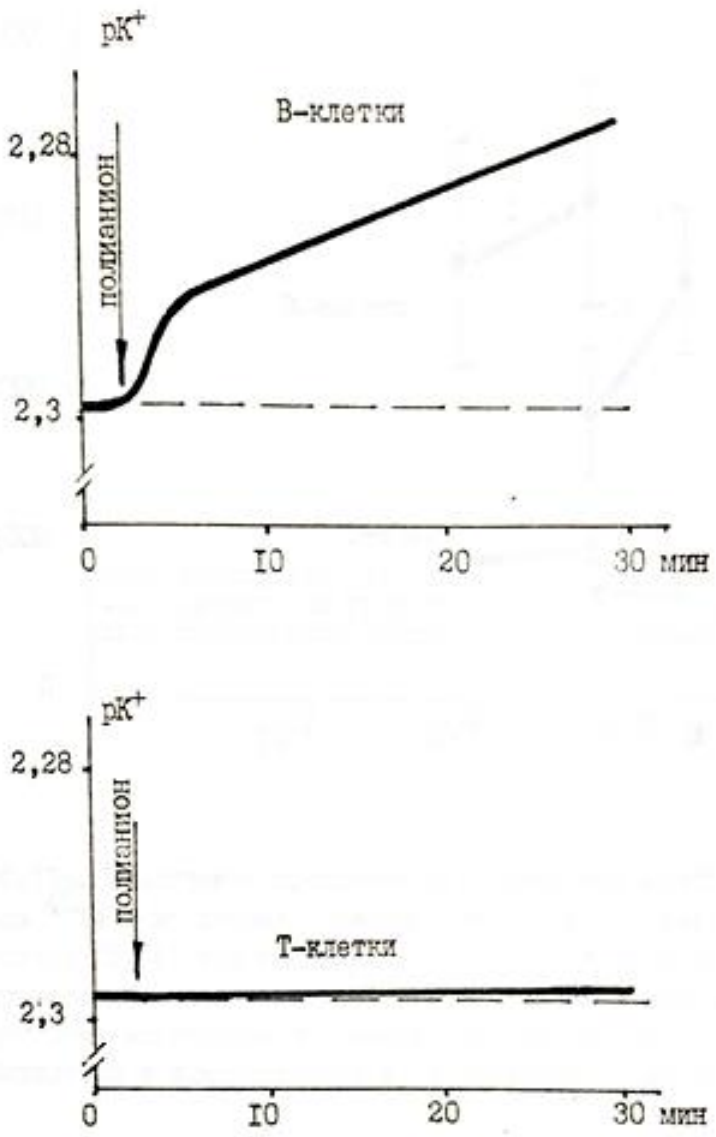


Figure. 15 Activation by polyanion of ion-transporting ATPhase s in the membrane of T - and B - lymphocytes. The x axis is the final concentration of polyacrylic acid in vitro (μg/ml). On the ordinate axis is the activation coefficient of the total cellular ATPhase (the level of ATPh, the consumer activity of cells immediately before their activation by polyion, is taken as 1.0). The data obtained from the activation of a fraction of T-cells of the spleen (1), a fraction of B-cells of the spleen (2), and such T-cells of the spleen pre-treated with neuraminidase (3) are presented.

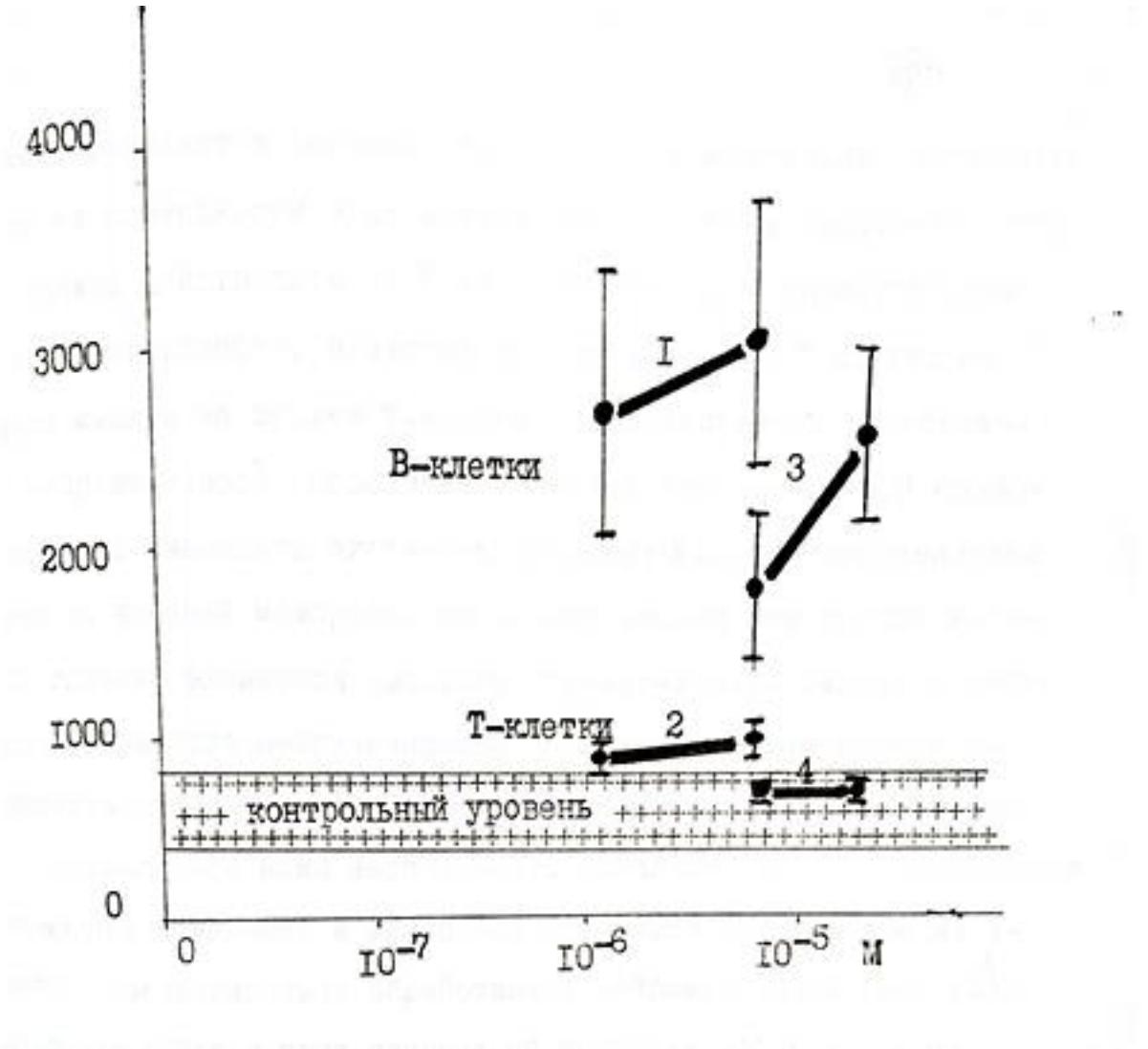


Figure. 15 c. Increased permeability of the cell membrane to the 45Ca isotope in cultures of B lymphocytes (1, 3) in contrast to T lymphocytes (2, 4) under the influence of polyacrylic acid (1, 2) or dextran sulfate (3, 4). The ordinate is the radioactivity (cpm) of cell extracts. The abscissa axis is the polyanion concentration in the incubation medium during cell activation in vitro.Treatment of T cells with neuraminidase can dramatically reduce the density of terminal N - acetylneuramine groups on the outer membrane. Since these groups are the main element of the high negative charge of the surface, treatment with neuraminidase can greatly reduce the density of the negative charge on the surface of T lymphocytes.

Our experiments showed that PAA induces pronounced changes in the ion transport system of mature T cells pre-treated with neuraminidase (Fig. 15). Activation of ATPhase s in these cells was not inferior to the effect of PAA on B lymphocytes and reached 135% of the initial level. Modification of ion transport in the thymocyte

membrane also occurred effectively. That is, the effect of the polyanion is not limited only to the pool of B cells.It is quite possible that it has an activating effect on T lymphocytes with a slight negative surface charge. Only mature T cells, which have a high density of negatively charged groups on the outer membrane, are not sensitive to the activating effect of PAA.

From the point of view of the initial task of this experimental study, it is important that the membranotropic effect of the polyanion took place only on those cells (B - lymphocytes) that responded by activating divisions. In T cells of the spleen that did not divide in response to the addition of the polyanion, we did not observe early membrane effects.

2. Immunostimulating effect of membranotropic substances of non-polymeric nature.

Let us assume that a change in membrane permeability is a key process that activates the cell's response to the polyanion and, therefore, determines the immunostimulating properties of the polyanion. Based on this assumption, it can be assumed that even in the absence of a polymer, it is possible to activate the reaction of lymphocytes and the immune response with the help of nonpolymeric substances that increase the permeability of the cell membrane.

Based on this assumption, we studied the immunomodulatory properties of several membrane-active effectors - levorin, gramicidin S, nystatin. The substances used had different chemical structures, but were similar in their affinity for cell membranes and in their ability to increase the permeability of phospholipid membranes to ions [10, 35]. We tested the mitogenic effect of these substances in cultures of lymphoid cells in vitro and the immunostimulating effect upon induction of antibody synthesis in vitro.

Mitogenic action.

The addition of nystatin or levorin to a suspension culture of mouse cells led to an increase in the intensity of DNA synthesis (Fig. 16). The incorporation of 3H-thymidine in the period between 48-72 hours after activation of the cultures increased compared to the control level by 3-7 times. Such activation coefficients of DNA

synthesis are significantly inferior to the activation coefficients when using concanavalin A or lipopolysaccharide from intestinal bacteria. In the case of using the indicated “traditional” mitogens, the coefficients for stimulating the inclusion of 3H-thymidine

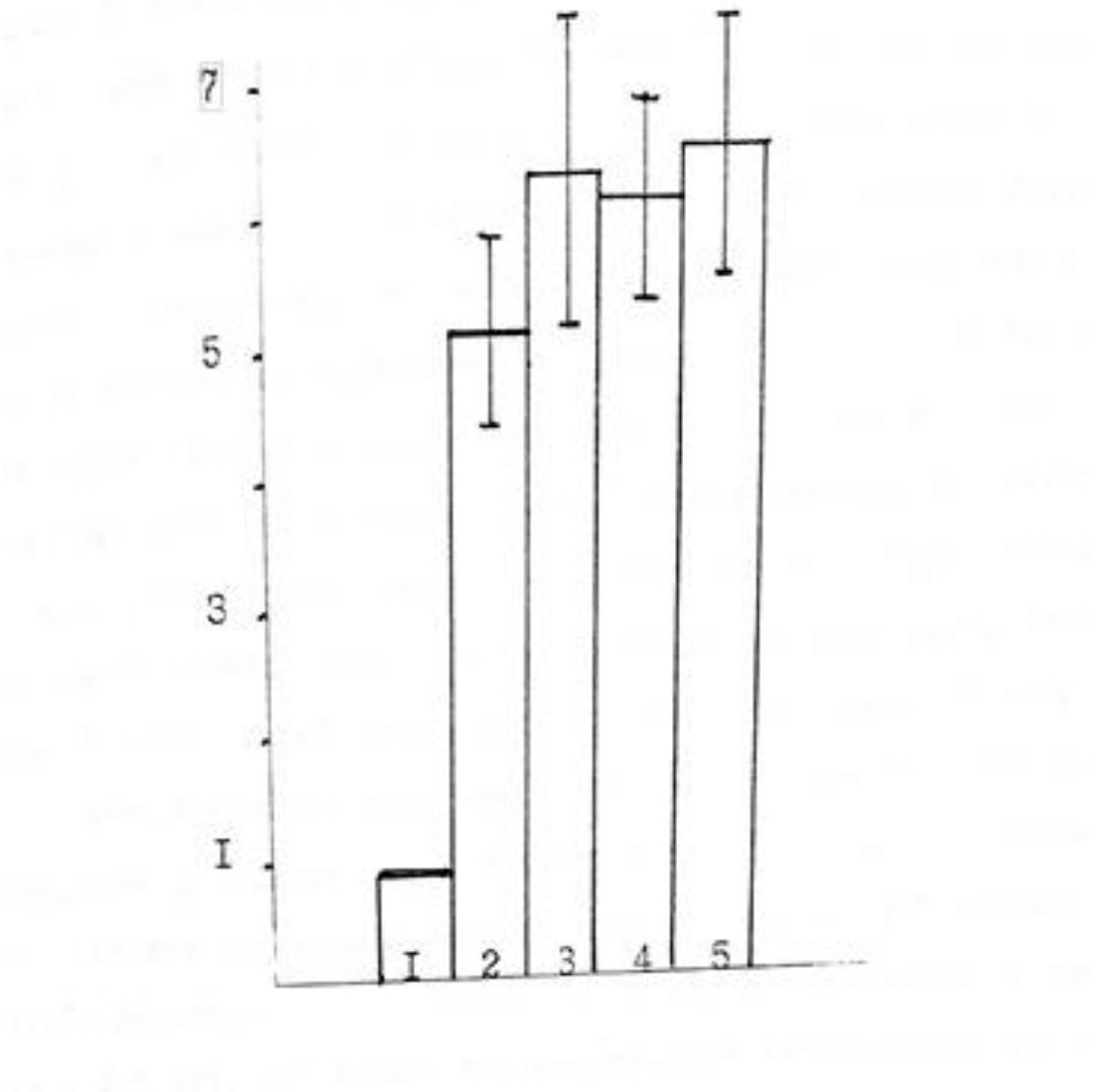


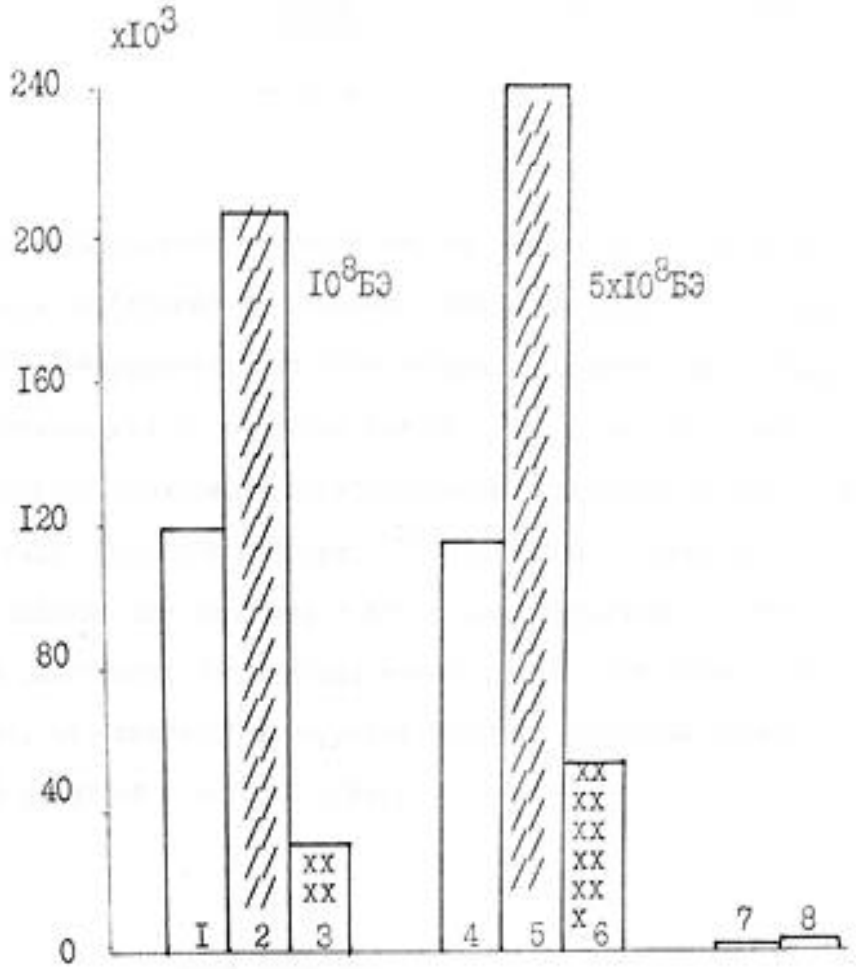
Figure. 16. Mitogenic effect of membrane-active substances, in vitro. On the ordinate - the coefficient of stimulation of the inclusion of 3H - thymidine in the DNA of cells; The value of the intensity of 3H-thymidine incorporation in control cultures (without mitogen) is taken as 1.0. The values of stimulation coefficients obtained in cultures of lymphoid cells from the spleen of mice in the presence of gramicidin (2), nystatin (3),

levorin (4) or ionophore A23187 (5) are presented in comparison with the control without mitogen (1).

reached 20-30. At the same time, it should be noted that when using polyions, a relatively weaker stimulation of DNA synthesis was previously noted. It was found that polyions induce the first stage of the mitogenic response of cells, exit from the Go- to G1-phase of the cell cycle. The G1 - S transition is weakly induced by polyions. In this sense, the mitogenic effect of membrane-active substances - levorin, gramicidin S and nystatin - is similar to the action of polyions. In addition, Ca2+ ionophore A23187 had a similar effect in our experiments. The effect of A23187 was to activate the inclusion of 3H-thymidine by 4-5 times, this was quite consistent with the literature data [152, 74, 75].

Stimulation of primary antibody synthesis in vitro.

In the model of antibody synthesis in mice in response to antigens of heterologous erythrocytes (in particular, sheep erythrocytes, EB), it is very convenient to test immunostimulants. After administration of gramicidin S in doses of 10 or 100 μg (per mouse) together with 108 EB, we found a significant change in the level of EB-specific antibody-forming cells (AFC) in the spleens (Fig. 17). A dose of 10 mcg stimulated the production of AOX, and a dose of 100 mcg significantly inhibited it. From the first experiments it was necessary to study the detailed dose-effect relationship.As a result, it was found that the administration of gramicidin together with EB in the dose range from 1 to 30 μg leads to a significant stimulation of antibody formation. Dosages of more than 50 mcg inhibit the production of AFC (Fig. 18). A similar dose dependence was found for levorin and nystatin. The differences were only in the ranges of optimal concentrations.Thus, it was found that the immunostimulating effect of levorin is manifested in doses from 0.01 to 0.2 mcg.


xI0^3
240
200
I60
I20
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40
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I
2
3
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5
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7
8
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5xI0^8БЭ

Figure. 17. Immunomodulatory effect of gramicidin S on the reaction of antibody formation against antigens of heterologous erythrocytes (BE, sheep erythrocytes). On the ordinate: accumulation in the spleens of mice of antibody-forming cells specific to BE antigens (4th day after immunization). Mice were immunized intraperitoneally with 108 BE (1, 2, 3) or 5x108 BE (4, 5, 6). Together with the antigen, mice were injected with 10 μg (2, 5) or 100 μg (3, 6) of gramicidin S. In groups 7 and 8, mice were injected with 10 or 100 μg of gramicidin without antigen, respectively.

Antigen dose.

The magnitude of the effect of stimulating antibody synthesis strongly depended on the dose of immunogen used. The greatest stimulation effects were observed when threshold doses of the antigen were used. Thus, with intraperitoneal injection of 106 BE mice (CBA x C57B1) F1 produced practically no specific AFCs (Fig. 19). A dose

of 107 BE induced the formation of 5-10 thousand specific AFCs in the spleen. And a dose of 108 BE is about 50 - 100 thousand AFC. After co-administration of gramicidin with BE, the coefficients of stimulation of the threshold response reached 20 - 50. For example, the production of AFC reached 10 thousand against 400-500 in the control. The average intensity response increased on average 7 - 8 times, from 5 - 10 thousand AFC to 35 - 80 thousand AFC. The reaction, close in level to the maximum, increased only 1.5 - 2 times (Fig. 20).

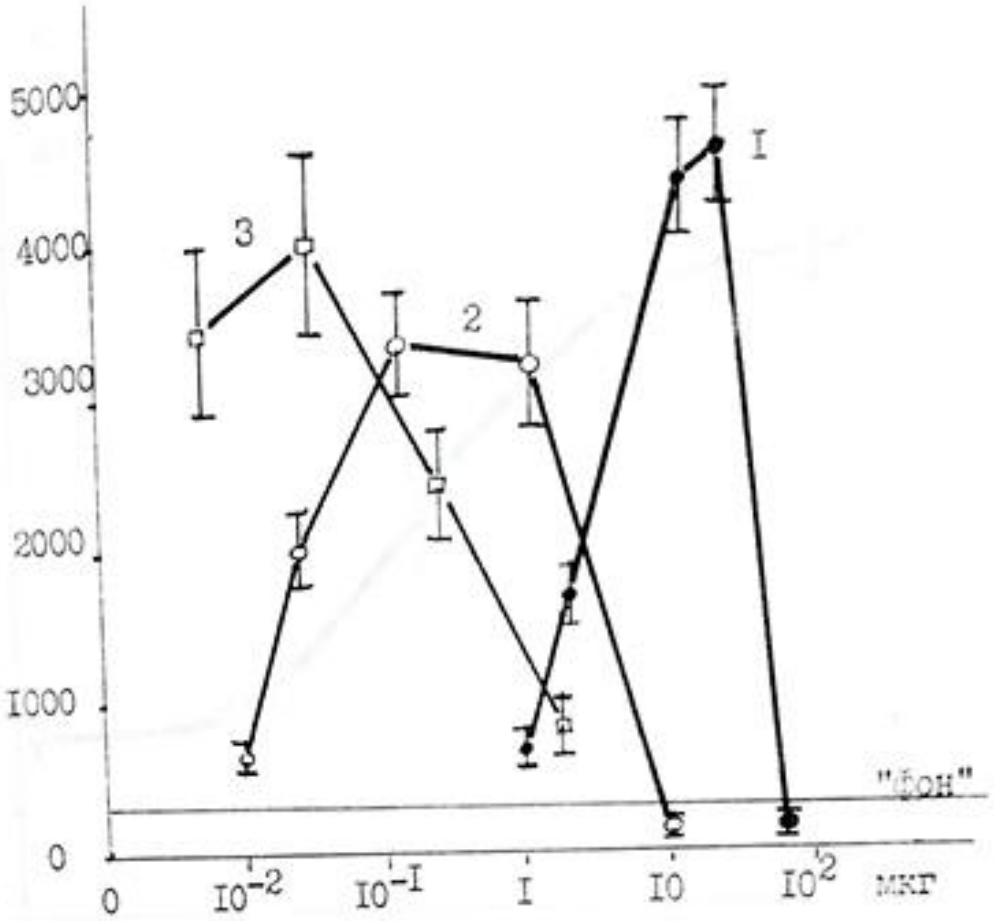


Figure. 18. Immunoadjuvant effect of substances that increase the permeability of the cell membrane. Heterologous (sheep) erythrocytes were used as an immunogen for immunization of mice in a suboptimal dose of 2 x 106. Gramicidin S (1), levorin (2) or gramicidin A (3) were used as an adjuvant. Along the axes: abscissa - dose of membrane-active effector administered together with the antigen (2 x 106 BE). The ordinate axis shows the content of antibody-forming cells in the spleen that are specific to BE antigens. “Background” is the level of immune response in the control to an injection of 2 x 106 BE without an adjuvant.

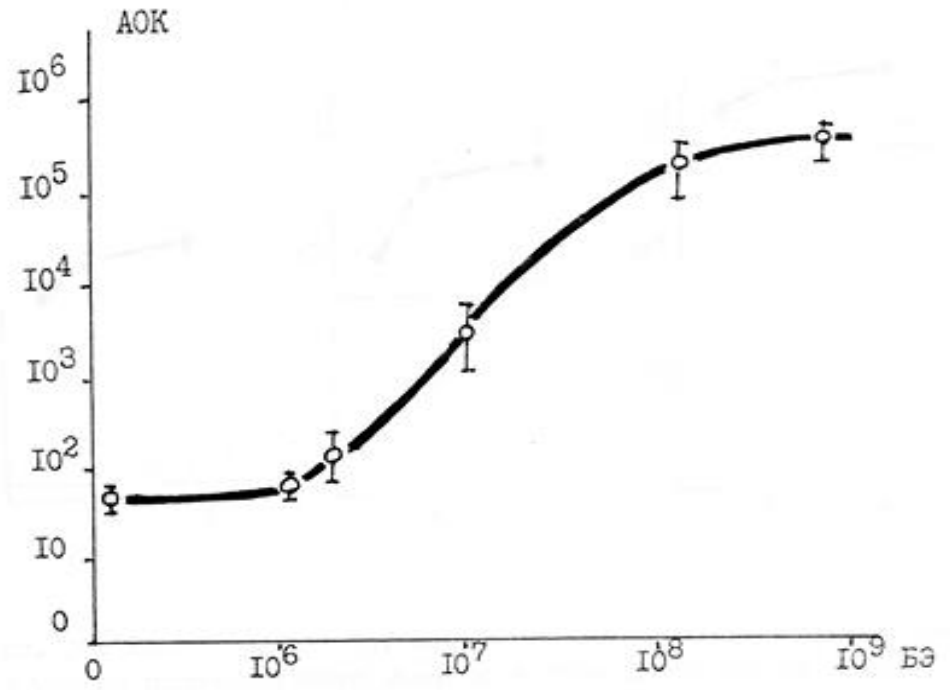
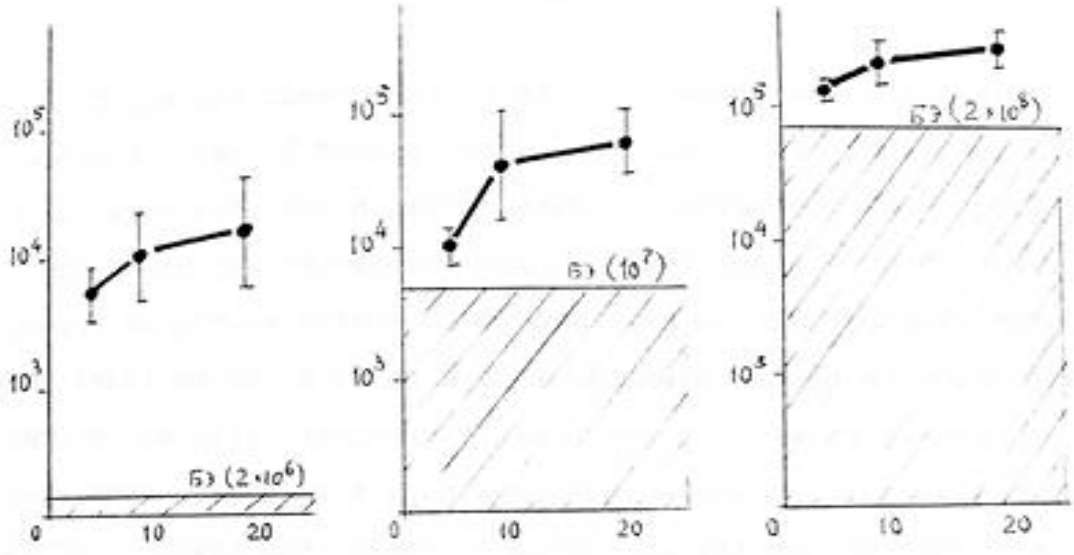


Figure. 19. Dependence of the level of antibody formation on the dose of antigen (AD) administered to mice. X-axis: antigen dose (number of BE cells per mouse). On



the ordinate: the content of antibody-forming cells (AFC) specific to BE antigens in the spleen of mice, 4 days after antigen administration.

Figure. 20. Dependence of the immunoadjuvant action of gramicidin S on the level of the stimulated immune response. Along the axes: ordinate - the number of antibody-forming cells specific to sheep erythrocyte antigens in the spleens of mice 4 days after immunization; abscissa - dose of gramicidin S (μg/mouse) administered together with the antigen when immunizing mice with one of the indicated doses of antigen (2 x 106 BE, or 107 BE, or 2 x 108 BE). The level of response in control animals immunized with the corresponding dose of BE without gramicidin is indicated by shaded bars.

Nature of the antigen

With the help of gramicidin S or levorin, it is possible to enhance the immune response not only to BE, but also to other antigens, both corpuscular and soluble. In particular, sheep erythrocytes, killed microbial bodies of S. typhimurium, water-soluble antigen (WSA) from BE, as well as water-soluble 0 - antigen from S. typhimurium were compared under identical experimental conditions (Fig. 21). The introduction of gramicidin with one of these antigens led to increased specific antibody synthesis. The level of stimulation of the response to microbial cells was close to the level of enhancement of the response to foreign erythrocyte cells (stimulation coefficient about 20). Noteworthy are the lower coefficients of enhancement of antibody synthesis during immunization with water-soluble antigens from the same foreign cells (BE or S. typhimurium). In the case of introducing a membranotropic agent together with a water-soluble antigen, the enhancement factors for antibody production did not exceed 3 - 5 (Fig. 21).The administration of immunoadjuvant doses of gramicidin S together with the p90 protein antigen (from the anthrax pathogen also led to more intense antibody formation compared to the response to the protein alone. Thus, the level (IEA - titer) of specific antibodies to p90 in mice immunized with a 3 kg mixture p90 and 10 μg of gramicidin S, reached 1: 30,720 by day 12 compared to 1: 1920 in mice immunized with only 3 μg of p90 without an adjuvant.

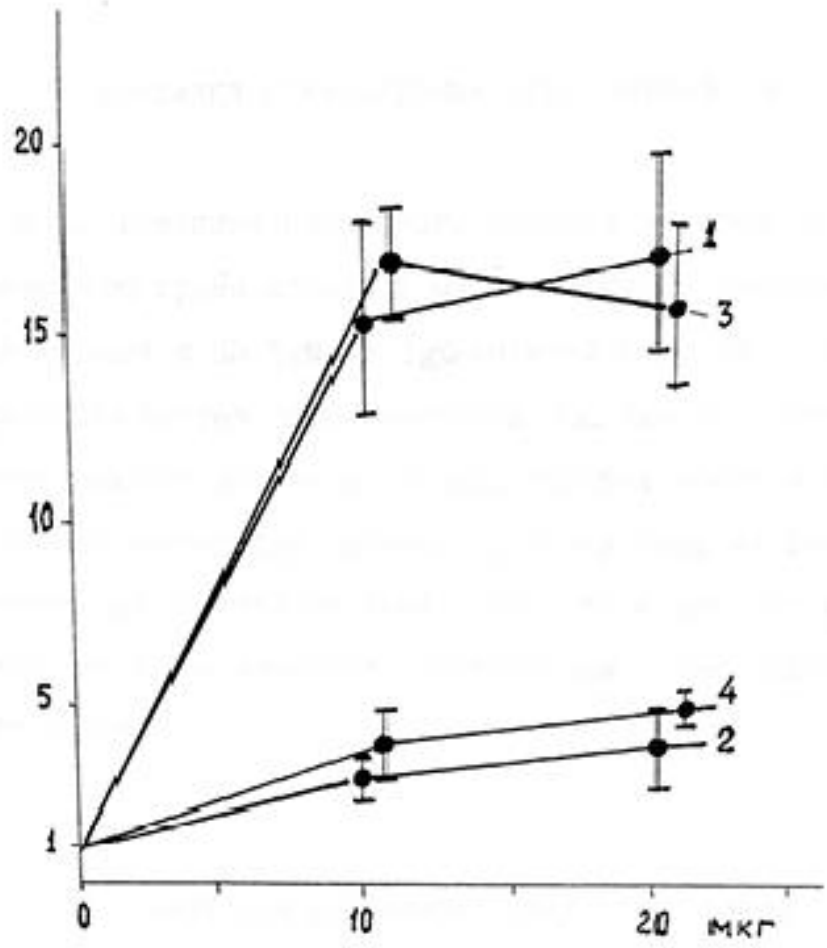


Figure. 21. Immunoadjuvant effect of gramicidin S upon induction of antibody synthesis using corpuscular (1, 3) or water-soluble (2, 4) antigens. On the y-axis: coefficient of stimulation of antigenogenesis (the level of antigenogenesis in the corresponding control during immunization with antigen only is taken as 1.0); The x-axis is the dose of gramicidin S administered together with the antigen. Immunization: 1 – 2 x 106 BE; 2 - 1 mg of water-soluble antigen from BE isolated using the Simon method; 3 - 100 μg of microbial mass of killed salmonella (S. typhimurium); 4 - 1 μg 0 - antigen from the same salmonella.

Dynamics of stimulated antibody synthesis

After inducing the primary synthesis of antibodies against BE and enhancing it with gramicidin S, we studied the dynamics of the synthesis of IgM and IgG antibodies specific to BE (Fig. 22). Stimulation affected the synthesis of both Ig isotypes. At the same time, the dynamics of the synthesis of each of the isotypes was similar to the

control. If in the control the synthesis of IgM antibodies reached a peak on the 5th day, and the synthesis of IgG antibodies on the 7th day, then when stimulated with gramicidin, the maximum values of IgM and IgG were observed at the same time.

Stimulation of the secondary immune response.

Essential for practice is the possibility of using immunostimulants with repeated injections of the antigen. We investigated the possibility of enhancing the secondary immune response to BE using gramicidin S and levorin. It turned out that to enhance the synthesis of antibodies, these membrane-active agents can be administered either during primary immunization, or during repeated immunization, or twice during the primary and repeated administration of the immunogen (Table 2). The highest level of synthesis of secondary IgG antibodies was observed when a membranotropic substance was administered at the time of the first immunization or twice, during the first and second immunization.Moreover, we did not observe the summation of stimulation effects with a two-time administration of a membrane-active agent compared to the response levels when gramicidin was administered either only during the first or only during the second immunization.

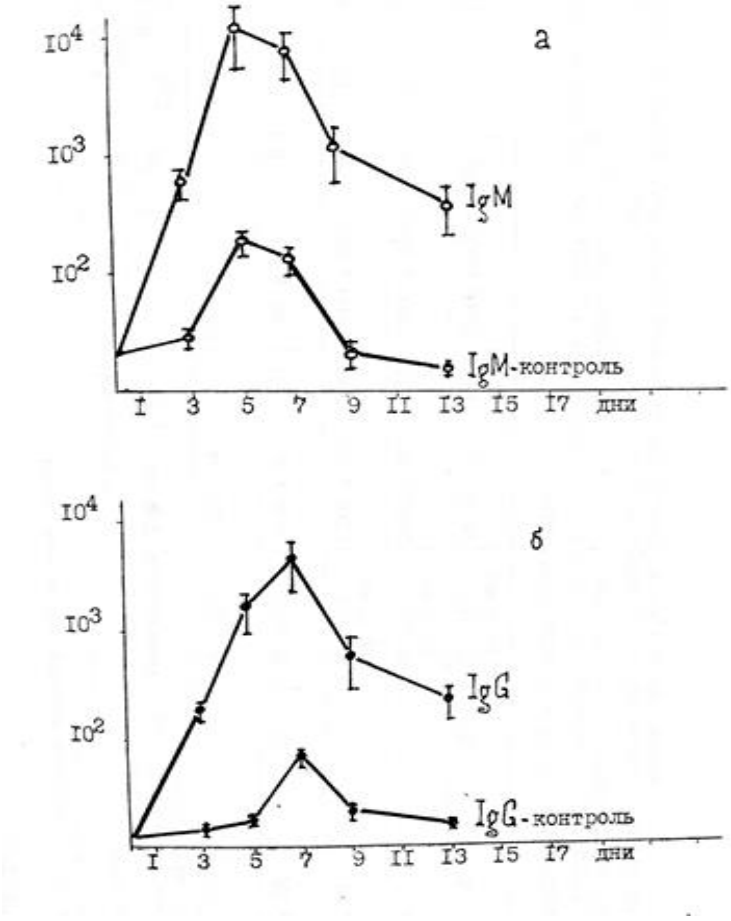


Figure. 22. Dynamics of accumulation of IgM - (a) and IgG - (b) antibody-secreting cells in the spleen of mice immunized with 2 x 106 BE (control) or a mixture of 2 x 106 BE plus 10 μg of gramicidin S. Along the axes: ordinate - number of antibody producers per spleen; abscissa – days after immunization of mice.

Effect of gramicidin S on the formation of specific immune memory a nd induction of a secondary immune response.

Primary immune response			**Secondary immune response**					**Immune memory coefficient**	
Immunization	Number of antibody producers in the spleen on the 4th day	Kst	Reimmunization	IgM - AFC	Kst	IgG - AFC	Kst	IgM	IgG
2 x 10^6	100 ± 37	-	BE 2 x 10^6	120 ± 44	-	128 ± 51	-	1,2	1,3
BE 2 x 10^6 Grc 10 mcg	2300 ± 396	23	BE 2 x 10^6	2046 ± 407	17	992 ± 113	8	1	1
BE 2 x 10^6	150 ± 29	-	BE 2 x 10^6 Grc 10 mcg	17860 ± 3329	160	12444 ± 287	96	110	100
BE 2 x 10^6	136 ± 49	-	BE 2 x 10^6 Grc 20 mcg	21050 ± 589	175	47990 ± 10200	390	160	375
BE 2 x 10^6 Grc 10 mcg	2005 ± 345	20	BE 2 x 10^6 Grc 20 mcg	15780 ±	130	35600 ±	273	7,8	17,5

				3100		6400			

Notes: Grts – gramicidin S; BE – sheep erythrocytes.

Kst is the stimulation coefficient, how many times did the immune response increase as a result of the administration of gramicidin S together with BE in relation to the corresponding control when immunized with BE only.

Immune memory coefficient - the ratio of the level of the secondary to the level of the primary response in the same group of mice (the number of IgM - and IgG - AFCs during the primary response were approximately equal)

It is interesting to note the following. When using very low threshold doses of the immunogen (106 EB twice with an interval of 25 days), the primary and secondary reactions in the control were extremely small. Moreover, the secondary reaction did not exceed the primary one (only 100 - 200 AFC per spleen). The use of the optimal immunostimulating dose of gramicidin S only during the first administration of the antigen led to the development of a very strong secondary response to the threshold dose of the antigen. About 50,000 IgG-AFCs were produced in response to the second injection of antigen.

We observed a significant stimulation of the production of IgG antibodies during a secondary immune response to the p90 protein antigen (Figure 23). Administration of 10 μg of gramicidin S together with 3 μg or 30 μg of p90 antigen induced a 3-10 times more intense antibody response than with the introduction of antigen alone.

In this case, it is also noteworthy that the dynamics of antibody formation enhanced by gramicidin S are not fundamentally different from the dynamics of antibody accumulation in the control group immunized with antigen without gramicidin S. While the dynamics are maintained, the maximum reaction increases significantly (Fig. 23).

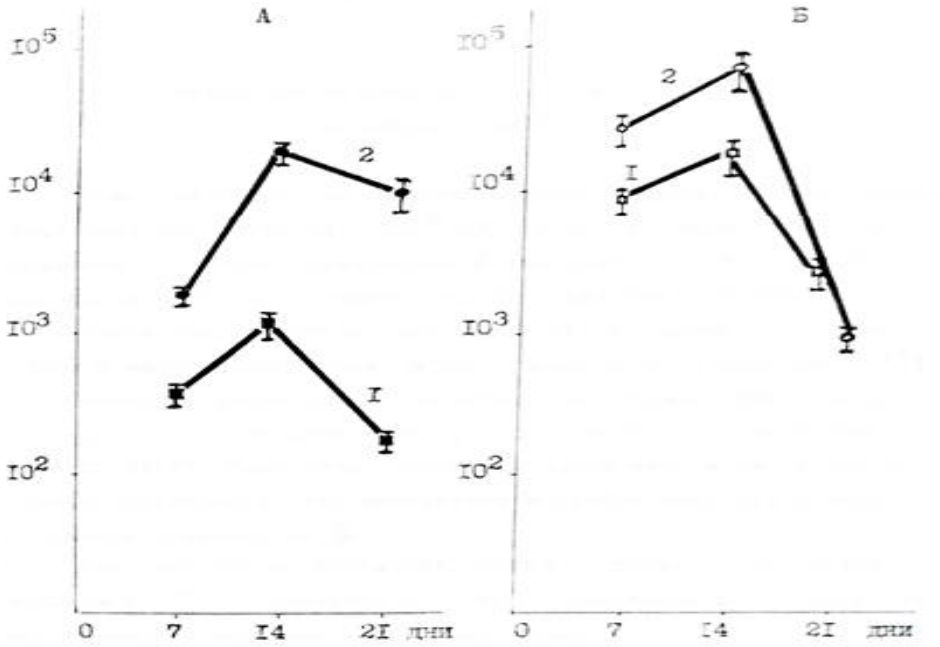


Fig.23.

Stimulation of antibody formation to the p90 protein antigen from the anthrax pathogen using gramicidin S. Along the axes: ordinate - level (ELISA - titer) of antibodies to p90 in the serum of immunized mice; abscissa - duration after repeated immunization (1) with p90 protein or (2) with a mixture of p90 + gramicidin S. For immunization, doses of p90 were used: 3 μg/mouse - (A) or 30 μg/mouse (B). The dose of gramicidin in both cases is 10 μg/mouse.

Stimulation of antibody genesis in mice and different inbred lines.

In a model of antibody production in response to immunization with suboptimal doses of antigen (2 x 106 BE), we investigated the possibility of enhancing the immune response in mice of high- and low-reactive genotypes (to this antigen) using gramicidin S.

It was previously known that CBA mice (H-2k haplotype) are highly responsive to EB, and C57B1 mice (H-2b haplotype) are low responsive to EB. It was also known that genes with a high level of response dominate in the first generation hybrids (CBA x C57B1) F1. It was necessary to understand to what extent it was possible to stimulate a genetically determined weak immune response using gramicidin S.

During immunization, CBA, C57B1, and (CBA x C57B1) F1 mice were injected with 2 x 106 BE together with 10 μg of gramicidin S. After 4 days, the content of

antibody-producing cells in the spleen was examined. The results are presented in Figure 24. It can be seen that with the help of gramicidin S it is quite possible to enhance the response in the low-responding genotype C57B1. Gain rates were the same as those observed in high responder CBA mice. The level of response and the level of stimulating effect of gramicidin in F1 hybrids from crossing high- and low-responding parents were almost the same as in mice of the highly responsive genotype.

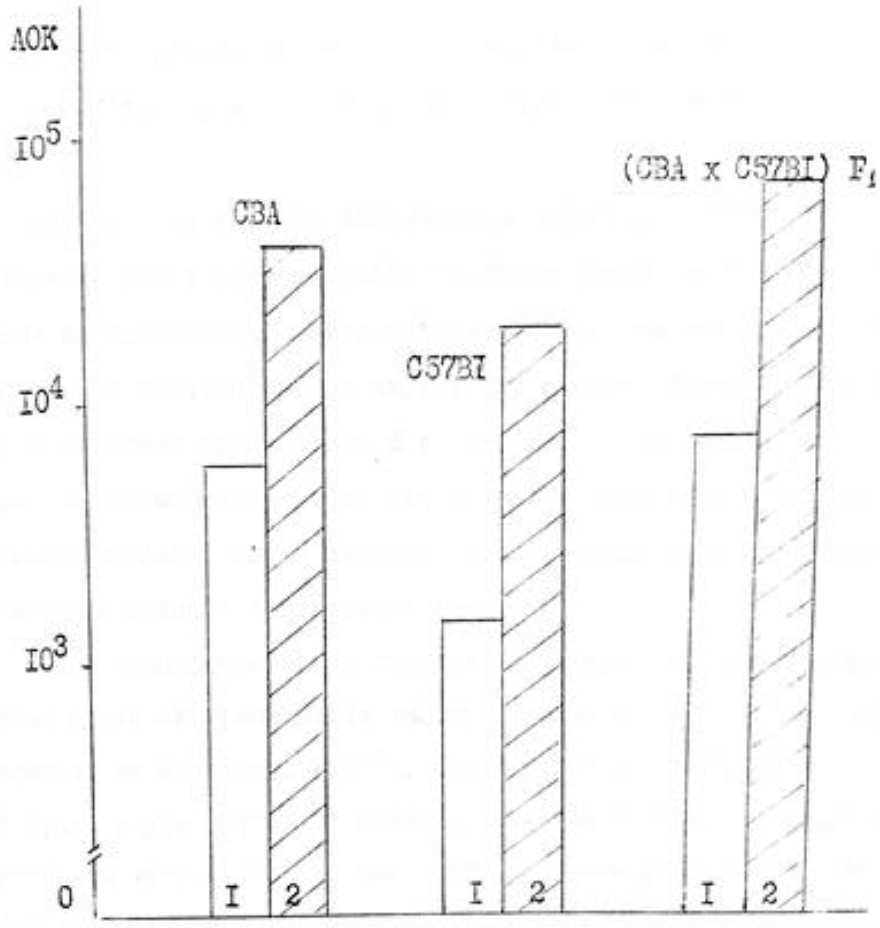


Figure. 24. The effect of gramicidin S on antibody formation against BE antigens in mice of highly responsive (CBA) or low-responsive (C57B1) genotypes, and such in first-generation hybrids. The ordinate shows the number of antibody producers to BE after 4 days. after immunization of mice with 107 BE (columns 1) or a mixture of 107 BE plus 10 μg of gramicidin S (columns 2).

Stimulation of local anti-infective

immunity in a mouse model of cutaneous leishmaniasis.

The process of cutaneous leishmaniasis is characterized by a sluggish, long-term course due to local anti-infective immunity. Therefore, we tried to stimulate local immunity using the official gramicidin paste. To assess the contribution of the antibiotic action of gramicidin S, monomycin ointment was used in one of the control groups as the most effective anti-leishmania drug among the antibiotic agents used to treat cutaneous leishmaniasis.

Mice were experimentally infected with leishmaniasis (highly viral 2 MB strain of Leushmania major) by injecting a culture of the pathogen into the skin of the right ear. Pathological manifestations consisted of slowly occurring local inflammation with the formation of a destructive skin ulcer. In mice that did not receive treatment, the process of ulceration ended with scarring of the ulcer only after 123.9 ± 2.9 days. At the same time, in more than half of the mice, partial or complete destruction of the tissue of the infected ear occurred.

Medicinal ointments (2% gramicidin or 10% monomycin) began to be used from the moment an open ulcer appeared on the skin of the ear. The surface of the ulcer was carefully covered with a thin layer of ointment. The procedure was repeated daily. The duration of the healing process was significantly reduced due to treatment.In the group of mice treated with monomycin, the ulcers healed after 97.3 ± 2.1 days. The percentage of mice with partial or complete destruction of ear tissue decreased to 25% (and control without treatment

more than 50%). Gramicidin S had a significantly stronger therapeutic effect. The healing time of ulcers decreased to 75.0 ± 2.6 days, complete destruction of the ear was not observed in any of the 152 mice studied, partial destruction of the ear was observed in only 10% of mice.

The significantly more effective therapeutic effect of gramicidin compared to monomycin indicates that the effect of gramicidin is determined not only by its antibiotic activity. There is enough reason to assume that gramicidin S has a local immunostimulating effect, which, in combination with the antibiotic effect, gives a very good therapeutic effect for cutaneous leishmaniasis.

In general, testing of non-polymeric membrane-active substances that increase the permeability of cell membranes to ions has shown that with the help of such effectors it is possible to effectively stimulate the immune response in vivo and activate the division of lymphocytes in vitro. Therefore, increasing the permeability of the cell membrane to ions is sufficient to activate the lymphocyte response and the immune response in general.

That is, the increase in lymphocyte membrane permeability induced by polyanions cannot be considered a concomitant effect, but is directly related to the mechanism of activation of the response of lymphoid cells. This conclusion is indirectly confirmed by the data presented in 6.1, according to which activation of lymphocytes took place only when the polyanion modified ion transport at the membrane level (B-lymphocytes). The absence of the polyanion effect at the level of ion transport in the membrane was accompanied by the absence of a proliferative response of cells (mature T lymphocytes).

Chapter 6. DISCUSSION

As part of a comprehensive program for studying the molecular mechanism of activation of the immune response by water-soluble polyelectrolytes (R.M. Khaitov, R.I. Ataullakhanov and co-authors), we performed part of the work. We studied the role of ionic permeability in activating the response of immunocompetent cells. First, the correlation between the ionophore-like and mitogenic effects of polyanions on subpopulations of lymphoid cells was studied. Secondly, we analyzed the immunomodulatory properties of nonpolymeric compounds that, like polyelectrolytes, have the ability to increase the permeability of the cell membrane to ions.

The influence of negatively charged polyions - polyacrylic acid and dextran sulfate - on the ion-conducting properties of the outer membrane of lymphoid cells was studied. Previous experiments have proven that the immunostimulating effect of the polyanion is determined by its direct effect on immunocompetent cells [22, 34, 23].As we reported in the literature review, it was found that polyion effectively induces the release of lymphocytes in the initial phase of the cell division cycle (G1 phase). In the presence of macrophages, the next step occurs, the G1 – S transition. The process of

beginning DNA synthesis is induced by polyanions much less strongly than by lectins and lipopolysaccharides [22]. It was theoretically substantiated that the mechanisms of activation of the lymphoid cell response should be sought at the level of the outer cell membrane [33]. The search for changes in such important membrane regulatory systems as the lipid matrix system and the cyclase enzyme system did not lead to the discovery of significant rapid changes under the influence of the immunostimulating polyion [5, 2].

As follows from the data presented in our work, polyanions induce very rapid and significant rearrangements in the membrane ion transport system. The passive flows of K+ from the cell and Ca2+ into the cell clearly increase. This increase is not a consequence of blocking the work of membrane ion transporting ATPhase s. Consequently, the polyanion induces an increase in the permeability of the cell membrane to ions. The kinetics of changes in membrane permeability are described. In general, two facts attract attention: the instability of the rate of ion leakage in the first 3-4 minutes after exposure and the temporary nature of the change in membrane permeability.The latter is significant, since after 35-40 minutes the leakage of ions practically stops. This indicates the existence of cellular mechanisms of protection against the ionophore-like effect of the polyanion. Quantitative assessment of the polyanion-induced loss of K+ by cells in comparison with the loss of K+ during lysis of the same cells allows us to consider the ionophore-like effect of the polyanion to be relatively “mild”.At least, when using non-toxic immunostimulating doses, as a result of the polymer-induced increase in cell membrane permeability, lymphocytes lost in 35-40 minutes approximately 1/1000 of the amount of K+ ions that is released when the same cells are lysed by saponin.

Along with changes in the permeability of the cell membrane to ions, we found activation of membrane ATPhase s transporting Na+, K+, Ca2+ against their concentration gradient. Comparison of the kinetics of activation of ion-transporting ATPhase s with the kinetics of changes in membrane permeability shows a clear coupling between these events. The time of the beginning of changes, the period of instability and the period of stabilization of the changed parameters coincide (Fig. 5

and Fig. 12).Considering that the activity of (Na+, K+) - and Ca2+ - ATPhase s of cell membranes is regulated, first of all, by the level of the corresponding ions on both sides of the membrane, we consider the change in membrane permeability to be primary, and the activation of ATPhase s to be secondary. An increase in cell membrane permeability leads to a decrease in transmembrane ion gradients. Inside the cell, the concentration of Ca2+ and Na+ increases, the level of K+ decreases.

This serves as a signal for the activation of the corresponding membrane "pumps", the work of which is aimed at restoring the normal ratio of ion concentrations in the cytoplasm and the environment for the cell. Consequently, we consider the polyanion-induced activation of (Na+, K+) - and Ca2+- ATPhase s to be compensatory, aimed at eliminating disturbances associated with an increase in the permeability of the cell membrane to ions.

When studying the mechanisms of activation of the response of lymphoid cells to lectins, many authors paid attention to changes in membrane ion transport. In the literature review, we reviewed the factual material on this issue. Some authors recorded activation of (Na+, K+)-ATPhase in lymphocytes when exposed to lectins [44, 53].Others - activation of Ca2+ - ATPhase under similar conditions [18]. Still others discovered an increase in the permeability of the cell membrane to amino acids, sugars, and nucleosides [34]. Still others paid attention to an increase in the concentration of Ca2+ in the cytosol (however, such works began to appear in large numbers only in recent years [32, 57, 59]. In each case, the researchers attributed the found change to a key role in triggering the cell response. Our data did not contradict these studies.Moreover, they make it possible to link all of the listed effects - changes in permeability, accumulation of Ca2+ in the cell and activation of ATPhase s. We believe that the primary change is permeability. The increased cation fluxes are partially compensated by the work of activated ATPhase s. But despite this, the concentration of ions may change and, in particular, the level of intracellular calcium may increase.

The detected changes in membrane ion transport in our work (under the influence of a polyanion) and in the work of other researchers (under the influence of

lectins) do not in themselves prove the key role of this change in the activation of the cell's response to external influences. We paid special attention to this issue in our experimental work. It turned out that modification of membrane ion transport systems occurs only in those cells (B lymphocyte) that respond to the polyanion by activating division. If the cells did not react to the polyanion by dividing, as was the case with mature T-lymphocytes, then we did not observe any changes in the membrane transport of ions (Fig. 15).This linkage between early membrane rearrangements and the later physiological response of cells indirectly indicates the importance of increasing ion permeability in the mechanism of triggering the response of lymphocytes and, consequently, in the mechanism of stimulation of the immune response by polyanions.

This conclusion is supported, not indirectly, but directly, by the results of testing the immunostimulating properties of non-polymeric membrane-active substances (Chapter 6). Our experiments show that with the help of substances that increase the permeability of the cell membrane to ions, in particular, levorin, nystatin, gramicidin S, it is possible to simulate the immunostimulating effect of the polyanion both in vivo and in vitro.

Increasing the permeability of the cell membrane to polyanions with the help of these substances induced activation of the initial phase of lymphocyte division in vitro, similar to polyanions (Fig. 16). Membrane-active substances are especially effective not so much as mitogens, but as stimulators of antibody synthesis in vivo (Fig. 18, 21).Co-administration of gramicidin or levorin with the antigen led to a strong stimulation of antibody formation. The production of antigen-specific antibody producers increased on average from 3 to 30 times. The level of stimulation depended on the level of the stimulated response (Fig. 20). A weak immune reaction upon introduction of threshold doses of antigen was stimulated by membranotropic agents 20-30 times. The initially high reaction could be increased by no more than 2-3 times. The inverse dependence of the stimulation effect on the level of the stimulated response is presented in Fig. 25.

In our work, we characterized in detail the parameters of stimulation of antibody synthesis using one of the agents used - gramicidin S. It was shown that the dynamics of stimulated synthesis of IgM and IgG antibodies were essentially no different from the control (Fig. 22). This apparently indicates that the immune reaction to the antigen, in the case of joint administration with a membrane-active agent, develops according to the same patterns and mechanisms as in the case of the introduction of only the antigen.+

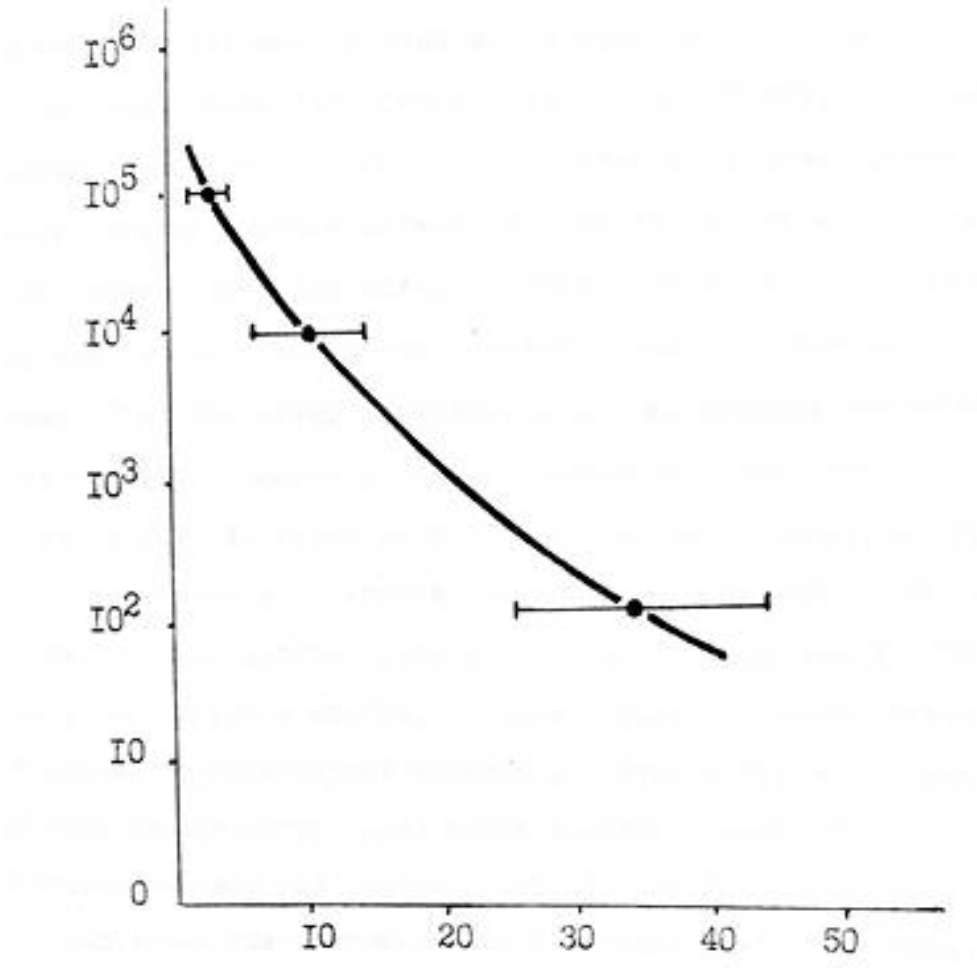


Figure. 25. Dependence of the immunoadjuvant action of gramicidin S on the level of the immune response undergoing stimulation. The x-axis is the coefficient of stimulation of the immune response when 10 μg of gramicidin S is administered together with different doses of antigen (BE). The stimulation coefficient was determined as the ratio of the number of antibody-secreting cells (ASC) in the experiment (immunization with BE plus gramicidin) to the number of antibody-

secreting cells in the control (immunization with BE only). The y-axis is the number of AFC in the control with the introduction of different doses of BE.

Apparently, increasing the permeability of the membrane to ions effectively serves as a “second signal” for cells that recognize the antigen. However, a rigorous study of the mechanisms of enhancing the immune response by membrane-active substances, in our opinion, remains to be done. For now, we can only state that the membrane-active agents used are strong immunostimulants. It is important to note that with their help it is possible to most effectively enhance a weak reaction to threshold doses of antigen. In addition, it is better to use corpuscular versions of antigens rather than soluble ones. It is quite possible that the latter fact indicates the activating effect of membrane-active agents on phagocytic cells, the function of which is especially important when introducing corpuscular antigens. It is also important that with the help of membrane-active gramicidin S the secondary immune reaction is very strongly activated (Table 2, Fig. 23).

The discovery of the immunostimulating properties of membrane-active substances is, in our opinion, of great importance for laboratory and, possibly, veterinary and medical practice. The range of immunostimulants today is still extremely insufficient. We hope that membrane active agents will be widely used as immunoadjuvants. The method consists in introducing one or more groups of membrane active gramicidin S into the structure of a nontoxic, inert with respect to the cell membrane, inactive as an immunoadjuvant water soluble polymer using a covalent bond.The synthesis products - neutral polymers containing membrane active gramicidin S - have good solubility in aqueous media, extremely low toxicity and very high immunostimulating activity.

In particular, a conjugate of neutral dextran and gramicidin S (named gradex) has been synthesized and is being actively studied [9]. Gradex provides 10-20-fold stimulation of a weak immune response, degrades in vivo, and is non-toxic. Moreover, it is effectively used as an immunostimulating carrier for antigens.

Currently, a significant increase in the immune response to hapten, serum proteins, viral and microbial antigens has been shown if they were attached to Gradex.

Thus, as follows from the presented data, we have discovered a new class of immunostimulating substances in the form of membrane-active agents. From the point of view of the tasks formulated at the beginning of the dissertation (section “Introduction”), it is important that to trigger the response of lymphoid cells in vitro and to enhance the immune response in vivo, it is quite sufficient to increase the permeability of the cell membrane to ions.Our findings prove that the polyanion-induced increase in cell membrane permeability can serve as a key link in the mechanism of triggering the response of lymphoid cells. It follows from this that the rearrangements in ion transport discovered in our work at the level of the membrane of lymphoid cells underlie the process of activation of lymphocytes by the polyanion, and ultimately, stimulation of the immune response.

Above, we described the work of foreign researchers, which attaches great importance to modification of membrane ion transport during activation of lymphocytes by lectins. In the last 2-3 years, reports have appeared proving the importance of ionic permeability of the membrane in the mechanism of activation of B-lymphocytes by antibodies to Ig [48], T-lymphocytes by antibodies to the antigen receptor [55] and T-lymphocytes when interacting with antigen-presenting cells [3]. In general, the current understanding is that activation of a lymphoid cell by various ligands begins with modification of the permeability of its outer membrane for ions.

Many schemes and hypotheses about the activation of lymphocytes have been proposed. Let's look at the most typical of them. Some authors [45] attach key importance to switching the activity of (Na+, K+)-ATPhase . It is assumed that the transition of (Na+, K+)-ATPhase to a mode of increased activity ultimately leads to the initiation of cell division. On the contrary, inhibition of (Na+, K+)-ATPhase activity serves as the initial stage of activation of terminal differentiation of the lymphocyte.

Other researchers [55] prefer the flow of Ca2+ from the extracellular environment into the cell as a mediator signaling the interaction of the lectin with the cell membrane. As is known, Ca2+ can trigger many important enzymatic reactions

inside the cell. In recent years, more and more specialists are leaning towards precisely this mechanism of activation of the cell response [48, 54-59, 61].

The interaction of lectins, bivalent antibodies, lipopolysaccharides with the outer cell membrane leads to the formation of ion-conducting structures in the membrane. According to the authors, in all cases, bi- and multivalent ligands are capable of redistributing integral membrane proteins into microaggregates, a state of 10 or more protein particles. In these aggregates, conditions are created for the formation of ion-conducting pores at the protein-protein interface.

A similar hypothetical scheme was proposed by Norcross [61] in relation to the mechanism of activation of the T killer cell precursor. As is known, activation of these cells occurs when T-cell receptors recognize an antigen in combination with histocompatibility proteins. Until now, the need for such double recognition was unclear. Norcross suggested that the meaning of double recognition is to stabilize the connection between the receptor and the recognized structure.

Extending the lifetime of the T-receptor complex with its associate (MHC + antigen) promotes the accumulation of a large number of such complexes in the contact zone between the T-cell and the antigen-presenting cell. These complexes consist of “recognition” molecules and “recognition” molecules, which belong respectively to the T cell and the antigen-presenting cell. There is an aggregation of the T cell receptor in the plasma membrane, and, as Norcross suggests, membrane domains (T3M) T - cellular receptors, clustering, form ion-conducting channels. It is the appearance of channels and a weaker increase in membrane permeability that marks the beginning of a restructuring of cellular metabolism.

Apparently, passive ion flows are quickly compensated by an increase in the active transport of the same ions using ATPhase s. However, the very fact of imbalance is already sufficient for signaling about the ligand and for the initiation of a response by the cell.

R. V. Petrov and co-authors believe that disturbance of ionic equilibrium initiates the first stages of activation of cell division, in particular, the synthesis of RNA and protein macromolecules is activated, and the expression of certain proteins on the cell surface

is enhanced. For example, the content of Ia molecules on the cell membrane increases, and special proteins appear - receptors for growth and differentiation factors. Acceptance of growth factors triggers DNA synthesis and replication.

n general, it appears that the mechanisms for initiating a cell response to exogenous influences are much more complex than is presented in any of the above-mentioned hypotheses. For example, we did not at all consider the role of such important processes as activation of membrane cyclase enzymes and protein kinases, activation of membrane phospholipases and the formation of diacylglycerols, inositol triphosphate, lysolecithin, arachidonic acid and many, many others.

At present it is difficult to give preference to any of the mentioned events. Most likely, they are different parts of complex biochemical pathways triggered by the ligand. Nevertheless, without a doubt, changes in ion transport at the level of the plasma membrane are one of the key events in triggering the response of lymphocytes by various ligands, including polyanions.

CONCLUSION

Our work, in general, allowed us to establish two fundamentally important facts: a) the correlation between the membrane-active and immunostimulating effects of polyanions (PAA and SD) on subpopulations of T and B lymphocytes; b) the possibility of stimulating immunity with the help of membrane-active substances that are not polyanions. Both facts can be considered as indirect evidence in favor of the key role of changes in membrane permeability during the activation of lymphocytes by polyanions.

At the same time, data on the immunostimulating effect of official membrane-active substances: gramicidin S, levorin and nystatin have independent scientific and practical significance. They allow us to recommend the use of these substances as immunoadjuvants for the induction of antibody genesis against antigens from pathogens of various infections.At the same time, the work presents detailed data on the dose dependencies of the immunoadjuvant effect of gramicidin S, on the dynamics of the production of IgM and IgG antibodies, on the possibility of enhancing both the

primary synthesis of antibodies and immune memory, as well as secondary production of antibodies, on the dependence of the severity of the immunoadjuvant effect on the level of the modified reaction (on the dose of the immunogen) and on the structural features of the antigens under study (soluble or corpuscular). All this is information that can be useful for a more targeted use of gramicidin S and its analogues in practice. The results obtained in this work should be developed in subsequent studies. This is also confirmed by successful attempts to use gramicidin S to stimulate the immune response against antigens of salmonella and the causative agent of anthrax, together with the significant therapeutic effect of gramicidin S in cutaneous leishmaniasis, which is characterized by a protracted sluggish course due to weak local immunity.

1. There is a direct correlation between the ionophore-like and mitogenic effects of the polyanion on lymphocytes. Polyacrylic acid and dextran sulfate cause a rapid change in the ion-transporting properties of the plasma membrane of B lymphocytes and activate the division of these cells. In a suspension of T-lymphocytes, these same polyanions induce neither changes in membrane ion transport nor activation of cell division.

2. Membrane-active compounds - gramicidin S, nystatin and levorin - effectively model the immunostimulating effect of polyanions. In in vitro cell culture, these substances activate lymphocyte division. When membrane-active compounds are co-administered with an antigen in vivo, multiple stimulation of the synthesis of specific antibodies occurs.

3. The fold increase in antibody production by gramicidin S depends on the level of the immune response. Weak antibody synthesis can be enhanced 20-30 times or more. The immune response of medium intensity can be enhanced 5-7 times. A high immune response, close to the maximum, is stimulated by gramicidin S 1.5-2 times.

4. Using gramicidin S, you can stimulate both primary and secondary synthesis of antibodies. At the same time, the production of IgM and IgG antibodies increases equally. The dynamics of stimulated antibody synthesis do not differ from the dynamics of the control response to the antigen (without garmicidin S).

5. Membrane-active immunoadjuvant - gramicidin S - is suitable for stimulating the production of antibodies to various antigens: heterologous erythrocytes, water-soluble membrane antigen from heterologous erythrocytes, killed microbial cells S, soluble O-antigen from Salmonella, as well as soluble protein antigen (p90) from the pathogen Siberian ulcers.

REFERENCES

1. Artemyev M.M. Classification of mosquitoes (Diptera, Psychodidae, Phlebotominae): Dis. Doctor of Biology Sci. – M., 2022—.

2. Ataullakhanov R.I., Abdullaev D.M. Increased permeability of the plasma membrane of lymphocytes for mono- and divalent cations and low-molecular metabolites under the influence of mitogenic polyanions. - Bill.experimental.biol. and Med., 1984, 7, 81-84.

3. Ataullakhanov R.I., Khaitov R.M., Gubarev M.I. Participation of macrophages in the reaction of lymphocytes to polyion-immunostimulant. - Immunology, 2019, 5, 26-29.

4.Baranets M.S., Species composition and distribution of mosquitoes (Diptera, Psychodidae, Phlebotominae) of Central Asia. Ponirovsky E.N., Kadamov D.S.// Med. parasitol. – 2015. - No. 4. – S.

5.Wolf K., Johnson R., Sumond D. Dermatology according to T. Fitzpatrick. Atlas-directory. – M., Praktika, 2007. – 1228 p.

6. Dolmatova A.V. On the main factors determining the epidemiological significance of individual species of mosquitoes (Phlebotominae) in foci of leishmaniasis.//Med.paritol. No. 3, p. 298.

7.European guide to the treatment of dermatological diseases / ed. HELL. Katsambasa, T.M. Lottie; lane from English – 2nd ed. – M.: MEDpressinform, 2009. – 736 p. 3.45.

8. Zhakhongirov Sh.M., “Analysis of changes in the epidemiological situation in cutaneous leishmaniasis in the regions of Uzbekistan.” Kovalenko D.A., Abdiev F.T. etc.//Problems of biology and medicine, No. 3-2017, pp. 45-

9.Leishmaniasis. – WHO publication. – No. 375. – 2014.

10. Majidov A.V. Stimulation of interleukin-2 production with synthetic polyelectrolytes. - In the book. "Current issues" of modern histology", Moscow, 1984, p. 35.
11. Muratov T.I., “Modern epidemiological aspects of cutaneous leishmaniasis in Uzbekistan” Achilova O.D., Suvonkulov U.T. // Journal Bulletin of the Tashkent Medical Academy No. 1, 2018, p. 28.
12. Paltsev M.A., Potekaev N.N., Kazantseva I.A., Lysenko A.I., Lysenko L.V., Chervonnaya L.V. Clinical and morphological diagnosis of skin diseases: atlas. – 2nd ed., stereotypical. – M.: Medicine, 2005. – 432 p.
13.Petrov R.V., Khaitov R.M., Liozner A.L. etc. Fully synthetic peptide-polyion complexes have a protective effect against influenza infection. - Immunology, 2016, 1, 29-32.
14. Ponirovsky U.N., Arid ecosystems. Darchenkova N.N// Moscow-2005 – T. 11 – No. 28 – P.39-50.
15. Potekaev N.N., Akimov V.G. Differential diagnosis and treatment of skin diseases. – M.: GEOTARMEDIA, 2016. – 456 p.
16. Rabbimova N.T., “Modeling the processes of spread of cutaneous leishmaniasis in Uzbekistan” Suvonkulov U.T., Muratov T.I., Malikov M.R. // Doctor's Bulletin No. 3, p. 78.
17. Sviridov B.D., Skvortsov V.Y., Ataullakhanov R.I., Nekrasov A.V. Synthetic high molecular weight immunostimulants based on modified vinylpyrrolidone copolymers. - Immunology, 2015, 2, 25-27.
18. Sergeev V.P. Parasitic diseases of humans / V.P. Sergiev, Yu.V. Lobzin, S.S. Kozlov. – St. Petersburg: Foliant, 2011. – 608 p.
19. Sokolovsky E.V., Mikheev G.N., Krasnoselskikh T.V. and others. Dermatovenerology: a textbook for students of higher institutions. prof. honey. education / ed. E.V. Sokolovsky. – SPb.: SpetsLIT, 2017. – 687 p.
20.Strachunsky, L.S. Practical guide to anti-infective therapy / L.S. Strachunsky, Yu.B. Belousov, S.N. Kozlov. – NIIAH SGMA, 2007. – 420 p.

21. Suvonkulov U.T., Modern characteristics of the natural focus of zoonotic cutaneous leishmaniasis in the Mubarek district of the Kashkadarya region of Uzbekistan. Abdiev T.A., Usarov G.H., // Infection, immunity and pharmacology." Tashkent-2019. Page 45

22. Suvonkulov U.T., Etiology of cutaneous leishmaniasis in Endemic regions of Uzbekistan using the example of the Jizzakh region. Achilova O.D., Muratov T.I. Epidemiology and infectious diseases" ed. "Medicine". Volume 24 No. 3 -2019 art. 123

23. Khaitov R.M., Ataullakhanov R.I. Mitogenic effect of poly-acrylic acid on lymphocytes. II. Kinetics of proliferation of lymphocytes of T- and B-sub-classes. - Immunology, 1982, 4, 30-32.

24. Shuikina E.E. Possibility of antibiotic therapy for leishmaniasis / E.E. Shuikina [and others]. // Med. parasitol. – 2009. – No. 3, – P. 45–47.

25. Ajdary S., Riazi-Rad F., Alihomommadian M.H. et al. Immune response to Leishmania antigen in anthroponotic cutaneous leishmaniasis. Parasite Immunol. 1999; 21: 423-431.

26. Alexander J, Bryson K. T helper (h) 1/Th2 and Leishmania: paradox rather than paradigm. Immunol. Lett. 2005 Jun 15; 99(1):17-23.

27. Almeida R.P., Brito J., Machado P.R. et al. Successful treatment of refractory cutaneous leishmaniasis with GM-CSF and antimonials. Amer. J. Trop. Med. Hyg. 2005; 73(1): 79081.

28.Anthony R.M., Rutitzky L.K.I., Urban J.F. et al. Protective immune mechanisms in helminth infection. Nat. Rev. Immunol. 2007; 7; 975-987.

29. Arimand R., Fard S., Saberi S., et al. Antigenic profile of heat-killed versus thimerosal-treated Leishmania major using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Adv Biomed Res. 2015; 4:128.

30. Aronson N, Herwaldt BL, Libman M, et al: Diagnosis and treatment of leishmaniasis: Clinical Practice Guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH).Clin Infect Dis 63 (12):e202-e264, 2016. doi: 10.1093/cid/ciw670.

31. Barbosa J.F., de Figueiredo S.M., Monteiro F., et al. New Approaches on Leishmaniasis Treatment and Prevention: A Review on Recent Patents. Recent Pat. Endocr. Metab. Immune Drug Disco. 2015 Sep 21.

32.Berman J.D. Human leishmaniasis: clinical, diagnostic and chemotherapeutic developments in the last 10 years. Clin. Infect. Dis. 1997; 24:684-703.

33.Chakravarty J., Sundar S. Drug resistance in leishmaniasis. J. Globe. Infect. Dis. 2010; 2(2):167-176.

34. Choi B.S., Kropf P. Evaluation of T cell responses in healing and nonhealing Leishmaniasis reveals differences in T helper cell polarization ex vivo and vitro. Parasite Immunol. 2009; 31:199-209.

35.Clem, Angela A: Current Perspective on Leishmaniasis. Journal of Global Infectious Diseases. May 2010, Vol. 2 Issue 2, p125.

36. Costa D. L., Carregaro V., Lima-Júnior D. S., et al. BALB/c mice infected with antimony treatment refractory isolate of Leishmania braziliensis present severe lesions due to IL-4 production. PLoS Negl Trop Dis. 2011 Mar 1;5(3):e965.

37. Croft S.L., Olliaro P. Leishmaniasis chemotherapy – challenges and opportunities. Clin. Microbiol. Infect. 2011. 17 (10): 1478-1483.

38. Desjeux P. Information on the epidemiology and control of the Leishmaniasis by country of territory. WHO, 1991, Switerland, Geneva.

39.Haas N., Hauptmann S., Paralikoudi D. et al. Interferongamma treatment induces granulomatous tissue reactions in a case of localized cutaneous leishmaniasis. Am. J. Dermatopathol. 2002, Aug; 24 (4):319-323.

40.Harms G., Zwinngenberger K., Chahade A.K. et al. Effects of intradermal gamma-interferon in cutaneous leishmaniasis. Lancet. 1989; 1(8650): 1287-1292.

41.Kunzler B. Cutaneous leishmaniasis: the efficacy of nonantimony treatment in the austere environment. Using cryotherapy, thermotherapy and photodynamic therapy as an alternative method of treatment. J. Spec. Oper. Med. 2013; 13(4):40-45.

42. Launois P., Louis J.A., Milon G. The fate and persistence of Leishmania major in mice of different genetic backgrounds: an example of exploitation of the immune system by intracellular parasites. Parasitology. 1997; 115 Suppl: S25–S32

43. Lazarski Ch.A., Ford J., Katzman Sh.D. et al., IL-4 attenuates Th1-associated chemokine expression and Th1 trafficking to inflamed tissues and limits pathogen clearance. PloS One. 2013; 8(8):e71949.

44. Lessa H.A., Machado P., Lima F. et al. Successful treatment of refractory mucosal leishmaniasis with pentoxifylline plus antimony. Am J Trop Med Hyg. 2001 Aug; 65(2):87-89.

45.Maizels R., Hewitson J.P., Smith K. Susceptibility and immunity to helminth parasites. Current Opinion in Immunology. 2012; 24: 459-466.

46. Matos I., Mizenina O., Lubkin A. et al. Targeting Leishmania major antigens to dendritic cells in vivo induces protective immunity. PLoS one, 2013; 8(6): e67453.

47. Mock D. J., Hollenbaugh J. A., Daddacha W. et al. Leishmania induces survival, proliferation and elevated cellular dNTP levels in human monocytes promoting acceleration pf HIV-co-infection. PloS Pathog. 2012; 8(4):e1002635

48. Moreno E., Schwartz J., Fernandez C. et al. Nanoparticles as multifunctional devices for the topical treatment of cutaneous leishmaniasis. Expert Opinion on Drug Delivery. 2014. –Vol.11, N 4. – 579-597.

49. Neumayr A.L., Morizot G., Visser L.G. et al. Clinical aspects and management of cutaneous leishmaniasis in rheumatoid patients treated with INF-α antagonists. Travel Med. Infect. Dis. 2013; 11(6):412-420.

50. Olliaro P., Vaillant M., Arana B. et al. Methodology of clinical trials aimed at assessing interventions for cutaneous leishmaniasis. PLoS Negl Trop Dis. 2013;7(3):e2130. doi: 10.1371/journal.pntd.0002130. Epub 2013 Mar 21.

51. Reiner S.L., Locksley R.M. The regulation of immunity to Leishmania major. Ann Rev Immunol. 2020; 13:151-177.

52. Rezaei Riabi T., Sharifi I., Miramin Mohammadi A., Khamesipour A. et al. Evaluation of a Possible Synergistic Effect of Meglumine Antimoniate with Paromomycin, Miltefosine or Allopurinol on in Vitro Susceptibility of Leishmania tropica Resistant Isolate. Iran J Parasitol. 2013 Jul;8(3):396-401.

53. Sacks D., Noben-Trauth N. The immunology of susceptibility and resistance to Leishmania major in mice. Nat Rev Immunol. 2002; 2:845–858.

54. Santos J.B., de Jesus A.R., Machado P.R. et al. Antimony plus recombinant human granulocyte-macrophage colonystimulating factor applied topically in low doses enhances healing of cutaneous Leishmaniasis ulcers: a randomized, double-blind, placebo-controlled study. J Infect Dis. 2004 Nov 15;190(10):1793-6. Epub 2004 Oct 18.

55.Savoia D. Recent updates and perspectives on leishmaniasis. J Infect Dev Cries. 2015 Jul 4;9(6):588-96.

56. Schwarz T., Remet K., Nahrendorf W. et al. T-cell derived IL-10 determines leishmaniasis disease outcome and is suppressed by a dendritic cell based vaccine. PLOS Pathog. 2013; 9(56): e1003-476.2013).

57. Scott P., Artis D., Uzonna J., Zaph C. The development of effector and memory T cells in cutaneous leishmaniasis: the implications for vaccine development// Immunol Rev. 2019; 201: 318–338.

58. Taheri A.R., Govonlo V.M., Nahidi Y. et al. Plasma levels of interleukin-4 and interferon-γ in patients with chronic or healed cutaneous leishmaniasis. Iran J. Basic Med. Sci. 2014; Mar; 17 (3):216-219.

59. Tripathi P., Singh V., Naik S. Immune response to leishmania: paradox rather than paradigm. FEMS Immunol Med Microbiol 2020; 51:229–242.

60. Valencia C., Arévalo J., Dujardin J.C. et al. Prediction score for antimony treatment failure in patients with ulcerative leishmaniasis lesions. PLoS Negl Trop Dis. 2012; 6(6): e1656. doi: 10.1371/journal.pntd.0001656. Epub 2012.

61. Castellano L.R., Filho D.C., Argiro L., Dessein H., Prato A., Dessein A., et al. Th1/Th2 immune response.