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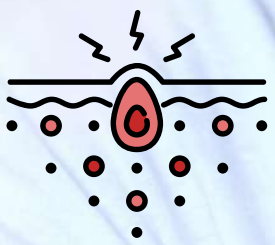
STIMULATION IMMUNITY IN A MODEL OF SKIN LEISHMANIASIS



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STIMULATION IMMUNITY IN A MODEL OF SKIN LEISHMANIASIS

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MONOGRAPH

India– 2022

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

A 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 are sufficient grounds to suggest that gramicidin S has a local immunostimulatory effect, which, in combination with antibiotic action, gives a very good therapeutic effect in cutaneous leishmaniasis.

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CONTENT

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LIST REDUCED

ATP - adenosine triphosphate

ELISA - enzyme immunoassay

KLA - antibody-forming cells

PAA - polyacrylic acid

BPA - water soluble antigen

FOREWORD

Data on the immunostimulating effect of official membrane-active substances: gramicidin S are of independent scientific and practical importance. 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 paper presents detailed data on the dose dependences of the immunoadjuvant action 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 on the secondary production of antibodies, on the dependence of the severity of the immunoadjuvant effect on the level of the modified reaction (from the dose of the immunogen) and on the structural features of the studied antigens. All this is information that may be useful for more targeted use of gramicidin S and its analogues in the practice of treating 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 Salmonella antigens and the anthrax pathogen, together with a 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 for residents of the magistracy and clinical residents.

INTRODUCTION

The introduction of water-soluble polyacids or polybases into the body can lead to the 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 successfully used 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 progress has been made in the analysis of the participation of subpopulations of immunocompetent cells in the polymer-enhanced immune response.

This monograph indicates a set of works on the study of molecular mechanisms of activation of the response of an immunocompetent cell with a polyelectrolyte. We had to determine the degree of participation of the ion-transporting 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 immunity by non-polymeric ion permeability inducers in leishmaniasis.

Chapter 1. LITERATURE REVIEW

Before proceeding to a description of the experiments carried out by us, it is necessary to consider in more detail the facts established before us. First, about what is known about the immunostimulating effect of polyelectrolytes. Secondly, about the role of membrane transport of ions in the mechanism of activation of lymphoid cells by lectins.

1. Stimulation of immunogenesis by polyelectrolytes.

Polyelectrolytes are soluble polymers of various structures that have multiple charges. It was shown that by introducing these substances into the organism of animals, it is possible to intensify the processes of hematopoiesis and immunopoiesis. Joint administration of a polyelectrolyte with an antigen leads to antibody production that is 3–5 times higher than when the antigen itself is administered (11, 32, 46). Stimulation of the end result of the reaction of the immune system is a consequence of the activation of specific links of immunopoiesis by the polymer. For example, administration of a polyelectrolyte in vivo significantly activates the migration 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 process of growth of hematopoietic colonies is intensified (13). Such important links of 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 polyelectrolyte into the mouse body (16). These migration processes are a necessary condition for the convergence of T and B cells within the same tissue, which allows these cells to interact. Moreover, exposure to polyelectrolyte intensifies the very 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 the

same experiments, polyelectrolytes stimulated B-cell response even in the absence of T-lymphocytes (21). There was an assumption about the direct activating effect of polyelectrolyte on B-lymphocytes. The data obtained were regarded as evidence of the possibility of replacing T-helpers with polyelectrolyte 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 it was impossible to exclude influences mediated through the hormonal, nervous, and other body systems. Therefore, experiments in cell cultures in vitro can be recognized as 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 (GI phase) was activated, and in the presence of macrophages, DNA synthesis (S-phase) was also activated (4a). The proliferative response of lymphocytes to polyion differs significantly from the response of cells to mitogenic lectins (34). The latter increase DNA synthesis by 20 - 30 times and induce a series of successive mitoses lasting several days. On the contrary, under the influence of polyion, DNA synthesis is activated no more than 3-7 times. Cells, if they do, then only one cycle of division. Rather, the polymer induces only the initial phases of the fission cycle, without creating conditions for its full completion. The reasons for the inferiority of the mitogenic signal of polyelectrolytes, apparently, are 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 weak polyclonal effect of polyelectrolytes on the maturation of B cells into Ig - secreting cells is also associated with the lack of the ability to induce a series of full divisions and secretion of differentiation factors (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. Upon induction of antibody synthesis in cell culture in vitro, the polyelectrolyte increased the number of antibody-secreting cells by 4–5 times (23). In vivo, antibody production upon co-administration of antigens with polyelectrolytes can be 3–7 times higher than the level of response to "pure" antigens (16).

Therefore, the polyelectrolyte, acting strongly on lymphoid cells, induces a strong adjuvant signal and a weak mitogenic one. In what follows, speaking of 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 with polyions

R.I. Ataullakhanov et al. have been studying the earliest molecular changes in lymphoid cells during their activation by polyions for a number of years. At 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 could be transmitted into the cell about the effect of an exogenous polyelectrolyte on 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. It was screened with fluorescent probes for membrane lipid viscosity and membrane lipid matrix surface area (5). In the first minutes after the treatment of lymphocytes and macrophages with polycation, a significant change in the morphology of the plasma membrane of the cell was found (44). The cell surface was smoothed: membrane protrusions, villi, and ridges were shortened. At the same time, the elementary "grain" of the membrane matrix noticeably enlarged on a significant part of the cell surface. An assumption was made about the formation of numerous

complexes of exogenous polymer molecules with molecules of the cell membrane. Membrane cryosurgery and cleavage electron microscopy revealed membrane protein microaggregates consisting of 5–50 or more intramembrane protein particles (5a). Clustering of membrane proteins occurred already in the first minutes after the impact of the polyion on the cells. At the same time, there was a change in the permeability of the cell membrane. First, this change was detected in the fluxes of labeled nucleosides, and then in the fluxes of Ca2+ and K+ (4). The authors proposed a hypothesis in which the change in permeability was associated with the aggregation of membrane proteins and flattening of the membrane configuration (44). According to the hypothesis, polyelectrolyte molecules form many electrostatic complexes with complementary charged groups of membrane proteins. Cross-linking of protein particles with polymer threads leads to the formation of protein clusters. Actually, protein clusters contain gaps and pores, due to which the permeability of the membrane increases. Moreover, the pores are heterogeneous and non-selective; ions, water, and small molecules up to nucleosides penetrate through them.

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

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

We had to study in the experiment the significance of one of the detected changes, an increase in the permeability of the membrane for ions. Therefore, one should consider in detail the literature data on the role of ion transport in the activation of the response of lymphoid cells, although not by polyelectrolytes, but by lectins.

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

The content of eukaryotic cells differs significantly from the extracellular environment in terms of the concentration of many ions. Differences in Na+, K+, Ca2+ and H+ ions are especially important for the life of the cell. Special enzyme systems function in the cell membrane, which ensure the creation and maintenance of the ionic identity of cells at a certain level. In particular, membrane enzymes (Na+, K+)- and Ca2+-transporting ATPases are those unique structures that penetrate the outer cell membrane and ensure the creation of concentration gradients of these cations. In addition, these enzymes continuously transport ions in the direction opposite to their conientration gradient, compensating for the diffusion of the same ions along the concentration gradient through water pores and ion channels. The latter are also represented by protein structures integrated into the lipid bilayer of the membrane.

The ionic identity of the cell in relation to the pericellular environment and differences in the content of ions between compartments of the cell are, first of all, critically important for many enzymes and, consequently, for the activity of many metabolic systems inside 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" 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 the cell's response to external stimuli. For example, for an egg cell, the fact that the membrane is damaged by a spermatozoon can serve as a signal for the activation of cleavage divisions. 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, the signal that induces the secretion of granules and the production of new portions of the secret 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, the 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 reaction of a lymphocyte 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 by turning into a secretory cell, or by a combination of the two indicated types of reaction.

In the modern literature, a lot of data has accumulated indicating the importance of ion transport for triggering the response of lymphoid cells to external signals.

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

a) Passive ion flows. Allwood et al. [36] were among the first to show that the action of PHA on human lymphocytes leads to an increase in Ca2+ entry into the cells. Similar observations were also 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 the permeability for Ca2+ occurs already in the first minutes after the action of the mitogen on the cell.

The increase in Ca2+ entry induced by mitogens depends on 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+ input also increases with an increase in the lectin concentration, and this growth is not limited to the region of low concentrations at which lectin has mitogenic properties, but continues with a further increase in the

lectin concentration, up to its toxic doses. This result was obtained in a number of works, in which Ca2+ input was studied over a wide range of changes in mitogen concentrations [60, 43, 26, 17]. Some authors [52, 59] observed a local maximum in the region of mitogenic lectin concentrations in the study of Ca influx; however, with a further increase in the mitogen concentration, the Ca2+ influx increased even more [33, 37]. It should be noted that some authors did not find significant changes in passive Ca2+ fluxes when using mitogenic concentrations of concanovalin A and PHA lectins [52, 42, 27]. In these works, a marked increase in Ca2+ entry was recorded when using concentrations exceeding the optimal immunogenic doses.

Similar to the change in the permeability for calcium, an increase in the passive transport of K+ was found in a number of works [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 medium into the cell (along the concentration gradient) in the literature. An increase in the Na+ flux can be indirectly judged by a certain increase in the Na+ concentration inside the cells and by an increase in the flux of the RB+ isotope [38, 39, 56].

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

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

In some works, the activation of (Na+, K+)-ATPase 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+-ATPase activity in the membrane of lymphocytes when they are exposed to mitogens is still far from a final decision. Some studies report 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 with the corresponding control. True, it should be noted that in most studies, Ca2+-ATPase 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 concentration of Ca2+ near the cytoplasmic segments of Ca2+-ATPase and, consequently, to the rapid activation of this enzyme to the maximum possible level.

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

Obviously, in order to judge relatively small physiological changes in the activity of ion-transporting ATPases, 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) The concentration of ions inside the cell.

When exposed to mitogens on lymphocytes, as noted above, the fluxes of cations significantly increase along the gradient of their concentration. At the same time, the activity of transport ATPases, pumping the same cations in the opposite direction, increases. 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. In some studies, an increase in Ca2+ concentration inside the cell is found, in others - a decrease, and in others - the intracellular Ca2+ content did not change [58].

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

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

A physiologically active dose of the immunostimulant causes a mild increase in membrane permeability, a slight increase in passive cation fluxes, which practically instantly turns on the compensatory activation of transport ATPases. 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, while the concentration of Ca2+ will increase. In the second case, the concentration of both cations - will remain unchanged. It is quite possible that the enhanced active fluxes will for some time exceed the value of the passive fluxes, which will lead, respectively, to a slight and temporary increase in the concentration of K+ or a decrease in the concentration of Ca2+ inside the cell. The most probable, according to the authors, are small fluctuations in the intensity of two oncoming flows, which is accompanied by relatively weak fluctuations in the concentration of ions inside the cell near the initial values. Apparently, such processes can be the reason for obtaining conflicting data by different experimenters.

Despite the above-mentioned contradictions in the published data, it should be noted that the question of the kinetics of the Ca2+ level in the cytosol after the activation of lymphocytes by lectin has been significantly clarified by recent studies [48, 54-59, 54, 52]. It has been established that ligands activating lymphocytes cause a faster increase in the Ca2+ concentration in the cytosol, followed by a gradual decrease in the Ca2+ level. 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 depots. Moreover, the latter effect is regulated through the activation of the membrane enzyme phospholipase C and the production of a second 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 ATPases.

Very often, researchers use two groups of facts as an argument in favor of the key role of ion transport in the activation of lymphocytes. 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 works on the study of the mitogenic response of lymphocytes in the presence of inhibitors of ion-transporting ATPases.

A decrease in the concentration of Na+ 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 required for the development of the response of lymphocytes to the mitogen [49, 37]. A number of works suggest that extracellular Ca2+ is required starting from the 10th hour of mitogen activation. In the 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 in the cell of its own reserves of Ca2+ (for example, the pool of Ca2+ associated with the membrane, 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 fluxes of these ions for triggering the cell's response to external influences. Removal of an important component of the medium can disrupt any metabolic processes in the cell, inhibiting its physiological reaction, while not affecting the initial moment of triggering this reaction.

The situation is similar with the "proof" of the key role of membrane ATPases in triggering the reaction of lymphoid cells. As noted above, ATPases that transport ions across the membrane are a critical organ of the cell. Without the normal functioning of ATPases, the full vital activity of the 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 ATPases (for example, inhibition of (Na +, K +) - ATPases 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 +) - ATPase in the mechanism of triggering the response of lymphocytes.

Effect of ionophores on functional activity lymphocytes.

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

Valinomycin, a cyclic depsipeptide, a 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+, the nigericin ionophore, also had a similar effect. The ionophore monensin, which transports Na+ in exchange for H+, also inhibited the proliferation of lymphocytes [42, 43]. It should be noted here that all the carriers listed above, penetrating into the cell, carry out the transport of the corresponding cations and 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 to respond to the mitogen. At the same time, it is

impossible to judge the significance of the effects of the same ionophores on the outer cell membrane in the initiation of a cellular response.

Ca2+ - ionophore A23187, an antibiotic that transports Ca2+ ions across the membrane [18], has a weak mitogenic effect in the culture of lymphocytes in vitro. The mitogenicity of the A23187 ionophore was considered as one of the main evidence of the functional role of Ca2+ entry in cell activation; therefore, a number of works 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+ studied, the ionophore X537A, had a mitogenic effect on cells [40].

Like other mitogens, the A23187 ionophore interacts with the cell membrane of lymphocytes and ultimately leads to DNA synthesis and cell division. Ionophore A23187 differs from lectins in some characteristics. It enhances DNA synthesis to a lesser extent than PHA and Con A [54], but stimulates RNA and protein synthesis to the same extent as other mitogens [32]. Ionophore A23187 also induces early biochemical changes characteristic of the onset of cell divisions: 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 what is the mitogen: Con A or ionophore A23187. Replacing Con A with an ionophore for a short time - the first 3 hours of mitogenesis (with subsequent washing of cells from the ionophore and the introduction of Con A) - does not reduce the intensity of DNA synthesis. If Con A is replaced by 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 Ca2+ concentration in the medium and loses its mitogenic properties at low concentrations of extracellular calcium (10–4 M) [43].

In general, the literature data presented indicate the undoubted importance of ion transport as one of the mechanisms for regulating the response of lymphoid cells to

the 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 et al., changes in cell membrane permeability upon activation of lymphocytes by polyions, it was necessary to investigate the significance of ion transport modification for activation not only and not so much of the response of lymphocytes in vitro, but also of the immune response in vivo. It was impossible to do this only on the basis of literary data. First, the mechanisms of cell activation by polyions remained unexplored. Secondly, data on the effect 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 can determine the stimulation of the immune response upon administration of the polyion in vivo. It is for this purpose that we started 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 RESEARCH METHODS

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

As an immunogen for the induction of primary or secondary synthesis of specific antibodies, heterologous erythrocytes obtained from the peripheral blood of a donor sheep, as well as killed microbial cells of Salmonella (S. typhimurium, strain 415) were used. Before use, heparinized blood erythrocytes were washed at least three times by centrifugation in 20 times the 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 I 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 a water-soluble p90 protein antigen from anthrax. A chromatographically homogeneous p90 antigen from the diagnosticum of the Stavropol Research Institute was used. The p90 antigen was dissolved in physiological NaCl solution and administered to mice intraperitoneally at a dose of 3 μg (per mouse). Re-immunization with the same dose of p90 was performed after 1 month. During the primary and secondary immune responses, blood was collected weekly from the orbital venous sinus in experimental mice. Blood from 5-7 mice within the same experimental group was pooled to obtain serum, 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 the immune response.

4-5 days after immunization of mice with lamb 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 spleen cell suspension was mixed with 2.5 ml of 0.65% agarose sol heated to 48°C. A 20% BE suspension was preliminarily added to the agarose sol at the rate of 27 μl per 1 ml of agarose. The agarose sol containing spleen and EB cells was gently mixed and poured into Petri dishes (diameter 100 mm) placed on a horizontal plane. After the

gel had 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. The plates were incubated for another hour at 37°C, after which the cups were analyzed or fixed with 4% formaldehyde solution and stored until counting. In oblique 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, rabbit antiserum specific to mouse immunoglobulins was added to the dishes before complement application. 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 register cells secreting antibodies to Salmonella antigens, the method of hemolysis in gel described above was used. But in this case, BEs were introduced into agarose, which were previously sensitized ("coated") with 0 - antigen from the same Salmonella. The sensitization of erythrocytes with 0 - antigen was carried out according to the method of VV Solovyov et al. 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 the 0-antigen on the surface of the BE, the latter were washed several times from the unbound antigen by centrifugation in a 20-fold volume of Hanks' solution.

After immunization of mice with p90 protein antigen (from anthrax), the intensity of the immune response was assessed by the accumulation of antibodies specific to p90 in the blood serum. For this, enzyme-linked immunosorbent assay (ELISA) was used. The optimal conditions for ELISA with the p90 antigen were selected (earlier) by A.I. Pereverzev.

When setting up the ELISA, we gradually carried out the following operations. 100 μl of p90 solution in 0.1 M carbonate-bicarbonate buffer pH 9.6 (final

concentration of p90 was 3 μg/ml) was added to the well of special 96-well Linbro panels (Flow Lab., Great Britain). Panels with antigen were left overnight at 4°C. Then the wells of the panels were washed three times with a special cleansing solution (0.15 M NaCl solution buffered with 0.01 M phosphate buffer pH 7.2-7.4 with 0.05% tween-20). The panels were washed using a Titertek Microplate Washer 120 (Flow Lab., UK).

After washing off the antigen that did not bind 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, then washed three times.

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

Upon completion of the incubation, the panels were washed three times and 100 μl of a solution of rabbit antibodies specific to (γ - chains of mouse immunoglobulins or rabbit antibodies specific to mouse IgM (Miles Scientific, USA) were added to the wells. Primary antibodies were added at a final dilution of 1:500 in incubation solution.Panels were incubated for 1 hour at 37°C.Primary antibodies were washed three times, and then 100 μl of a peroxidase-conjugated secondary antibody solution (Miles Scientific, USA) were applied.Panels were incubated at 37°C for 1 hour, washed three times , after which 100 μl of a substrate solution - 0.6 mg/ml orthophenylenediamine dihydrochloride (Sigma, USA) in 0.1 M citrate-phosphate buffer pH 5.0 with 0.015% hydrogen peroxide was added to the wells. The panels were incubated for 10 minutes in the dark The reaction was stopped by adding 50 μl

of 2 M sulfuric acid to the well of the panel The optical density of the solutions in the wells of the micropanels was measured at a wavelength of 492 nm on a special Titertek Multiscan MCC photometer (Flow Lab., UK). The ELISA results were analyzed on an IBM computer using a special program written by A.I. Pereverzev. This program made it possible to calculate the titers of specific antibodies in sera and the confidence intervals for their determination at P = 0.05.

2. Culture of lymphoid cells in vitro

Depending on the tasks to be solved, we used both short-term in vitro incubation of lymphoid cells for several hours and their longer-term cultivation for several days. A suspension of mouse lymphoid cells was prepared according to conventional methods. Mice were killed by cervical dislocation of the spine. Under aseptic conditions, the spleen and lymph nodes (inguinal, axillary, submandibular, mesenteric) were removed. 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 erythrocytes. After shock, the lymphocyte suspension was filtered through a layer of sterile cotton wool and additionally washed by centrifugation in a 20-fold volume of Hanks' buffered solution. Cell viability was assessed by microscopy in the presence of 0.1% trypan blue.

Short-term incubation was performed either in Hanks' buffered solution supplemented with 1% fetal bovine serum (FBS) and 50 U gentamicin, or in RPMI-1640 medium supplemented with 2 mM L-glutamine, 5 x 10-5 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% ETS. The cultures were kept in a special incubator in an atmosphere of 5% CO2 in the air.

In some experiments, the suspension of lymphocytes was divided into two fractions - enriched in T-cells and enriched in B-lymphocytes. Separation was carried out according to the method of M. Julius co-authors, using specially prepared nylon wool. Cotton wool Leucko-pack Leuckosyte Filter (USA) was boiled for two days in distilled water, changing the water several times a day. The cotton was then dried and weighed. A plastic column (height 140 mm, cross-sectional diameter 22 mm) was packed tightly with 3 g of dry nylon wool. The column was washed with a small amount of medium 199, supplemented with 5% ETS, placed for 30 minutes in a thermostat at 37°C. Then, 2 ml of a suspension of lymphocytes containing 50x10 living cells in 1 ml was introduced into the upper part of a vertically mounted column. Another 5 ml of the 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 warmed up to 37°C. The eluate was collected (first portion). After several cycles of mechanical compaction of nylon wool, the column was washed again with a medium that did not contain FTS. 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, the second fraction is cells that adhere to nylon wool. The cells resulting from this separation have been characterized previously. The fraction of non-adherent cells was shown to be rich in mature T-limits and almost free of B-cells. Conversely, the fraction of cotton-adhering cells contains mainly B-lymphocytes, and has a small admixture of nylon-adhering T-cells.

In our work, to control the quality of the 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 non-adherent 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 upon the addition of any immunostimulant in vitro, we studied 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 μCi per 1 ml. After 24 hours, the suspensions were filtered through Synpore No2 filters. The cells settled on the filter were washed with Hanks' solution and lysed with 0.5% trichloroacetic acid. The acid insoluble precipitate was washed with 96° ethanol. After drying, the filters with radioactive material were placed in scintillation liquid. The level of radioactivity of the filters was determined on a liquid scintillation β-counter.

3. Polymers.

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

Typically, polymer solutions were prepared the day before 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. Again, thoroughly mixed and sterilized by filtration through filters "Millipore" (USA) with a pore diameter of 0.22 μm. Prepared sterile polymer solutions were stored in a 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 Hank's buffered solution at a concentration 10–100 times higher than intended. This made it possible to add a small volume of a solution containing the polymer to the cell culture. As a rule, 10-100 μl of the polyion matrix solution was added to 1 ml of culture to create the intended final concentration.

4. Membrane-active effectors.

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

produced by Sigma (USA). Saponin is a membrane-active substance manufactured by Calbiochem (USA). Solutions of membrane-active substances were prepared immediately before use.

5. Cell Membrane Permeability Measurement for ions.

The permeability of the plasma membrane of lymphocytes for ions was determined by the change in the concentration of K+ in the extracellular environment, Ca2+ - in the cytoplasm.

Cell membrane permeability for Ca2+ was measured by the radiotracer method. For this, the 45Ca isotope was used. The 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 bovine serum. Salt 45CaCl was added to the cell suspension to a final concentration of 0.1 μCi 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 away from the extracellular isotope by centrifugation 2–3 times in a large volume of 199 medium. Then, the dense cell sediment was lysed with 300 μl of 5% perchloric acid to destroy the cells and extract the intracellular content. The extraction was carried out for 1-2 minutes, constantly shaking the samples. The extracts obtained were Centrifuged at 100 rpm for 10 minutes. Then, 5–10 μl of a saturated K2CO3 solution was added to 200 μl of the extract to neutralize perchloric acid. The extracts were left for a few minutes to settle insoluble "flakes" of potassium perchlorate. The light part of the extracts was taken for the measurement of radioactivity on the β - spectrometer "Intertechnique SL - 40". A dioxane scintillator was used (900 ml dioxane; 4 g PPO; 0.2 g POPOP; 60 g naphthalene). Extract samples were mixed with scintillation fluid 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 for K+.

Suspensions of lymphocytes were prepared using buffered Hanks solution. To do this, Hank's solution (NaHCO3) was supplemented with 20 mM HEPES buffer and 50 units of peniiillin 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 introduced into a special thermostated (37°C) cellmade of fluoroplast. The cell was equipped with a valinomycin electrode with selective sensitivity to K+ ions, as well as a double electric bridge connecting the measuring cell with the reference electrode EVL-1MZ [4]. The steepness of the characteristic of the valinomycin electrode in the range of potassium concentration from 10–4 to 1 M was 60 mV per order of K+ concentration. The selectivity of K+ /Na+ is not less than 104. The setup we used made it possible to continuously record the concentration of K+ in the extracellular medium with a record on an H-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 to the extracellular medium and to an increase in the level of K+ in the culture medium.

6. Study of active transport of ions, mediated (Na +, K +) - and Ca2 + - ATPases plasma membrane.

Скорость работы мембранных АТФаз, транспортирующих Na^+, K^+ или Ca^{2+}, оценивали методом ингибиторного анализа. С помощью использованного нами метода [1] можно было оценивать долю конкретной АТФазы в суммарной АТФазе клеток. В свою очередь, о суммарной скорости потребления АТФ и равной ей скорости производства АТФ судили по скорости потребления клетками кислорода. Как известно, скорость производства АТФ в

митохондриях находится в прямой пропорции со скоростью потребления O_2 митохондриальной системой окислительного фосфорилирования. Активация производства АТФ в митохондриях требует равного по значению увеличения скорости потребления O_2. В живых функционально полноценных клетках скорость производства АТФ равна скорости его потребления. Следовательно, по изменению скорости потребления O_2 клетками можно судить об изменению скорости производства АТФ митохондриями и равной ей скорости потребления АТФ всеми вместе взятыми клеточными реакциями, нуждающимися в энергии гидролиза АТФ.

Приведенные выше рассуждения верны при соблюдении двух условий. Во-первых, скорость потребления АТФ должна быть равна скорости производства АТФ. Это условие в наших экспериментах полностью соблюдалось. В течение всего периода измерения скорости потребления O_2 лимфоцитами уровень АТФ в исследованных клетках не изменялся [1]. Второе условие заключается в соотношении между валовым потреблением O_2 клетками и использованием O_2 в этих же клетках для нужд окислительного фосфорилирования в митохондриях. Дело в том, что в некоторых клетках значительная доля O_2 может потребляться для обеспечения процессов перекисного окисления или других немитохондриальных реакций. Например, такими клетками являются фагоциты, гепатоциты. Но лимфоидные клетки в наших экспериментах расходовали более 99% потребляемого O_2 именно для нужд окислительного фосфорилирования в митохондриях. Выключение "дыхания" митохондрий с помощью цианида натрия приводило к падению скорости потребления O_2 практически до нуля.

Измерение скорости потребления O_2 лимфоцитами мы проводили полярографическим, методом в специальной герметической ячейке, в которую был вмонтирован платиновый O_2 - электрод. В ячейку помещали 700 мкл

суспензии, содержавшей 2 – 3 x 10^7 лимфоцитов селезенки мыши (в расчете на 1 мл питательной среды).

Следует отметить, что вследствие восстановления O_2 на платиновом электроде при герметизации ячейки происходило поглощение O_2 самим платиновым электродом. Эффект поглощения O_2 электродом был значительно меньше поглощения O_2 клетками. Несмотря на это, эффект электрода измерялся и учитывался в каждом анализе. Для этого перед исследованием скорости поглощения O_2 суспензией клеток мы прописывали поглощение O_2 электродом. В каждое измерение "дыхания" клеток вносилась поправка на "эффект электрода".

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

O2 uptake by mouse spleen cells placed in a sealed polarographic cell. The time point 0 corresponds to the beginning of cell incubation in the sealed cell of the polarograph. O2 level equal to 0.2 mM corresponds to the initial content of incubation O2 in the medium at time 0 at a temperature of 37º C. O2 level equal to 0.02 mM (marked by a thin horizontal line) - the minimum possible oxygen concentration in the incubation medium placed in this polarographic cell for a given degree of sealing of the cell.

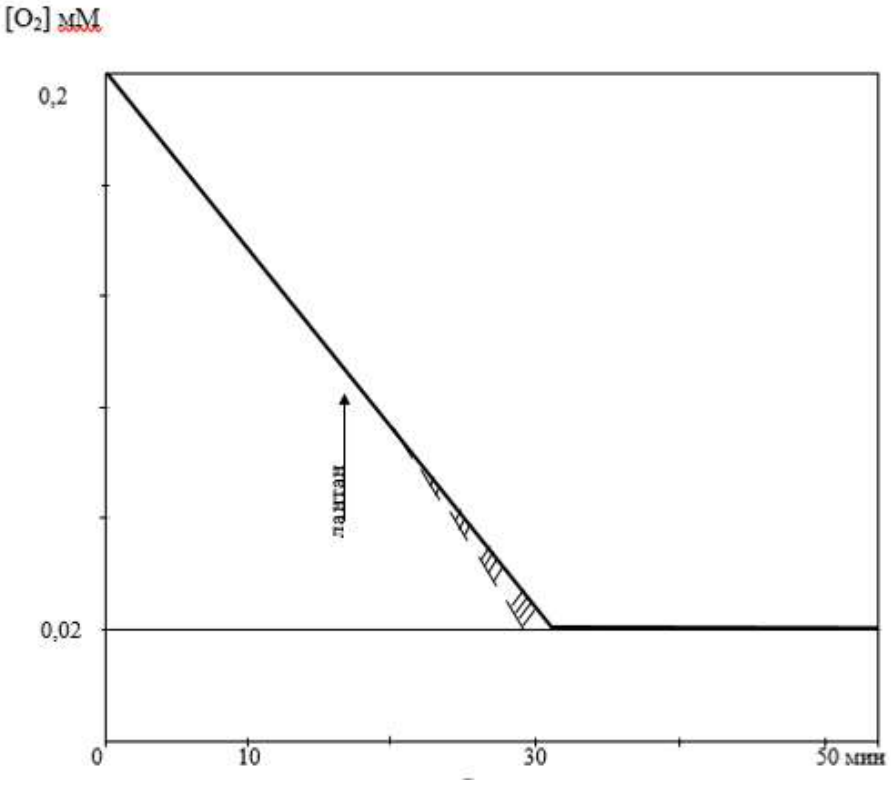
When incubating 2 - 3 x 107 lymphocytes in 1 ml of the medium, the cells consumed O2 at a stationary rate, reducing the O2 concentration to 0.02 mM approximately within 45-60 minutes (Fig. 1). This allowed us, in the course of continuous recording of the respiration of cells placed in a hermetic cell, to investigate the effect of the "injection" of some drug on the rate of consumption of O2 by cells. That is, we could measure the rate of O2 consumption by cells before exposure and in dynamics after exposure to cells with some substance. If cells were exposed to an ion-transporting ATPase inhibitor, then a decrease in the rate of O2

consumption in the first minutes after exposure to the inhibitor showed the share of this ATP-consuming process in the total consumption of O2 by the cell and, consequently, in the total consumption of ATP by the cell. Hereinafter, we will refer to this part as "the fraction of a given ATPase in total cellular ATPase".

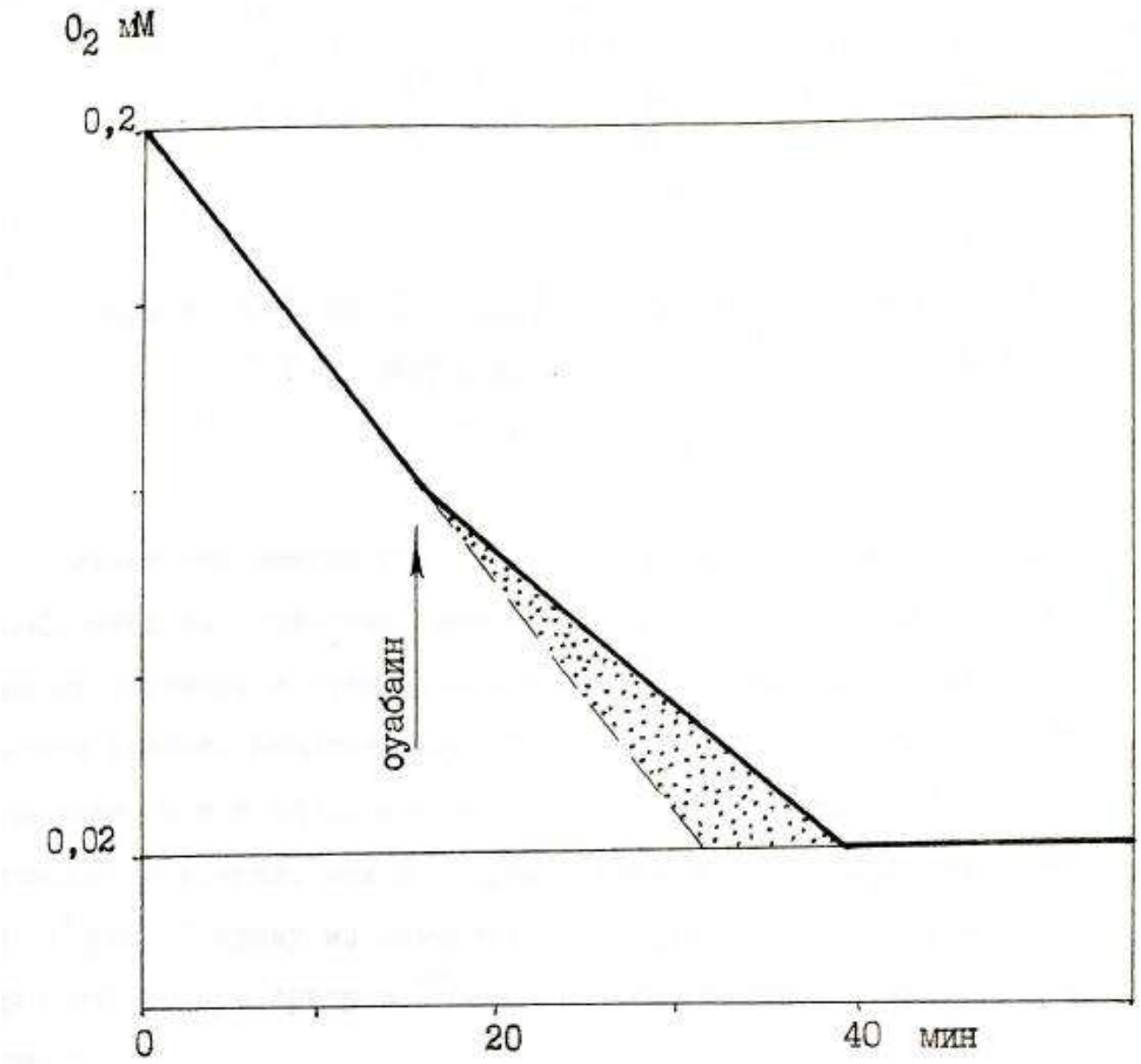
Thus, the effect of specific inhibitors of ion-transporting ATPases

on the intensity of O2 consumption by intact (non-activated) lymphocytes. A selective blocker (Na +, K +) - and ATPase - suabain at a final concentration of 10-4 M and a selective inhibitor of Ca2 + - ATPase - lanthanum (LaCl3) at a final concentration of 10-3 M were used. 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+ - ATPase, non-activated lymphocytes consume no more than 5% of the total ATP produced in the cell.

Specific inhibition of (Na+, K+) - transporting ATPase led to a decrease in the intensity of cellular respiration by an average of 18% (Fig. 3). That is, in non-activated lymphocytes, about 18% of the total ATP produced in the cell was used to work (Na +, K +) - ATPase of the plasma membrane.



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



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

RESULTS OF THE STUDY

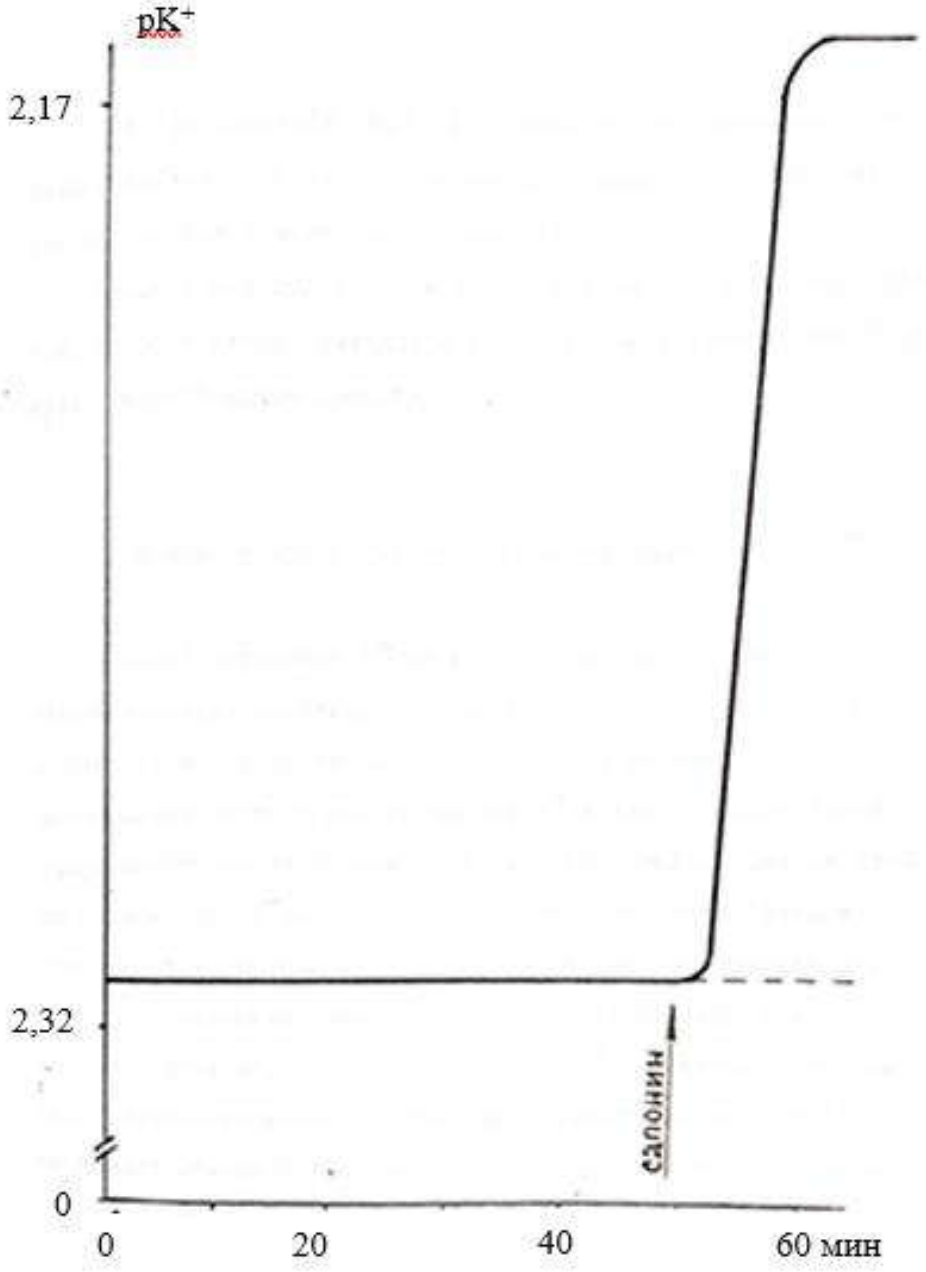
Chapter 3

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 content of ions inside the cells. The concentration of K+ in the extracellular medium is 30-35 times lower than in the cytosol. On the contrary, the concentration of Ca2+ inside the lymphocytes is less than in the incubation medium by at least 103 times. Therefore, we measured the passive fluxes of K+ from the cell to the extracellular medium and 45Ca from the incubation medium to the cytoplasm.

1. PASSIVE K+ FLOWS.

When incubating splenic lymphocytes 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+ fluxes 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, like



Rice. Fig. 4. Stability of the level of extracellular K+ in a suspension of lymphoid cells of the spleen during their incubation in a thermostatically controlled (37º C) cell with a valinomycin electrode. The abscissa shows the time of cell incubation in vitro, the ordinate shows the concentration of K+ in the extracellular medium (pK+ = - Ig [K+]). During 50 min and cell incubation, the concentration of K+ in the medium does not change. To illustrate the maximum release of K+ from cells, data are

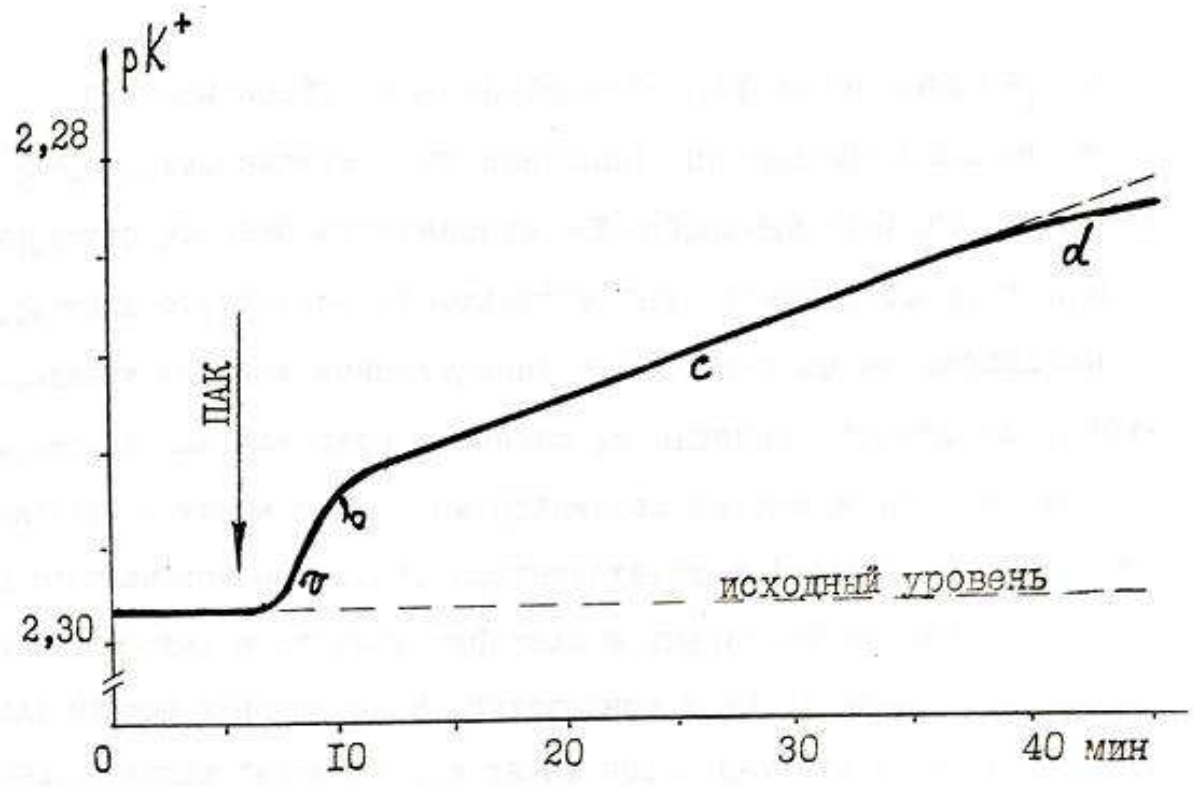
presented for complete cell lysis with saponin. The moment of lysis is marked with an arrow.

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

Influencing the polyanion (PAA) during cell incubation, we found a distinct increase in the concentration of K+ in the culture medium (Fig. 5).

MEMBRANE PERMEABILITY MEASUREMENT KINETICS FOR K+.

The process of K+ leakage from PAA-activated cells had a clearly non-linear kinetics. On fig. Figure 5 shows the kinetics of measuring the level of extracellular potassium after the introduction of PAA into a suspension of lymphocytes. Signs of K+ release from cells are already visible in the first minute after PAA exposure. The highest average rate of K+ outflow 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+ outflow, having somewhat decreased, stabilized. The steady state of K+ outflow rate was observed usually within 15-20 subsequent minutes (phase c). It was in this period that we could accurately measure the stationary 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.

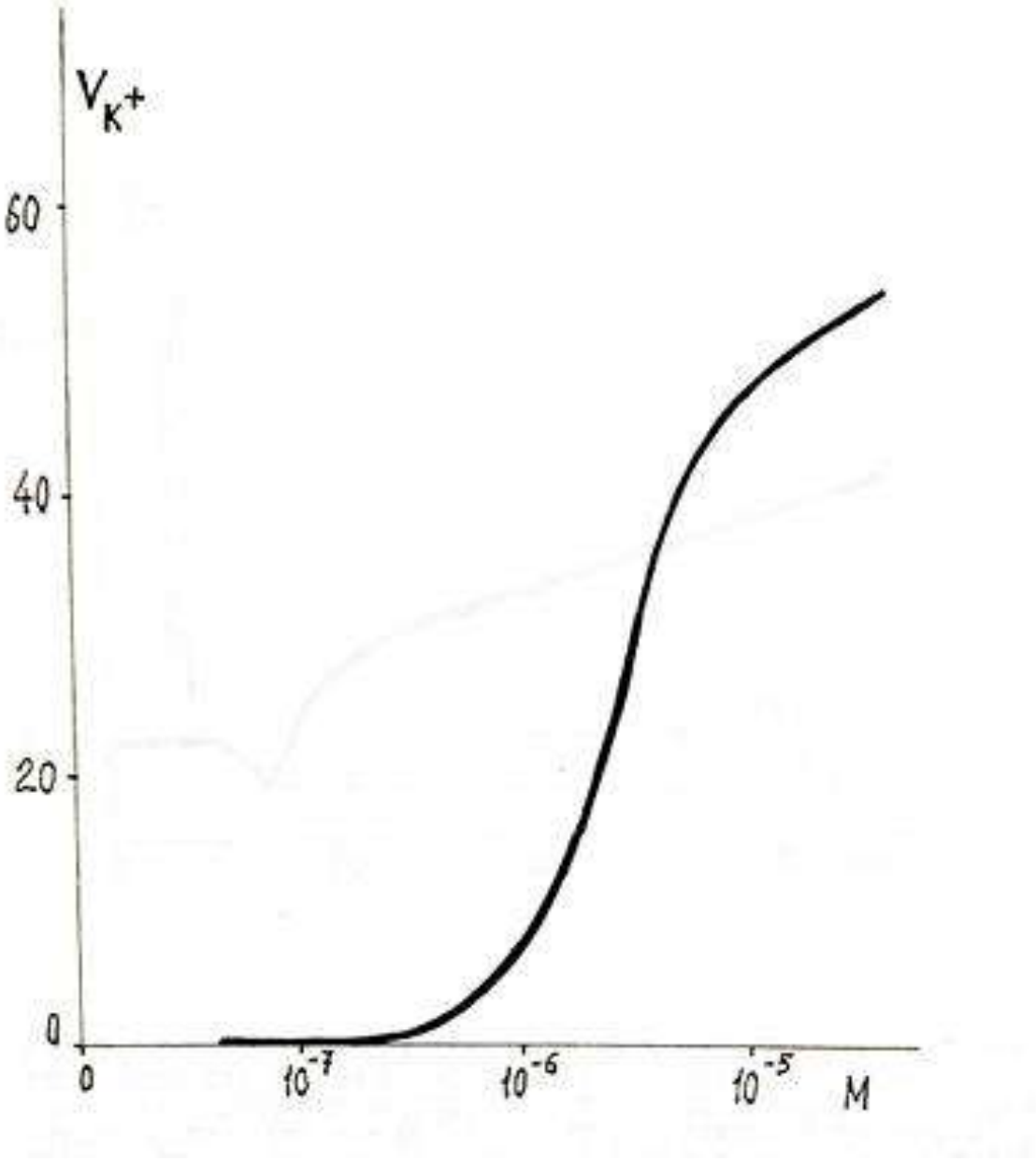


Rice. Fig. 5. Kinetics of increasing K+ flux from cells into the extracellular medium under the influence of a polyanion (polyacrylic acid, final concentration 10-6 M). The arrow indicates the moment of "injection" of the polyanion into the suspension of lymphoid cells. The phases are denoted by the kinetic curve as a, b, c, d (see text for explanations). On the axes: abscissa - in vitro cell incubation time; ordinate - K+ concentration in the extracellular medium (pK+ = - Ig [K+]).

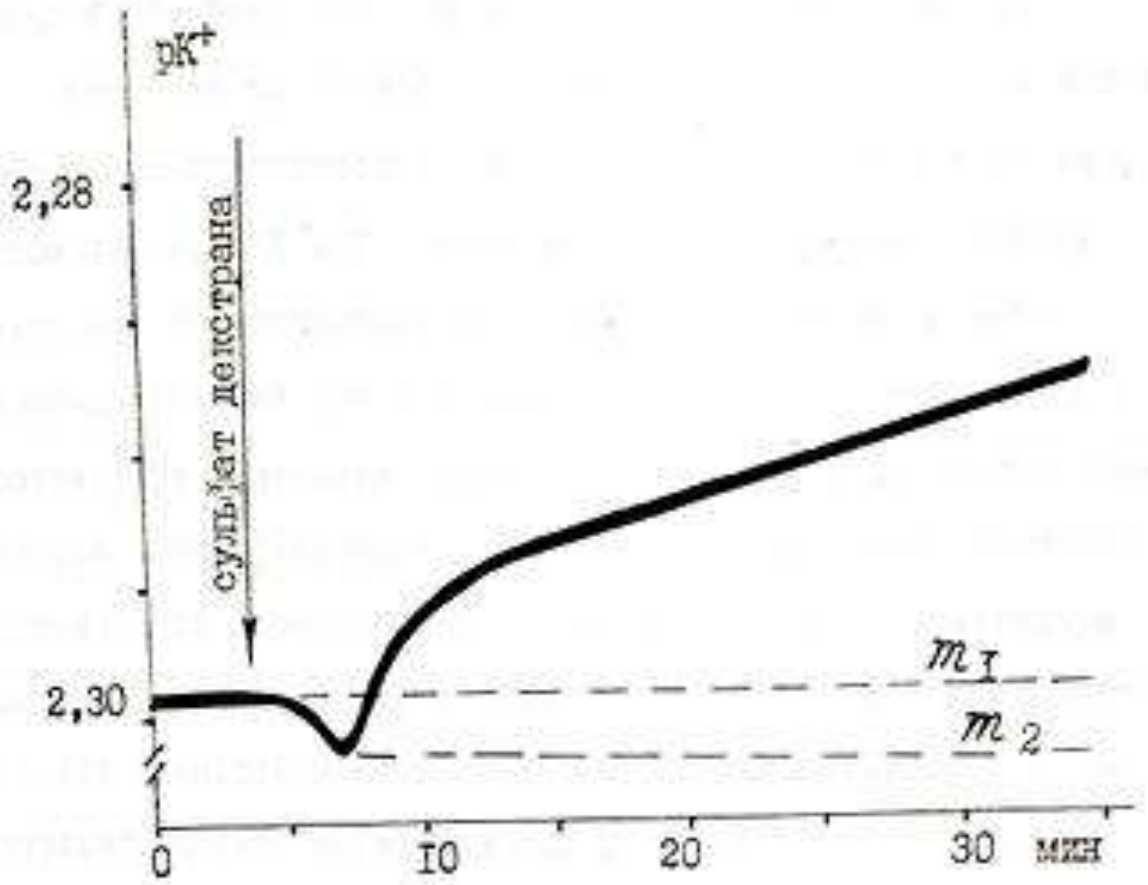
DOSE DEPENDENCY.

The intensity of PAA-induced leakage (VK) from the cells directly depended on the final concentration of the polymer. The nature of the dose dependence is S - shaped (Fig. b). It should be noted that there is no distinct "saturation". That is, when creating high concentrations of the polyanion, we did not observe the disappearance or significant decrease in the effect on an additional increase in the dose of the polymer. Most likely, we did not reach concentrations corresponding to the "plateau" on 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. For 1 hour, under the influence of the immunostimulating polymer, 100-1000 times less K+ was released from the cells into the medium than with the complete lysis of the same cells with saponin. In addition, as noted above, the effect of K+ leakage induced by the polyanion practically ceased already 35–40 minutes after the onset of exposure (see Fig. 5).

Like PAA, another polyanionic mitogen, dextran sulfate, also caused an increase in the permeability of the lymphocyte membrane for K+. The kinetics of K+ leakage was practically the same as when PAA was used (Fig. 7). Only the lag phase preceding phase "a" was not several tens of seconds, but 2-3 minutes. A similar PAA was the dose dependence when exposed to dextran sulfate. 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 manifests itself at molar concentrations an order of magnitude higher than when PAA is used (Fig. 8).



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

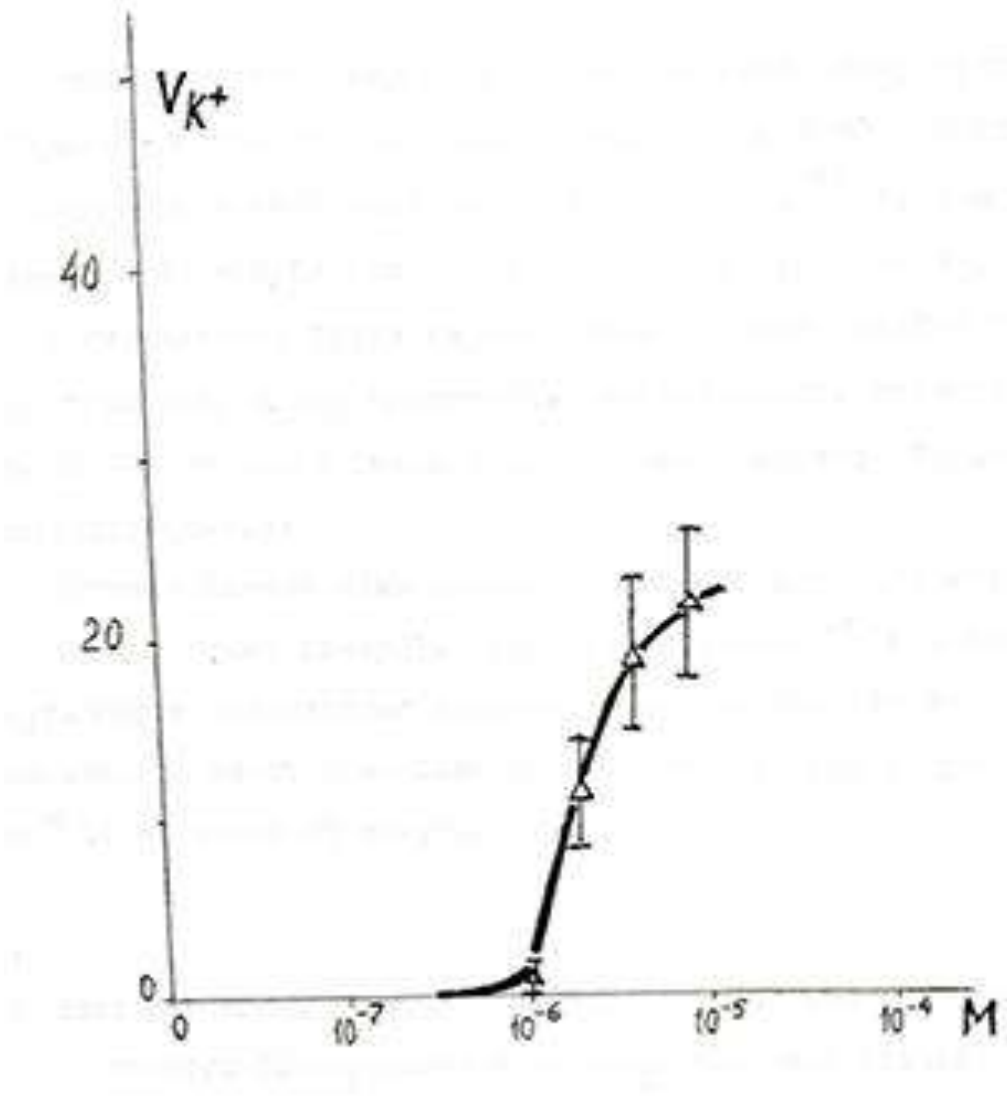


Rice. 7. Effect of dextran sulfate on the permeability of the lymphocyte membrane for K+. Along the axes: abscissa - the time of incubation of lymphoid cells, in vitro; ordinate – concentration of K+ in the extracellular environment (pK+ = - Ig [K+]). m1 is the initial level of K+ in the medium, m2 is 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 an 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 environment (regardless of the presence of cells). Therefore, when dextran sulfate was "injected" into a suspension of lymphocytes in the lag phase, we could observe a slight decrease in the level of K+ in the extracellular medium, and then the entire pattern of K+ leakage from cells, characteristic of a polyanion. The initial effect of a decrease in K+ in the medium could be avoided by first adding a small volume of 1 M solution of KS 1 to the dextran sulfate solution to "saturate" the dextran sulfate with potassium. Such a preliminary "saturation" of dextran sulfate with potassium did not affect the ionophore-like activity of the polyanion.

2. Passive Ca2+ flows

As 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 for 45Ca. Comparing this indicator in cultures of non-activated cells and in cell cultures activated by polyanion, it was possible to judge the effect of the immunostimulatory polymer on the membrane permeability for Ca2+.



Rice. 8. Dose dependence of the ionophore-like action of the polyanion, dextran sulfate, on the plasma membrane of lymphocytes. 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 from the VK+ values in the stationary portion of the kinetic curves recorded using various concentrations of the polyanion.

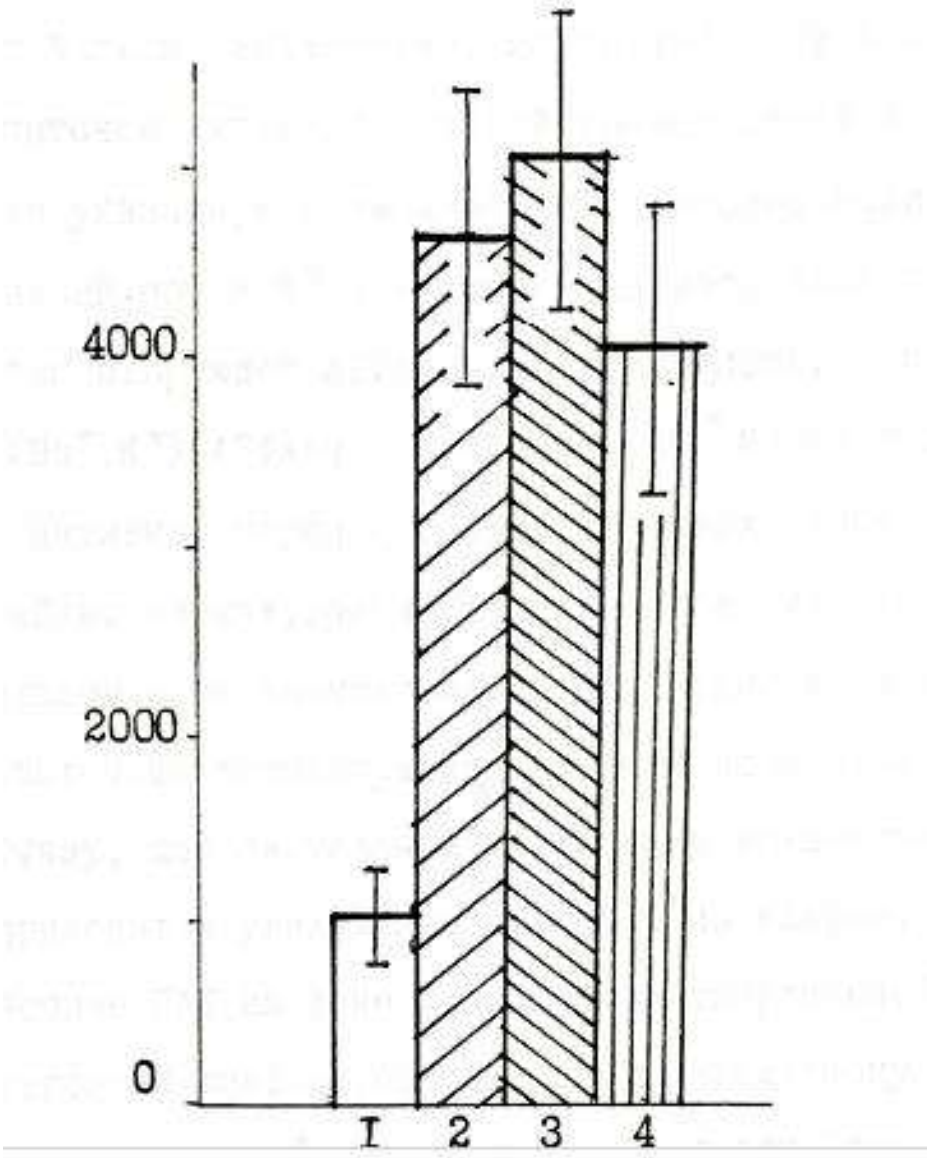
It turned out that mouse spleen lymphocytes incubated for 30-60 minutes in the presence of immunostimulatory concentrations of PAA contain several times more 45Ca isotope than the same lymphocytes incubated in the absence of PAA (Fig. 9).

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

The variant 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. Influence of PAA on the ionic permeability of the membrane under conditions of complete blocking of the ion-transporting ATPase.

The increase in passive fluxes of K+ and Ca2+ across the plasma membrane that we found could be a consequence of the inhibition of ion-transporting ATPases under the influence of the polyanion. In an intact cell, the flows of ions in the direction of their electrochemical gradient (passive flows) are precisely compensated by the active transfer of the same ions in the opposite direction. Transport of ions against the electrochemical gradient is carried out by membrane ATPases. Inhibition of the work of ATPases 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 flows. In fact



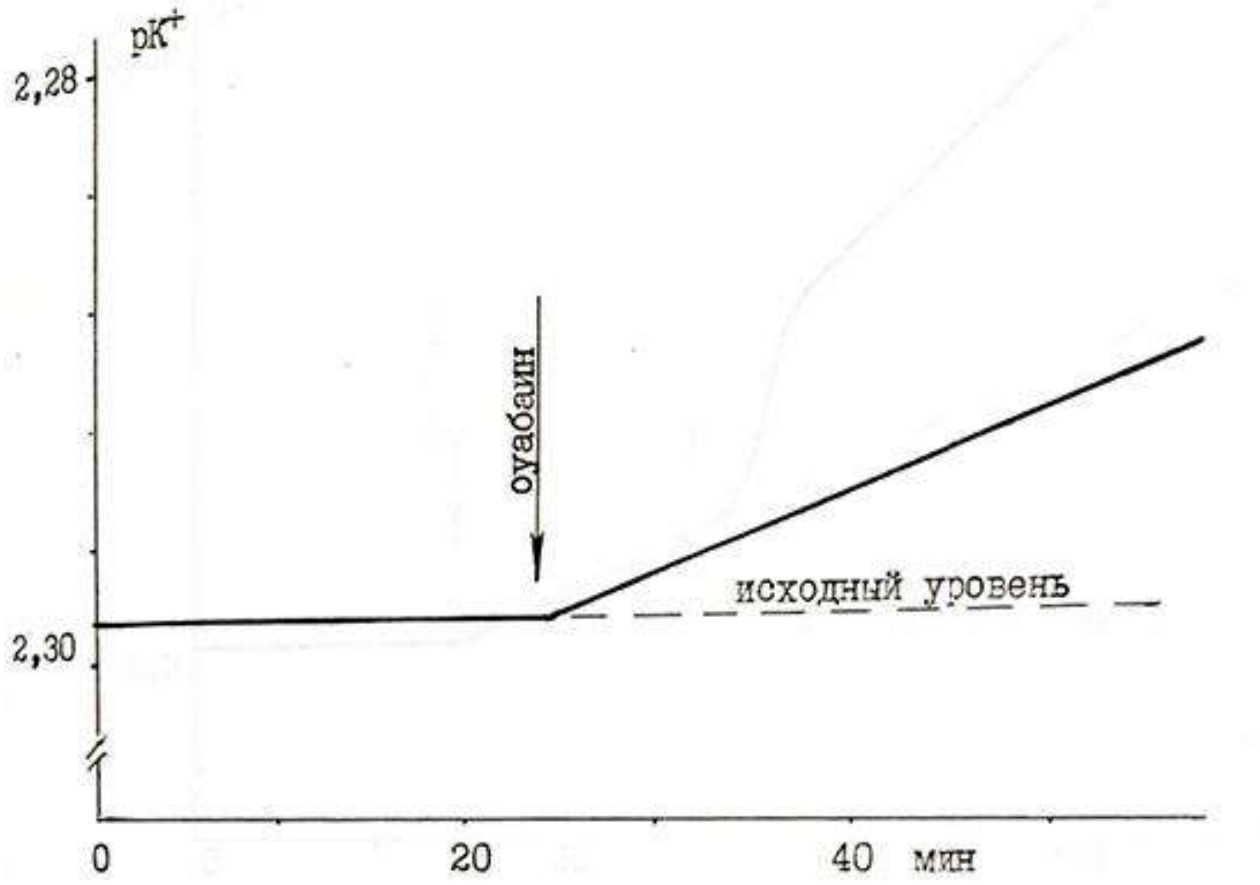
Rice. 9. Effect of polyanions on the incorporation of 45Ca from the medium into the cytosol of lymphoid cells. The y-axis shows the radioactivity of cell extracts (imp/min). Extracts were obtained from the following cell variants: (i) control non-activated culture of lymphocytes from mouse spleen; (2) cells activated with PAA (50 μg/ml); (3) cells activated with PAA (200 μg/ml); (4) cells activated with dextran sulfate (100 μg/ml).

passive flows could remain unchanged, but the compensation of these flows was blocked by the active work of transport ATPases.

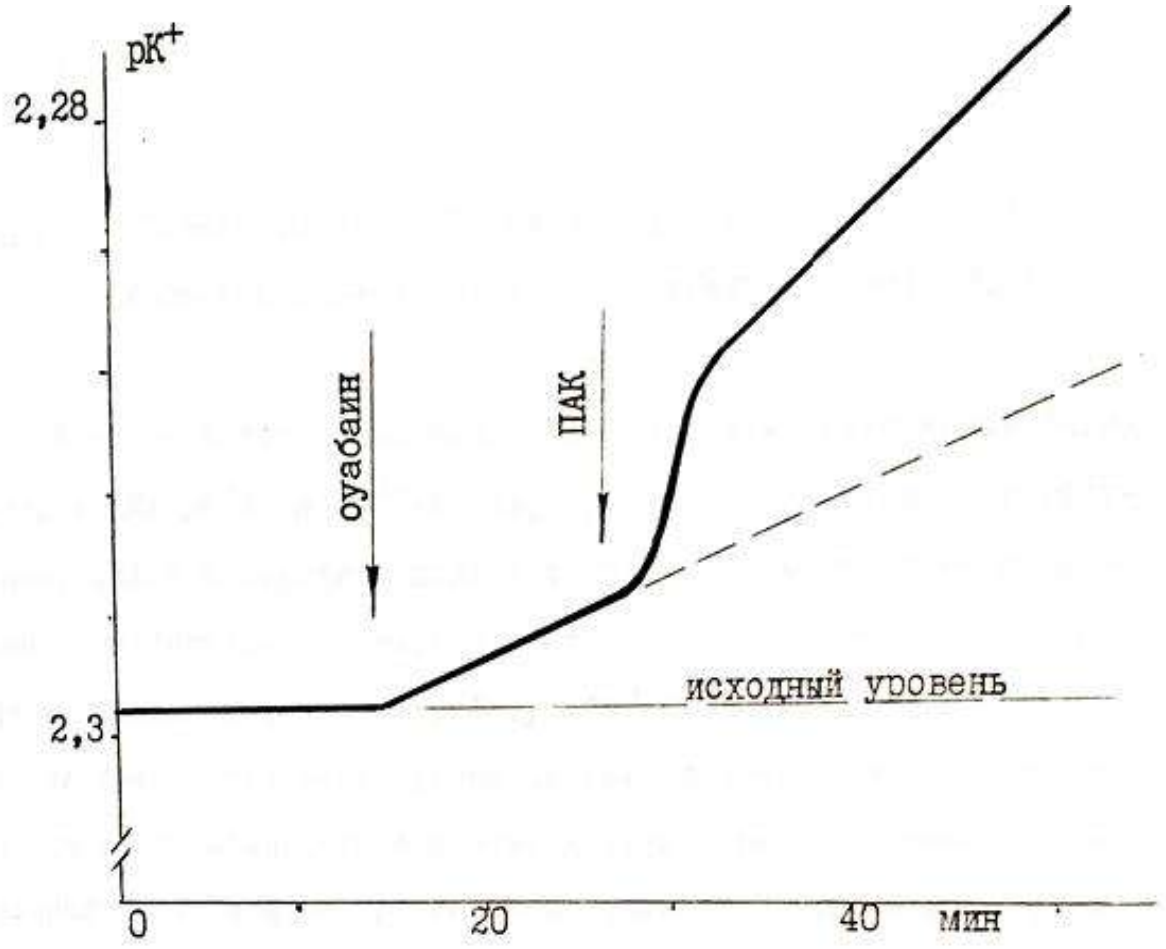
Considering this possibility, we studied the effect of PAA on the passive flow of K+ under conditions of complete blocking of the (Na+, K+)-transporting ATPase. Indeed, when the inhibitor of (Na+, K+)-ATPase - ouabain (10-4 M) is introduced, we completely "switch off" the active transfer of the corresponding 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+)-ATPase, and an additional increase in the concentration of the inhibitor does not increase the release of K+ from the cells. Despite this, the introduction of PAA against the background of a completely blocked (Na +, K +) - ATPase into a suspension of lymphocytes led to a noticeable increase in the passive flow of K + into the extracellular environment (Fig. 11). At the same time, the effects of PAA on intact and ouabain-treated cells were comparable in magnitude.

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

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



Rice. 10. Change in the level of extracellular K + with complete blocking of (Na +, K +) - ATPase by 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.



Rice. 11. Influence of PAA polyanion on cell membrane permeability under conditions of complete blocking of (Na+, K+)-ATPase by ouabain (10-4 M). Along the axes: abscissa - the 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 (Na, K) - and Ca - TRANSPORTING ATPASES TO THE LYMPHOCYTE MEMBRANE.

In Chapter 3, it was noted that with the help of selective venous inhibitors, (Na +, K +) - and Ca2 + - ATPases, ouabain and lanthanum, respectively, it is possible to determine the proportion of ATP consumed by these enzymes relative to the total cellular ATPase of intact lymphocytes. It turned out that up to 18% of the total ATP produced and consumed in these cells is consumed for the work of (Na+, K+)-ATPase in non-activated lymphocytes (Fig. 3). Such a significant "energy capacity" of the sodium-potassium pump of the outer cell membrane, which is significant on the scale of the cell, apparently indicates the importance of this mechanism in the viability of the cell. At the same time, this fact may be due to 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 minimized. And therefore the share of (Na +, K +) - ATPase in the total consumption of ATP in resting lymphocytes is quite large.

The process of active transport of Ca2+ against its concentration gradient requires significantly less energy consumption. The lanthanum-inhibited Ca2+-ATPase in resting lymphocytes consumes so little ATP that we were unable to measure it accurately. Taking into account the magnitude of the error of the method used, we can firmly believe that no more than 4-5% of the total ATP produced in these cells is consumed for the work of Ca2+ - ATPase in non-activated lymphocytes (Fig. 2.). Injection of a mitogenic polyanion - PAA - into a suspension of lymphocytes led to a significant activation of total ATP - the consuming activity of these cells. The rate of O2 consumption by cells, and hence the rate of ATP synthesis and consumption, increased under the influence of polyanion by an average of 1.3-1.5 times (Table 1). At the same time, inhibitory analysis showed that in such cells activated by the polyanion, a significant activation of the work of ion-transporting ATPases occurs. The inhibitory effect of lanthanum on the respiration of lymphocytes sharply increased. The rate of O2 consumption by lymphocytes, previously activated by PAA, decreased under the influence of lanthanum by 19-39% (Table 1, Figs. 12 and

13). Therefore, after exposure to the mitogenic PAA polyanion, a strong activation of the Ca2+-transporting ATPase occurred in the plasma membrane of lymphocytes. The work rate of (Na+, K+)-ATPase after the addition of PAA also increased, but to a lesser extent than Ca2+-ATPase. Thus, inhibition of ATPase in lymphocytes activated by PAA with ouabain (Na+, K+) 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 the total cellular ATPase upon the addition of PAA is provided only by the activation of (Na+, K+)- and Ca2+-ATPases. To do this, we measured the inhibitory effect of simultaneously added lanthanum and ouabain in suspensions of non-activated lymphocytes or PAA-stimulated cells. It turned out that the rate of consumption of O2 by cells decreases after the simultaneous in vitro injection of both inhibitors to 78–80% of the initial level. In cell suspensions previously activated with PAA.

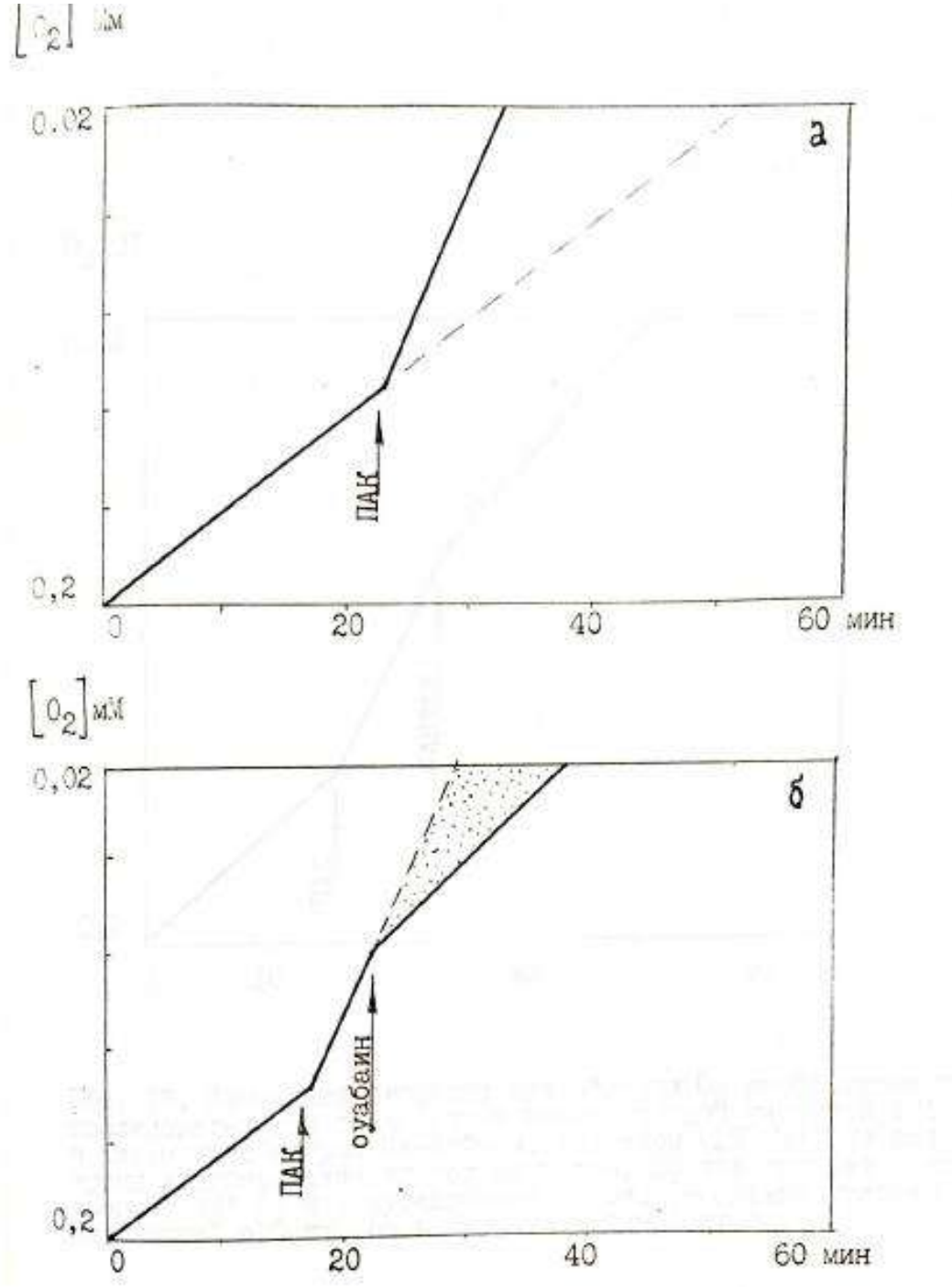
Table 1.

Effect of polyacrylic acid on the rate of O2 consumption by lymphocytes

Dynamics of V - O2 during continuous recording*	No. of a series of experiments				note
	1	2	3	4	
VISH, mM/h	0,130	0,104	0,117	0,125	-
"Injection" PAK in vitro, mgc/ml	50	50	200	200	-
VPAK, mM/h	0,166	0,135	0,176	0,183	-
(VPAK - VISH)/VISH, %	28	30	50	46	Indicators of total ATPase activation

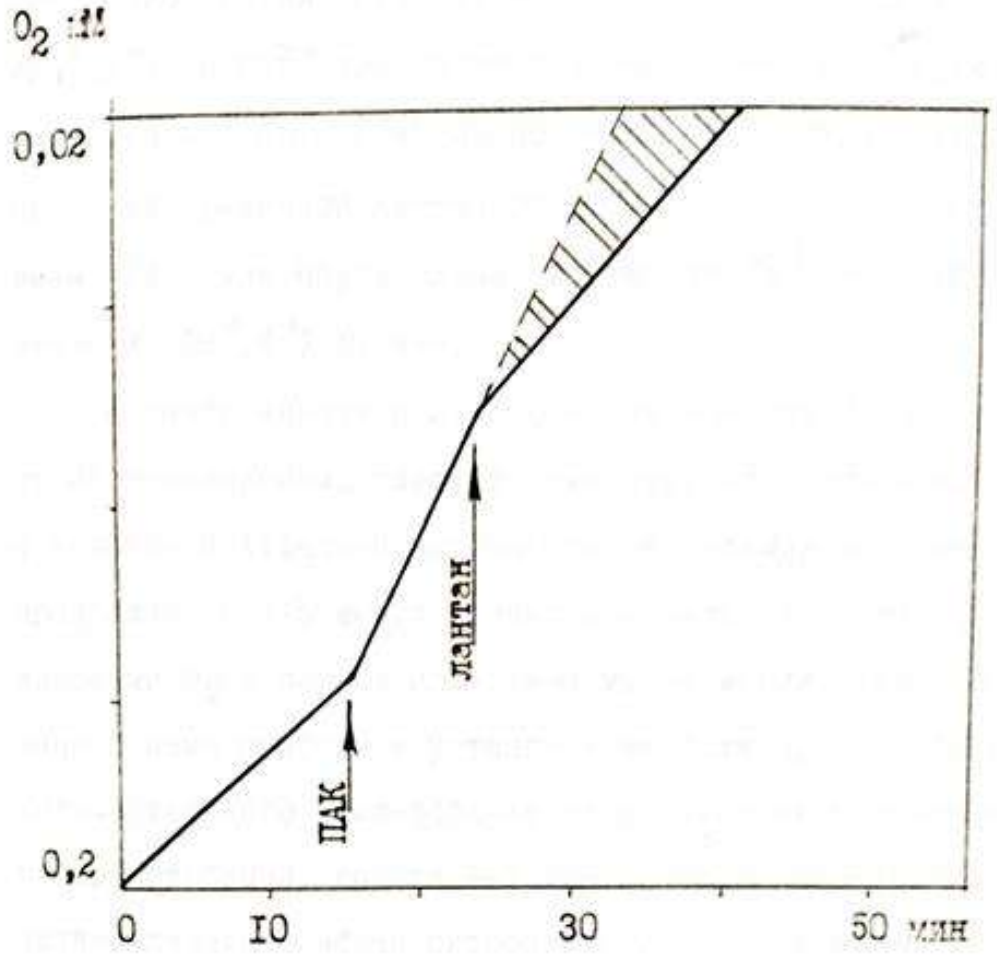
					under the action of PAA
Vlant, mm/h	0,134	-	0,130	-	-
(VPAK – Vlant)/VISH, %	25	-	39	-	Activation indices of Ca2+ dependent ATPase after PAA "injection"
(VPAK – Vlant)/VPAK, %	19	-	26	-	
Vlant/VISH, %	103	-	111	-	-
Vstr, mm/h	-	0,107	-	0,150	-
(VPAK – Vstr)/VISH, %	-	27	-	26	Activation indices (Na+, K+) - ATPases after "injection" of PAA
(VPAK – Vstr)/VPAK, %	-	21	-	18	
Vstr/Vpak	-	103	-	121	

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



Rice. Fig. 12. Changes in the rate of O2 consumption by lymphocytes upon exposure to: (a) polyacrylic acid (10-6 M) a or (b) sequentially polyacrylic acid (10-6 M) and then an inhibitor (Na+, K+) - ATPase, ouabain. Along the axes: abscissa, in vitro cell incubation time; ordinate, O2 concentration (mM) in the incubation

medium. The arrows indicate the moments of "injection" of effectors into the studied suspension of lymphocytes.



Rice. 13. Changes in the rate of O2 consumption by lymphocytes under sequential exposure to polyacrylic acid (PAA, 10-6 M) and then to the Ca-ATPase inhibitor, lanthanum (10-3 M). The abscissa shows the time of cell incubation in vitro, the ordinate shows the concentration (mM) of O2 in the incubation medium. The arrows indicate the moments of "injection" of effectors into the suspension of lymphocytes.

The total cellular ATPase reached 148% of the initial level. Consequently, after PAA treatment, in addition to (Na+, K+)- and Ca2+-transporting ATPases, some other ATP-consuming processes are activated. However, the vast majority of the increase in total cellular ATPase, which occurs under the action of PAA, was due to the activation of Ca2+ -ATPase and, to a lesser extent, (Na+, K+) -ATPase.

Regarding the kinetics of changes in ATPase activity after exposure to polyanion, it should be noted that activation began with fluctuations in ATPase activity. Fluctuations in ATPase activity lasted 3-5 minutes. We could not accurately measure the rate of O2 consumption by cells during the period of oscillations, since the instrumental setup we used was not adapted for the 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 ATPase activation by the polyion, directly depended on the dose of the polymer. When using immunostimulatory doses of PAA, as a rule, we did not observe ATPase activation 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 the cells. In addition, at high concentrations of polyacid, acidification of the medium becomes significant, with which many incomprehensible effects can be associated.

In general, it is clear that non-toxic immunostimulatory doses of PAA significantly and rapidly activate membrane ion-transporting ATPases. Another immunostimulating polyanion, dextran sulfate, had essentially the same effect [1]. We assumed [4] that the observed stimulation of the active transport of K+, Pa+, Ca2+ ions is a secondary phenomenon occurring as a result of an increase in the passive fluxes of the same ions. Apparently, the polyanion, interacting with the cell membrane, induces a rapid increase in its permeability for ions. A change in the

transmembrane gradients of ions, in turn, leads to a rapid activation of the work of the corresponding ATPases that transport the same ions across the membrane in the direction opposite to the passive flow. That is, the activation of ATPases is aimed at compensating for increased ion fluxes and restoring the initial ratio of ions between the cell and the environment. Of fundamental importance is the question of the significance of the detected changes in the permeability of the cell membrane for the activation of the physiological response of lymphoid cells to polyions. In other words, is the increase in the permeability of the cell membrane one of the key links in triggering the cell response to the polyanion, or is this change just one of the consequences of the interaction of the polymer with the cell, which is not directly related to the activation of the immune response. The next chapter of the dissertation is devoted to the analysis of this issue.

Chapter 5

PERMEABILITY OF CELL MEMBRANE FOR ACTIVATION OF LYMPHOCYTE RESPONSE TO POLYANION.

The polyanion induces a rapid increase in the permeability of the cell membrane for ions and a compensatory reaction of ion-transporting ATPases. What does it matter, key (to trigger the response of a lymphoid cell) or concomitant, not related to the process of activation of lymphocytes? To analyze this issue in the experiment, we used two approaches. First, we studied the effect of the polyanion on the membrane permeability of subpopulations of lymphocytes that react and do not react to the polyanion by proliferation in vitro. Secondly, we studied the immunomodulatory properties of substances of different chemical structure, which are not polymers, but are capable of increasing the permeability of the cell membrane for ions.

I. Влияние полианиона на Транспорт ионов в мембране Т- и В-лимфоиитов.

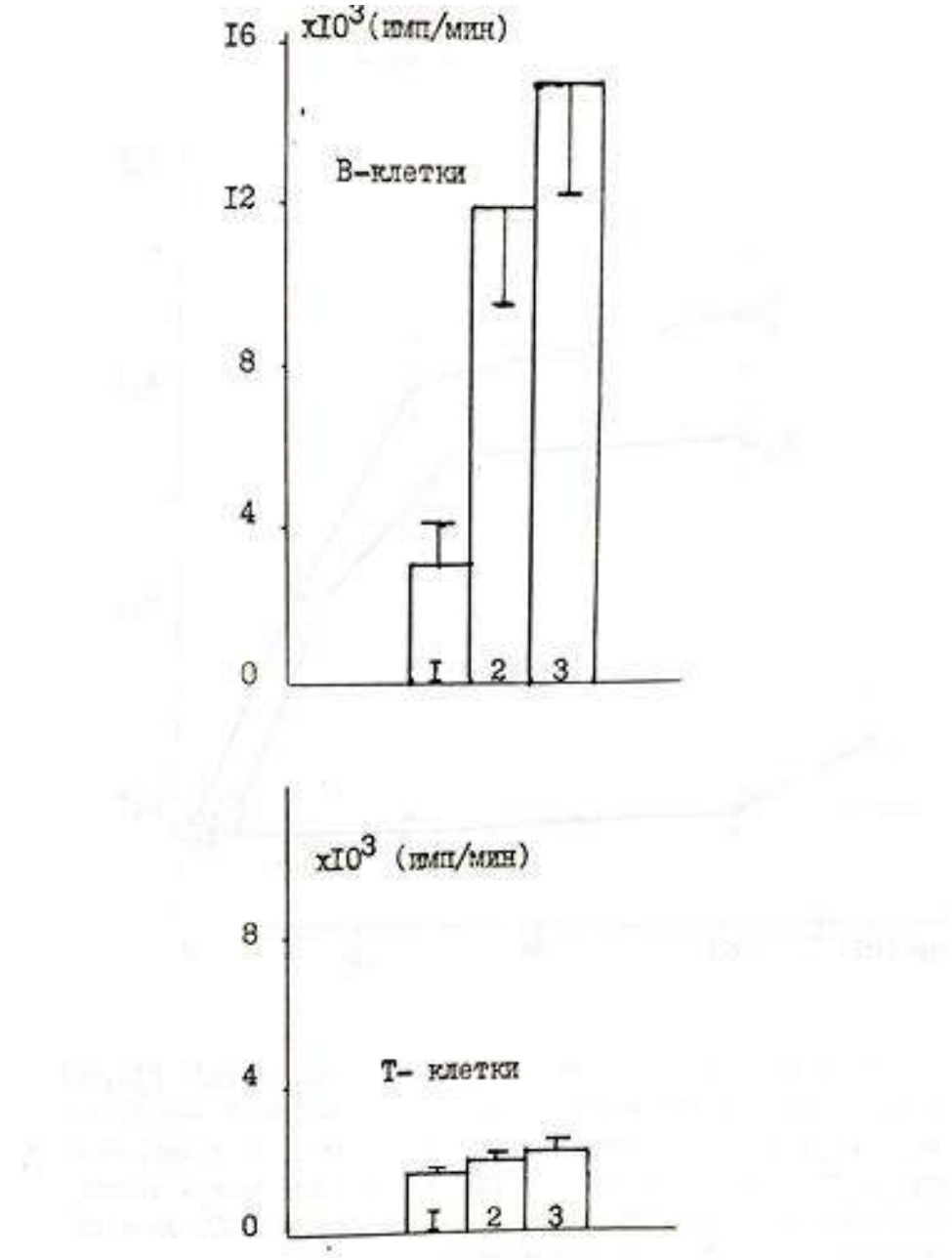
It is known that polyanions (PAA, dextran sulfate) exhibit the properties of B-cell mitogens and do not activate the response of T-lymphocytes [44]. Therefore, it was of interest to study the membrane-active effect of the polyanion on T- and B-lymphocytes separately.

As described in the Materials and Methods chapter, spleen 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 non-nylon-adhering cells. This fraction of non-nylon-adhering cells was poor in B-lymphocytes. We will conditionally 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 B-thracia, but not in the T-cell fraction (Fig. 14). This was in good agreement with previously published data [44].

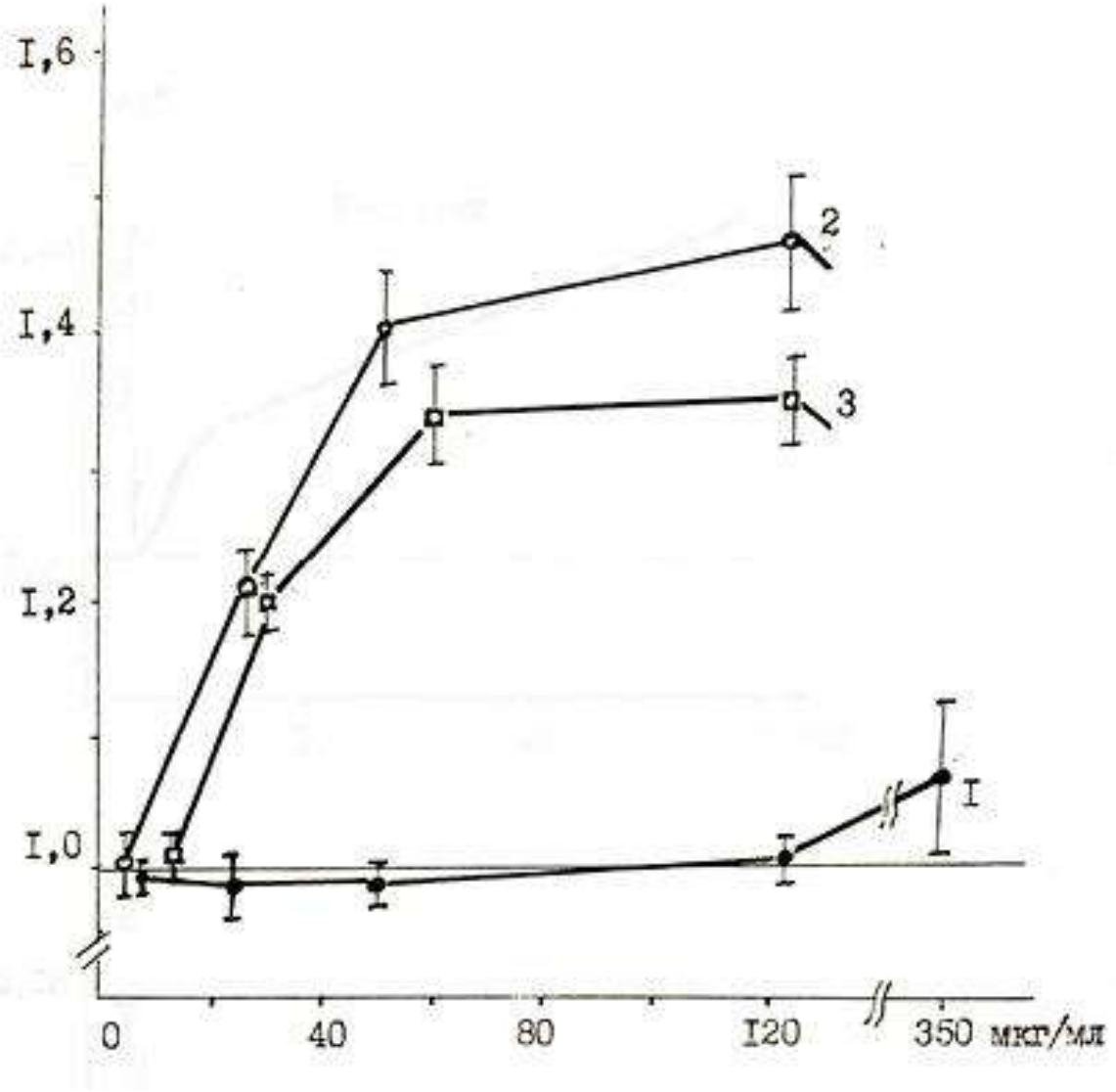
The study of the action of PAA at the level of membrane transport of ions also showed the selectivity of the polyanion in relation to B - lymphocytes. Thus, activation of ion-transporting membrane ATPases, as well as an increase in passive fluxes of K+ and Ca2+, were observed in our experiments only in a suspension of B-cells (Fig. 15 a, b, c,). Mature T-lymphoites from the spleen did not react to the addition of a polyanion by activation of membrane ion-transporting systems. In connection with the data obtained, the following working hypothesis was put forward [90]. It was based on numerous data on the differences between T - and B - lymphocytes in terms of surface electric 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 adherence of T - cells to a similarly charged substrate - plastic, nylon, etc. The working hypothesis that guided our work suggested that the absence of the 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 lower negative surface charge, in particular, on immature T - cells from the mouse thymus and on mature T - cells previously treated with neurons.

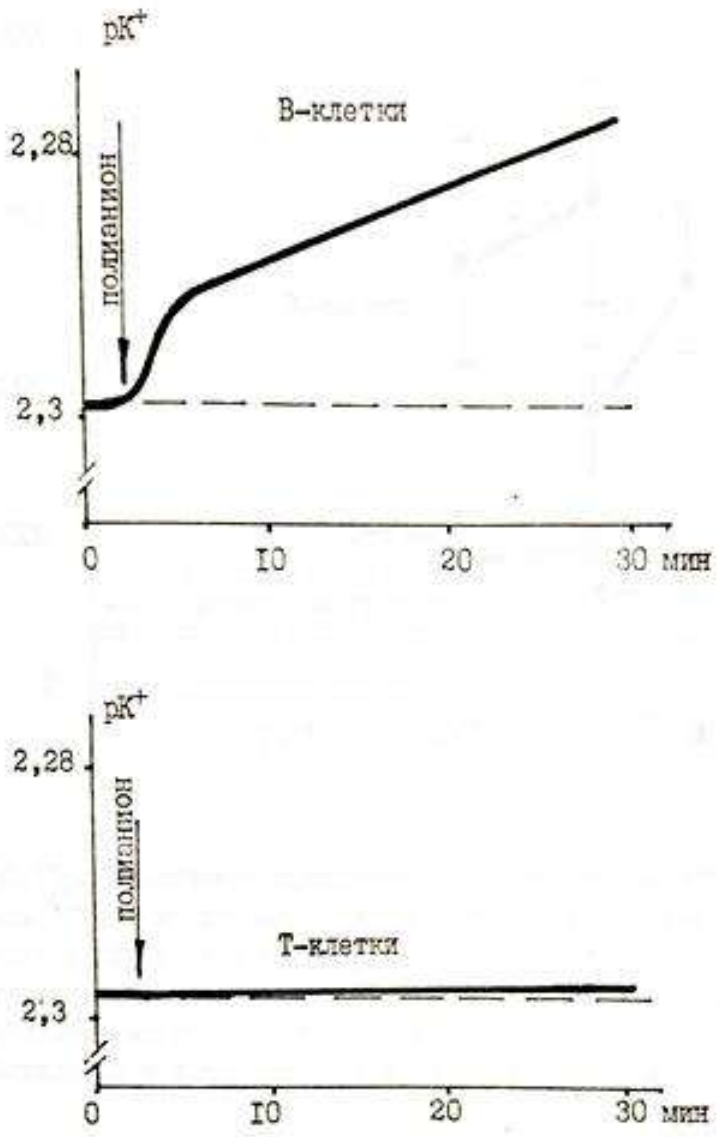


Rice. 14. Mitogenic effect of polyanions on enriched subpopulations of T- and B-lymphocytes in cell culture in vitro.

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



Rice. 15 Activation by polyanion of ion-transporting ATPases in the membrane of T- and B-lymphocytes. The abscissa shows the final concentration of polyacrylic acid in vitro (μg/ml). On the y-axis - the activation coefficient of the total cellular ATPase (the level of ATP - the consumer of cell activity immediately before their



activation by polyaion is taken as 1.0). The data obtained during the activation of the fraction of T-cells of the spleen (1), the fraction of B-cells of the spleen (2), and such T-cells of the spleen, previously treated with neuraminidase (3), are presented.

Rice. 15 b. Influence of polyanion on passive fluxes of K+ from cells into the extracellular medium in a suspension of the enriched fraction of B-lymphocytes (a) and T-lymphocytes. Along the axes: ordinate - concentration of K + (pK + = - Ig [K +]) in the extracellular environment; abscissa - cell incubation time in vitro (min).

The arrows show the moments of "injection" of polyacrylic acid (final concentration 10-6 M) into the suspension of lymphoid cells.

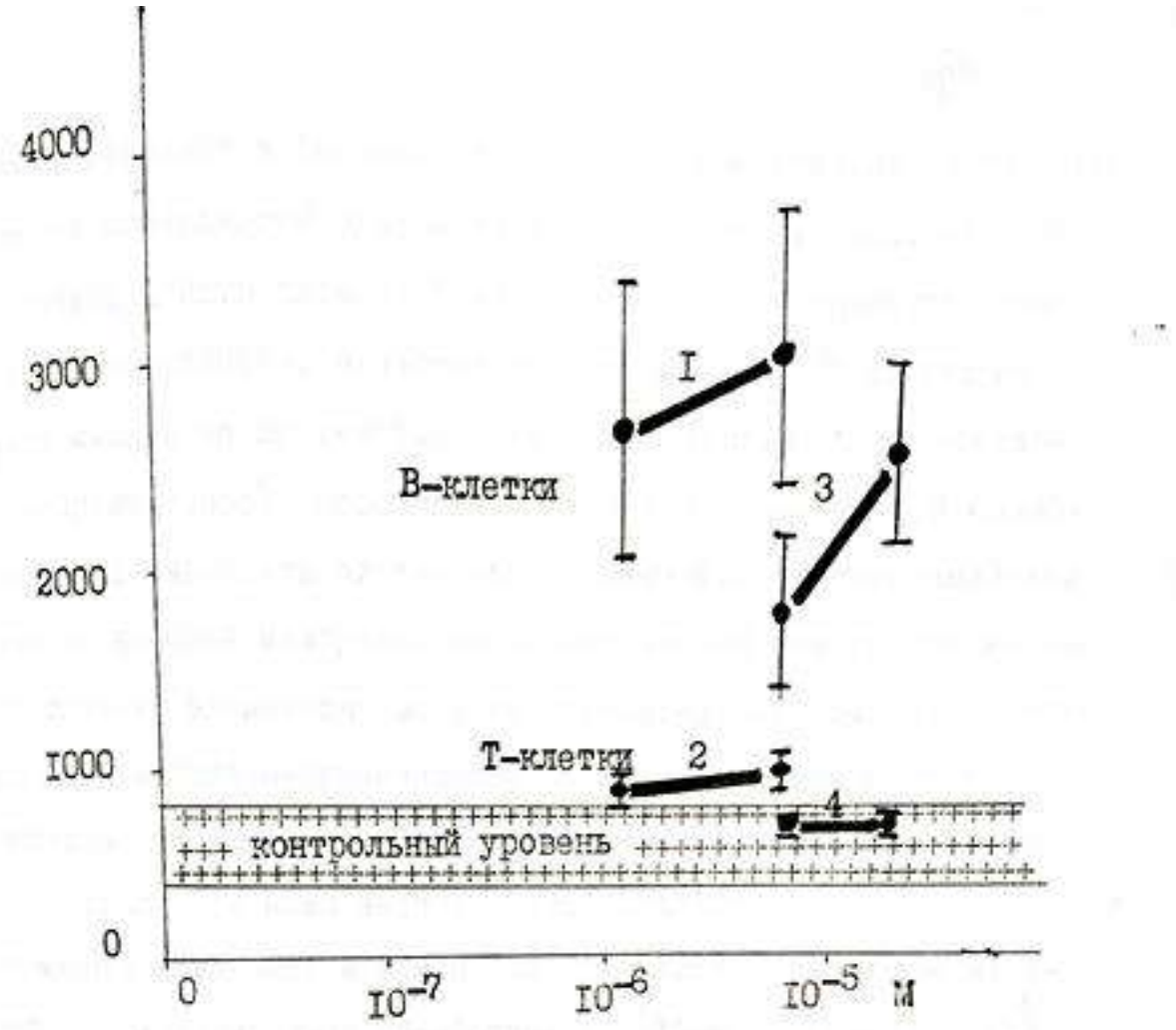


Fig. 15 c. An increase in the permeability of the cell membrane for the 45Ca isotope in cultures of B-lymphocytes (1, 3), in contrast to T-lymphocytes (2, 4), under the influence of polyacrylic acid (1, 2) or dextran sulfate (3, 4). The y-axis shows the radioactivity (imp/min) of cell extracts. The abscissa shows the concentration of the polyanion in the incubation medium during cell activation in

vitro. Treatment of T - cells with neuraminidase can dramatically reduce the density of terminal N - acetylneuramin groups on the outer membrane. Since it is these groups that are the main element of the high negative surface charge, treatment with neuraminidase can greatly reduce the negative charge density on the surface of T-lymphocytes.

Our experiments have shown that PAK induces pronounced changes in the ion-transporting system of mature T-cells pre-treated with neuraminidase (Fig. 15). The activation of ATPases in these cells was not inferior to the effect of PAA on B-lymphocytes and reached 135% of the initial level. The modification of ion transport in the thymocyte membrane was also effective. That is, the action of the polyanion is not limited to the pool of B cells. Its activating effect on T-lymphocytes with a small negative surface charge is quite possible. Only mature T cells, which have a high density of negatively charged groups on the outer membrane, are not sensitive to the activating action 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 to the activation of divisions. On T-cells of the spleen, which did not divide in response to the addition of a polyanion, we did not observe any early membrane effects either.

2. Immunostimulatory effect of non-polymeric membranotropic substances.

Let us assume that the change in membrane permeability is the key process that activates the cell response to the polyanion and, consequently, determines the immunostimulatory 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 non-polymeric substances that increase the permeability of the cell membrane. Based on this assumption, we

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

The introduction of nystatin or levorin into a suspension culture of mouse cells led to an increase in the intensity of DNA synthesis (Fig. 16). Inclusion of 3H - thymidine in the period between 48-72 hours after the activation of cultures increased in comparison with 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 bacteria of the intestinal group. In the case of using these "traditional" mitogens, the coefficients of stimulation of the incorporation of 3H-thymidine The introduction of nystatin or levorin into a suspension culture of mouse cells led to an increase in the intensity of DNA synthesis (Fig. 16). Inclusion of 3H - thymidine in the period between 48-72 hours after the activation of cultures increased in comparison with 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 bacteria of the intestinal group. In the case of using these "traditional" mitogens, the coefficients of stimulation of the incorporation of 3H-thymidine The introduction of nystatin or levorin into a suspension culture of mouse cells led to an increase in the intensity of DNA synthesis (Fig. 16). Inclusion of 3H - thymidine in the period between 48-72 hours after the activation of cultures increased in comparison with 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 bacteria of the intestinal group. In the case of using these

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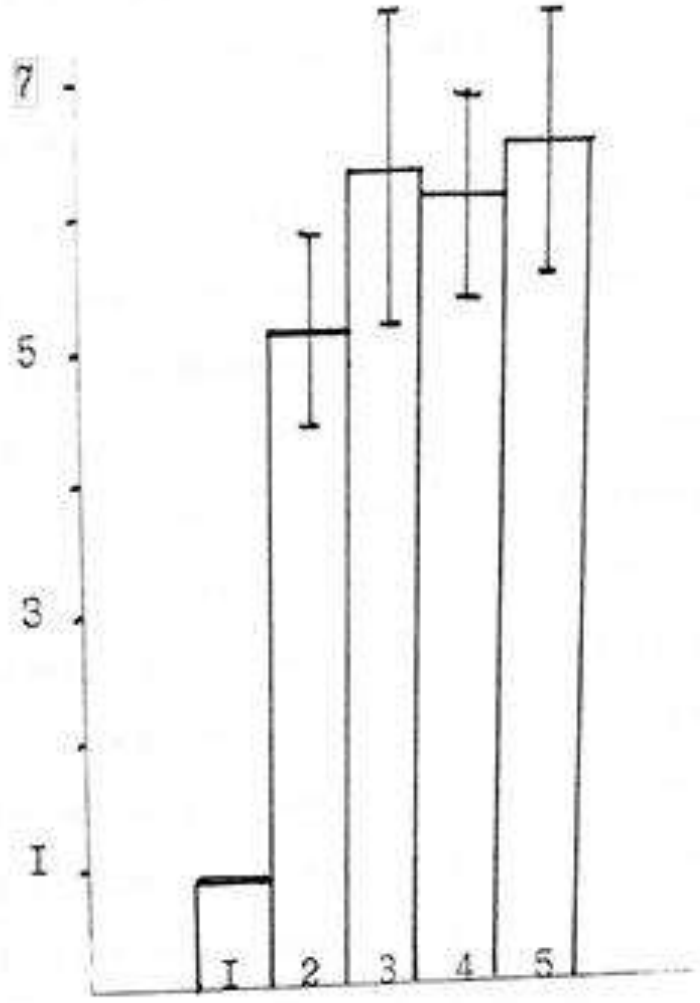


Rice. 16. Mitogenic action of membrane-active substances, in vitro. On the y-axis - the coefficient of stimulation of the inclusion of 3H - thymidine in the DNA of cells; 1.0 is the value of the intensity of inclusion of 3H - thymidine in control cultures (without mitogen). Shown are the values of stimulation 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) in comparison with the control without mitogen (1).

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

Stimulation of the primary synthesis of antibodies in vitro.

In the model of antibody synthesis in mice in response to antigens of heterologous erythrocytes (in particular, sheep erythrocytes, BE), it is very convenient to test immunostimulants. After administration of gramicidin S at doses of 10 or 100 μg (per mouse) together with 108 EBs, we found a significant change in the level of EB-specific antibody-forming cells (AFCs) in the spleens (Fig. 17). A dose of 10 μg stimulated the production of AFC, and a dose of 100 μg significantly inhibited it. From the first experiments followed the need 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 genesis. Doses of more than 50 mcg - inhibit the production of AOK (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 μg.

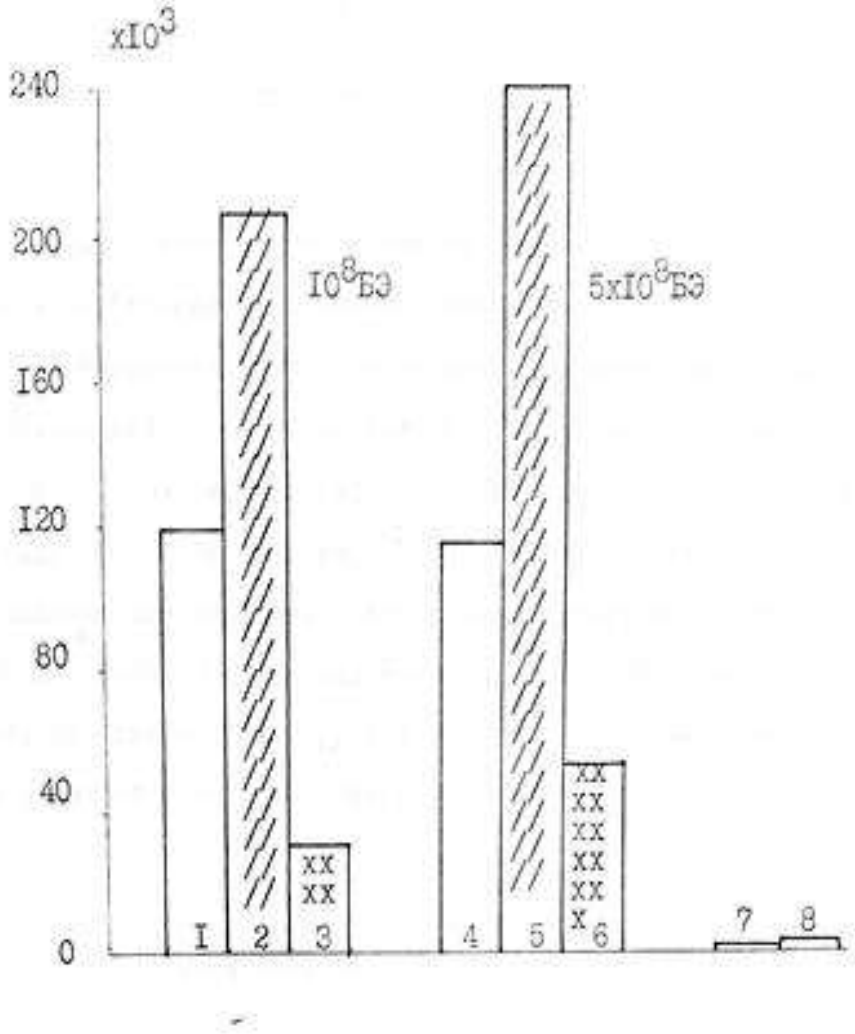
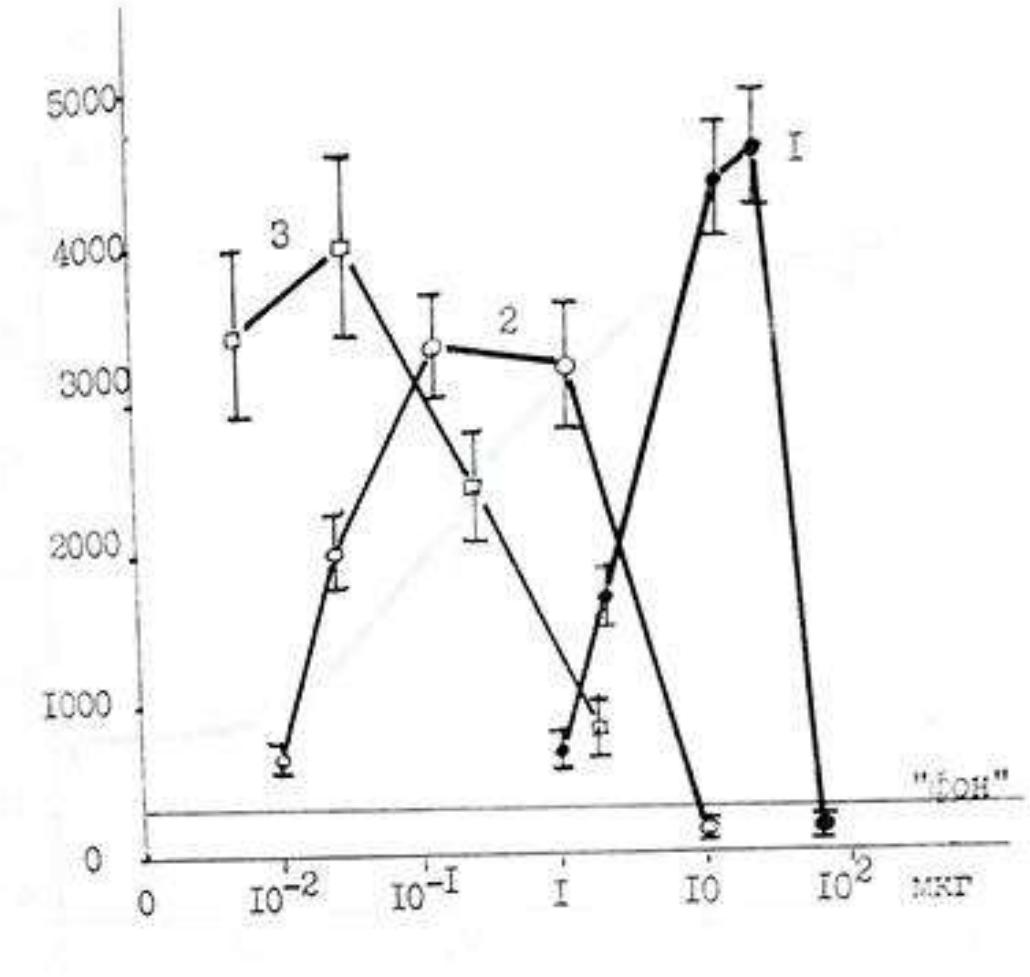


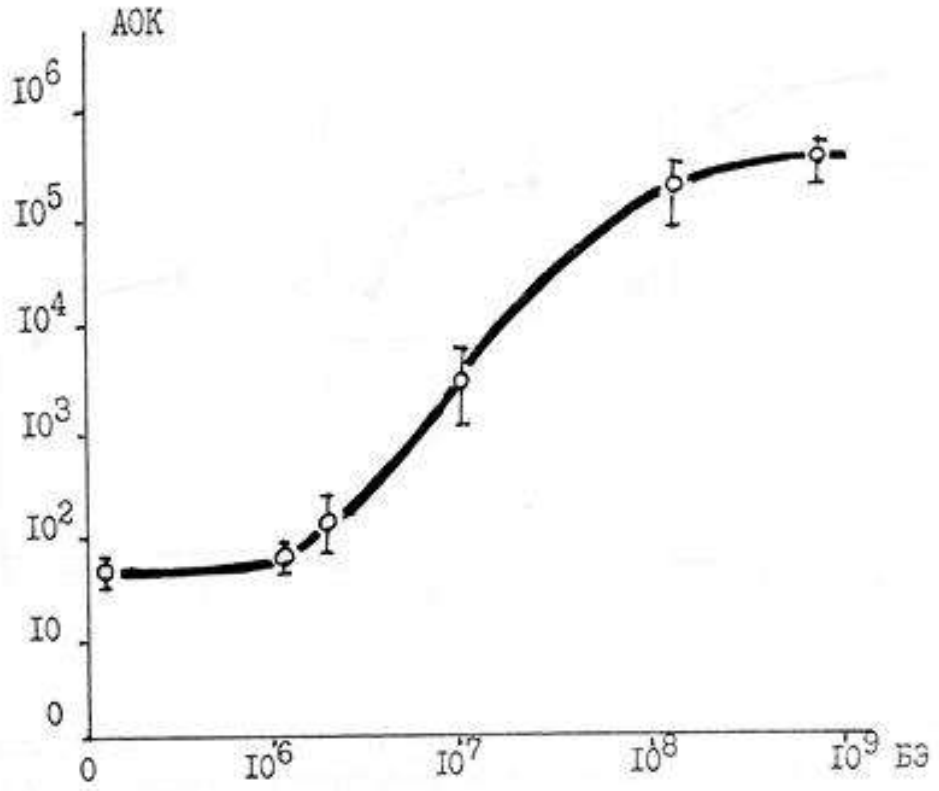
Рис. 17. Иммуномодулирующее влияние грамицидина S на реакцию антителообразования против антигенов гетерологичных эритроцитов (БЭ, sheep erythrocytes). Y-axis: accumulation in the spleens of mice of antibody-forming cells specific to BE antigens (day 4 after immunization). Mice were immunized intraperitoneally 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.

Dose of antigen.

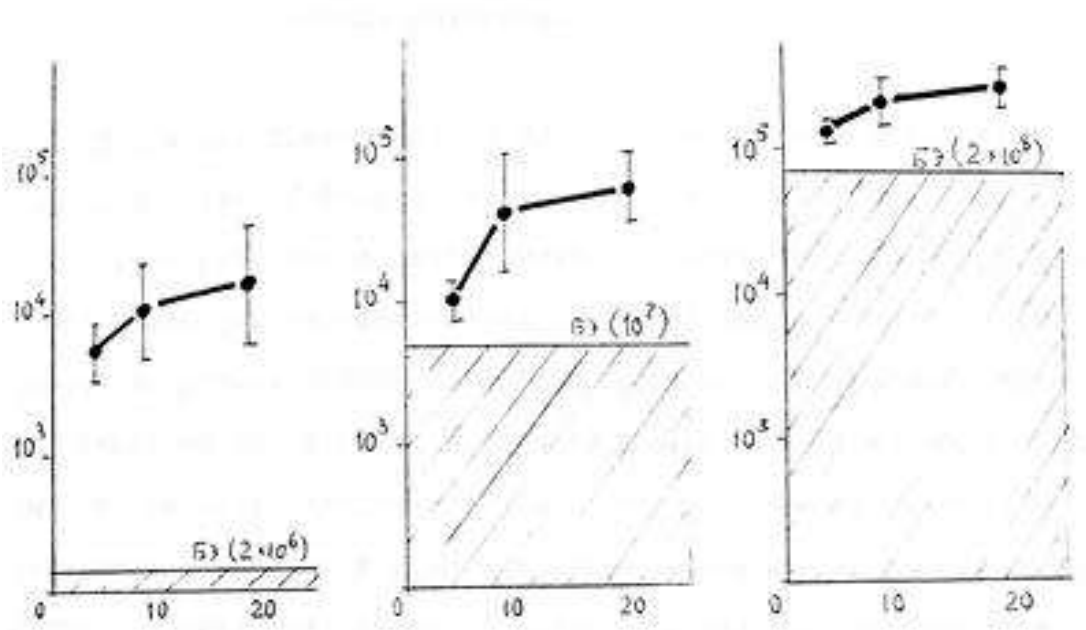
The magnitude of the antibody synthesis stimulation effect strongly depended on the immunogen dose used. The greatest stimulation effects were observed when using threshold doses of the antigen. Thus, intraperitoneal administration of 106 BE mice (CBA x C57B1) F1 practically did not produce specific AFC (Fig. 19). A dose of 107 BE induced the formation of 5-10 thousand specific AFCs in the spleen. And the dose of 108 BE is about - 50 - 100 thousand AOK. After the joint administration of gramicidin with BE, the stimulation coefficients of the threshold response reached 20-50. For example, the production of AFC reached 10 thousand against 400-500 in the control. The response of medium intensity 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).



Rice. 18. Immunoadjuvant action of substances that increase the permeability of the cell membrane. Heterologous (lamb) erythrocytes at a suboptimal dose of 2 x 106 were used as an immunogen for immunization of mice. Gramicidin S (1), levorin (2), or gramicidin A (3) was used as an adjuvant. Along the axes: abscissa - the dose of the membrane-active effector,введенная совместно с антигеном (2 x 10^6 БЭ). The y-axis shows the content in the spleen of antibody-forming cells specific to BE antigens. "Background" - the level of the immune response in the control to the injection of 2 x 106 BE without adjuvant.



Rice. 19. Dependence of the level of antibody formation on the dose of antigen (BE) administered to mice. Abscissa: antigen dose (number of BE cells per mouse). Y-axis: content of antibody-forming cells (AFC) specific to BE antigens in the spleen of mice 4 days after antigen injection.

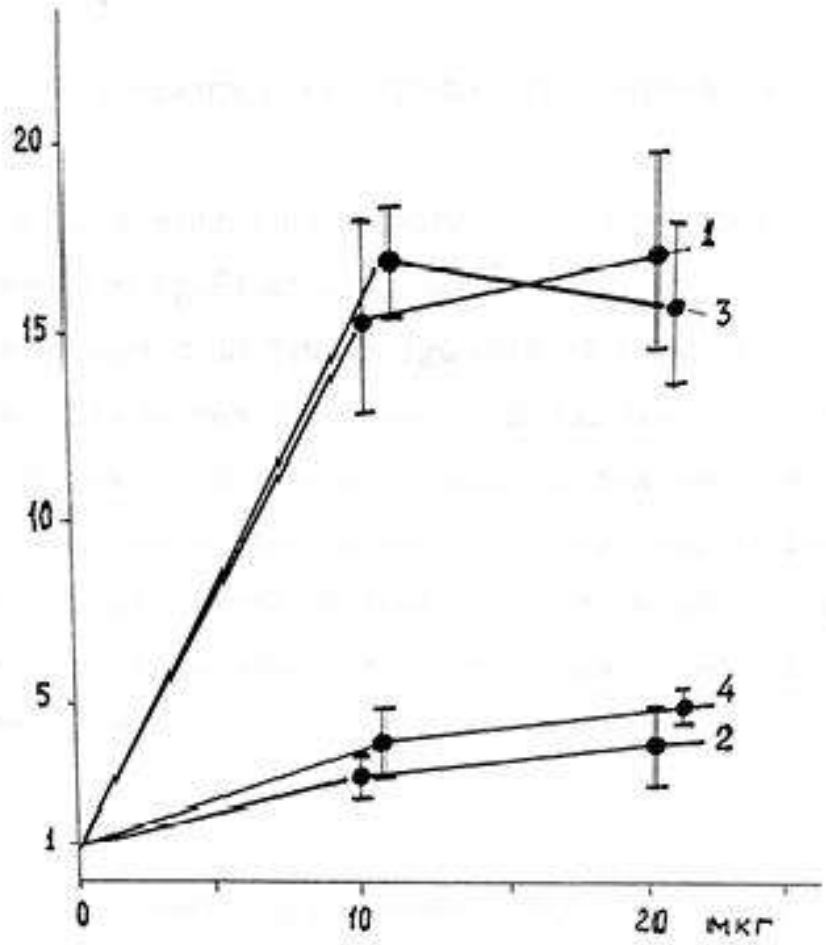


Rice. 20. Dependence of the immunoadjuvant action of gramicidin S on the level of stimulated immune response. Along the axes: ordinate - the number of antibody-forming cells specific to sheep erythrocyte antigens in the spleens of mice 4 days after immunization; abscissa - dose of gramicidin S (µg/mouse) administered together with antigen when mice were immunized 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 appropriate 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 S. typhimurium microbial bodies, water-soluble antigen (WPA) from BE, and 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 an increase in the specific synthesis of antibodies. 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 of about 20). Attention is drawn to 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 the introduction of a membranotropic agent together with a water-soluble antigen, the amplification factors for antibody production did not exceed 3–5 (Fig. 21).

The introduction of immunoadjuvant doses of gramicidin S together with the p90 protein antigen (from the causative agent of anthrax also led to more intense antibody formation, compared with the response to the protein alone. Thus, the level (ELISA - titer) of specific antibodies to p90 in mice immunized with a mixture of 3 kg p90 and 10 µg of gramicidin S reached 1:30720 by day 12 compared to 1:1920 in mice immunized with 3 µg of p90 alone without adjuvant.



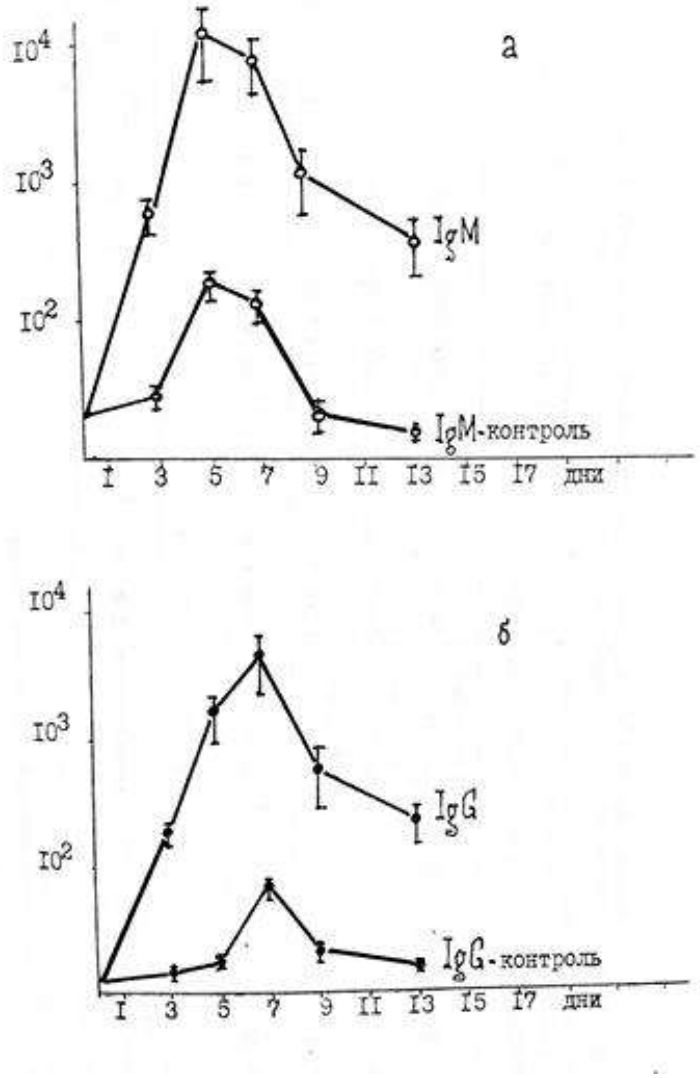
Rice. 21. Immunoadjuvant action of gramicidin S in the induction of antibody synthesis using corpuscular (1, 3) or water-soluble (2, 4) antigens. On the y-axis: the coefficient of stimulation of antibody genesis (the level of antibody genesis in the corresponding control was taken as 1.0 when immunized only with the antigen); on the abscissa axis - 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 by the Simon method; 3 - 100 mcg microbial mass of killed salmonella (S. typhimurium); 4 - 1 mcg 0 - antigen from the same Salmonella.

Dynamics of stimulated antibody synthesis

After the induction of the primary synthesis of antibodies against BE and its enhancement with gramicidin S, we studied the dynamics of the synthesis of IgM and IgG antibodies specific to BE (Fig. 22). The 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 one. If in the control synthesis of IgM - antibodies peaked on the 5th day, and the synthesis of IgG - antibodies on the 7th day, then during stimulation 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 primary and repeated administration of the immunogen (Table 2). The highest level of synthesis of secondary IgG antibodies was observed with the introduction of a membranotropic substance at the time of the first immunization or twice, at the first and second immunizations. Moreover, we did not observe the summation of stimulation effects with a two-fold administration of a membrane-active agent compared with the response levels when gramicidin was administered either only during the first or only during the second immunization.



Rice. Fig. 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 - the number of antibody producers based on the spleen; abscissa, days after immunization of mice.

Таблица 2.

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

Primary immune response			secondary immune response					Immune memory coefficient	
Immunization	The number of antibody-producers in the spleen on the 4th day	$К_{ст}$	Reimmunization	IgM - АОК	$К_{ст}$	IgG - АОК	$К_{ст}$	IgM	IgG
BE 2 x 106	100 ± 37	-	BE 2 x 106	120 ± 44	-	128 ± 51	-	1,2	1,3
BE 2 x 106 Hz 10 mcg	2300 ± 396	23	BE 2 x 106	2046 ± 407	17	992 ± 113	8	1	1
BE 2 x 106	150 ± 29	-	BE 2 x 106 Hz 10 mcg	17860 ± 3329	160	12444 ± 287	96	110	100
BE 2 x 106	136 ± 49	-	BE 2 x 106 Hz 10 mcg	21050 ± 589	175	47990 ± 10200	390	160	375

BE 2 x 106 Hz 10 mcg	2005 ± 345	20	BE 2 x 106 Hz 10 mcg	15780 ± 3100	130	35600 ± 6400	273	7,8	17,5

Notes: Grts, gramicidin S; BE - sheep erythrocytes.

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

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 - AFC in 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 responses in the control were extremely small. Moreover, the secondary reaction did not at all exceed the primary one (only 100-200 AFC per spleen). The use of the optimal immunostimulatory 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. Approximately 50,000 IgG-AFCs were produced in response to the second antigen injection.

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

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

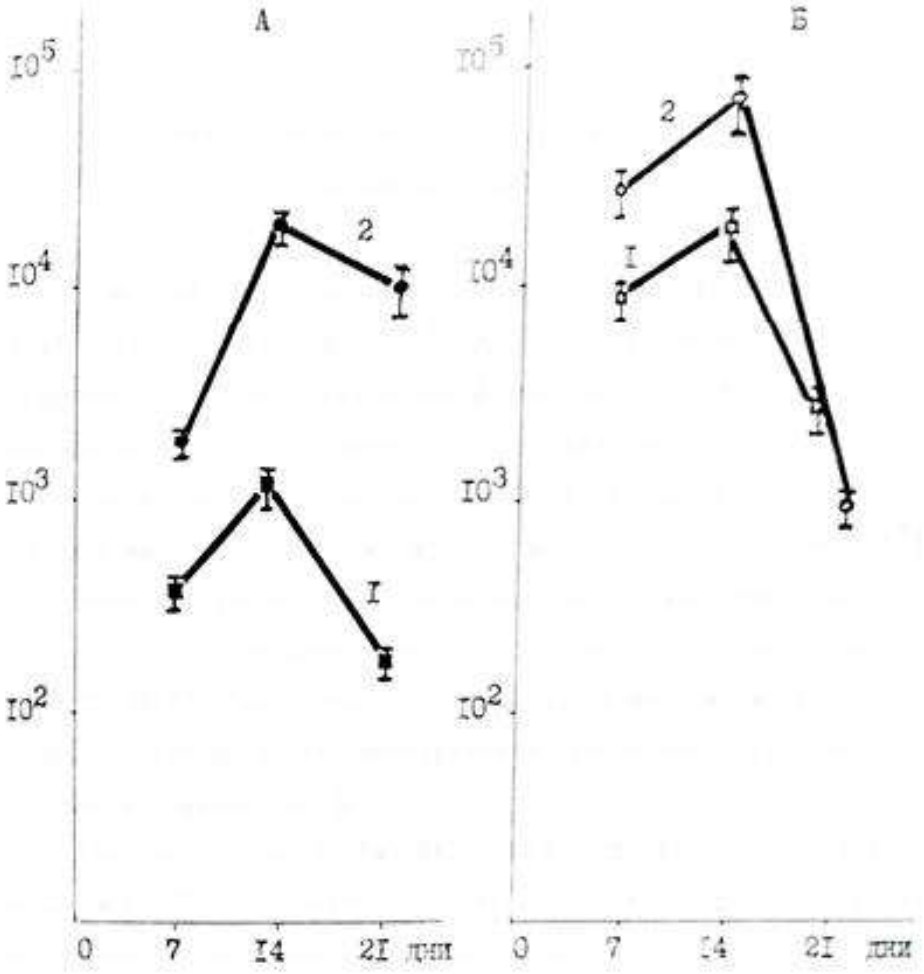


Fig.23. Stimulation with the help of gramicidin S of antibody formation to the p90 protein antigen from the anthrax pathogen. Along the axes: ordinate - level (ELISA - titer) of antibodies to p90 in the serum of immunized mice; abscissa - length after repeated immunization (1) with p90 protein or (2) with a mixture of p90 + gramicidin S. For immunization, doses of p90: 3 μg/mouse - (A) or 30 μg/mouse (B) were used. The dose of gramicidin in both cases was 10 μg/mouse.

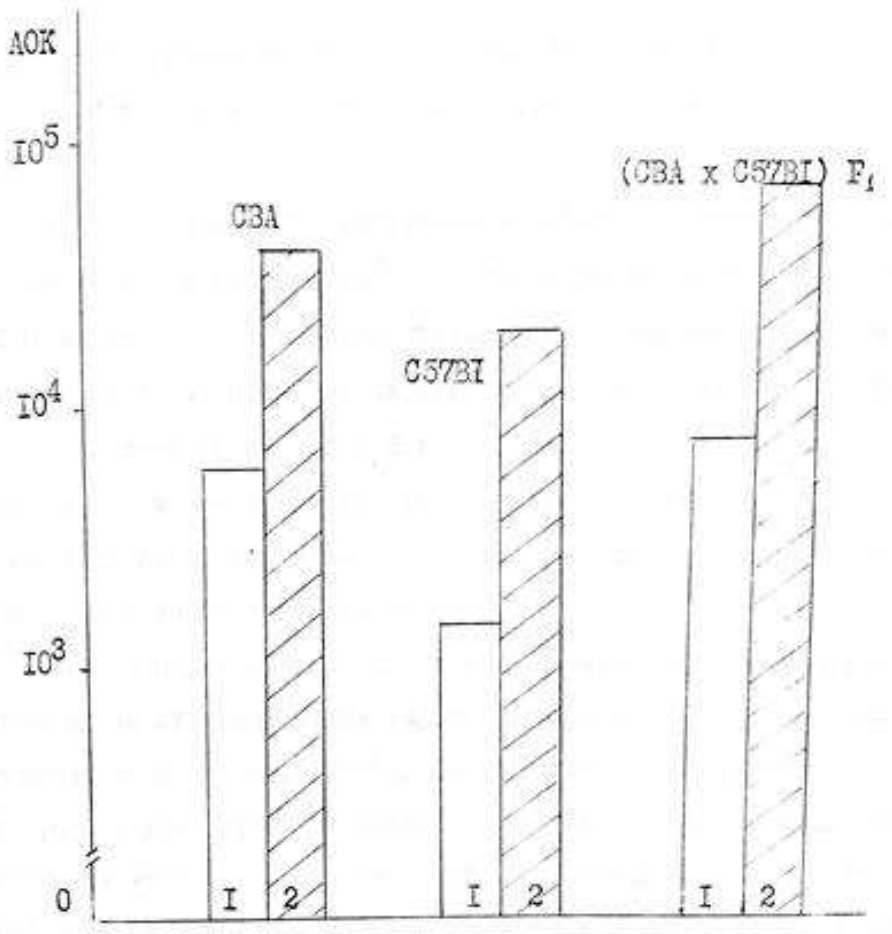
Stimulation of antibody genesis in mice of different

inbred lines.

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

It was previously known that CBA mice (H-2k haplotype) are highly responsive to BE, while C57B1 mice (H-2b haplotype) are low-responders to BE. It was also known that genes of a high level of response dominate in the first generation hybrids (CBA x C57B1) F1. It was necessary to understand to what extent it is possible to stimulate a genetically determined weak immune response with the help of 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 studied. The results are shown 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-responder C57B1 genotype. The amplification factors were the same as those of the highly responsive CBA mice. The level of response and the level of stimulatory effect of gramicidin in F1 hybrids from crossing high and low response parents were almost the same as in mice of the high response genotype.



Rice. 24. Influence 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 y-axis shows the number of antibody-producers to BE after 4 days. after immunization of mice with 107 BE (bars 1) or with a mixture of 107 BE plus 10 μg of gramicidin S (bars 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 course due to local anti-infective immunity. Therefore, we tried to stimulate local immunity with the help of the official gramicidin paste. To assess the contribution of the antibiotic action of gramicidin S in one of the control groups, monomycin ointment was used as the most effective anti-leishmania drug among antibiotics used to treat cutaneous leishmaniasis.

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

Therapeutic 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 the treatment. In the group of mice treated with monomycin, ulcers were scarred after 97.3 ± 2.1 days. The percentage of mice with partial or complete destruction of the ear tissue decreased to 25% (and the control without treatment

more than 50%). Gramicidin S had a significantly stronger therapeutic effect. The healing time for 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 - only in 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 are sufficient grounds to suggest that gramicidin S has a

local immunostimulatory effect, which, in combination with antibiotic action, gives a very good therapeutic effect in cutaneous leishmaniasis.

In general, the testing of non-polymeric membrane-active substances that increase the permeability of cell membranes for ions showed that such effectors can effectively stimulate the immune response in vivo and activate the division of lymphocytes in vitro. Therefore, an increase in the permeability of the cell membrane for ions is sufficient to activate the response of lymphocytes and the immune response in general. That is, an increase in the permeability of the lymphocyte membrane 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 the 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 to study the molecular mechanism of activation of the immune response by water-soluble polyelectrolytes (R.M. Khaitov, R.I. Ataullakhanov et al.), we have completed part of the work. We studied the role of ionic permeability for activating the response of immunocompetent cells. First, we studied the correlation between the ionophore-like and mitogenic effects of polyanions on subpopulations of lymphoid cells. Secondly, we analyzed the immunomodulatory properties of non-polymeric compounds, which, like polyelectrolytes, have the ability to increase the permeability of the cell membrane for ions.

The effect 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 shown that the immunostimulating effect of a 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 into the initial phase of the cell division cycle (G1 phase). In the presence of macrophages, the next step occurs, the transition G1 – S. The process of the beginning of DNA synthesis is induced by polyanions much weaker than by lectins and lipopolysaccharides [22]. It was theoretically substantiated that the mechanisms for triggering the response of a lymphoid cell should be sought at the level of the outer cell membrane [33]. The search for changes in such important regulatory systems of the membrane as the lipid matrix system and the system of cyclase enzymes did not lead to the discovery of significant rapid changes under the influence of the immunostimulatory polyion [5, 2].

As follows from the data presented in our work, polyanions induce very fast 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 ion-transporting membrane ATPases. Therefore, the polyanion induces an increase in the ion permeability of the cell membrane. The kinetics of changes in membrane permeability is described. In general, two facts attract attention: the instability of the ion outflow rate in the first 3-4 minutes after exposure and the temporary nature of the change in the membrane permeability. The latter is essential, since after 35-40 minutes the outflow of ions practically stops. This indicates the existence of cellular mechanisms of protection against the ionophore-like action of the polyanion. Quantification of the loss of K+ induced by the polyanion in comparison with the loss of K+ during lysis of the same cells allows us to consider the ionophore-like effect of the polyanion as relatively `mild'. At least when using non-toxic immunostimulatory doses, as a result of the

polymer-induced increase in the permeability of the cell membrane, lymphocytes lost approximately 1/1000 of the amount of K+ ions in 35-40 minutes, which is released during the lysis of the same cells by saponin.

Along with a change in the permeability of the cell membrane for ions, we found the activation of membrane ATPases transporting Na+, K+, Ca2+ against their concentration gradient. Comparison of the kinetics of activation of ion-transporting ATPases with the kinetics of changes in membrane permeability shows a clear linkage 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). Given that the activity of (Na +, K +) - and Ca2 + - ATPases of cell membranes is regulated primarily 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 ATPases to be secondary. An increase in the permeability of the cell membrane leads to a decrease in transmembrane ion gradients. Inside the cell, the concentration of Ca2 + and Na + increases, the level of K + falls. This serves as a signal for the activation of the corresponding membrane "pumps", the work of which is aimed at restoring the ratio of the concentration of ions in the cytoplasm and the environment, which is normal for the cell. Therefore, we consider polyanion-induced activation of (Na+, K+)- and Ca2+- ATPases to be compensatory, aimed at eliminating disorders associated with an increase in cell membrane permeability for ions.

When studying the mechanisms of activation of the response of lymphoid cells to lectins, many authors paid attention to changes in the membrane transport of ions. In the literature review, we reviewed the factual material on this issue. Some authors recorded the activation of (Na+, K+)-ATPase in lymphocytes when they were exposed to lectins [44, 53]. Others - activation of Ca2+ - ATPase under similar conditions [18]. Still others found an increase in the permeability of the cell membrane for amino acids, sugars, and nucleosides [34]. The fourth paid attention

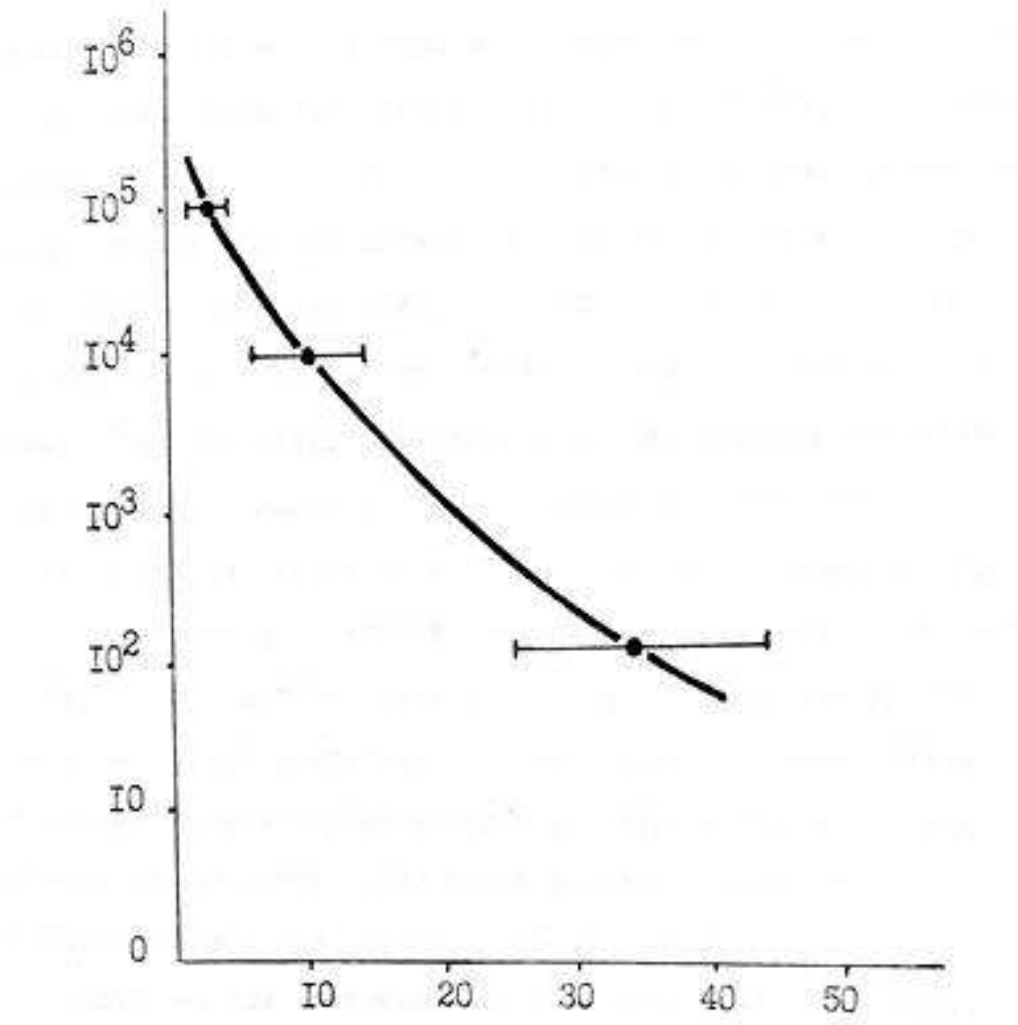
to an increase in the concentration of Ca2+ in the cytosol (however, such works began to appear in large numbers only in recent years [32, 57, 59]. In each case, the researchers attributed the found change to a key role in triggering the response of the cell. The data we obtained did not contradict these studies.Moreover, they make it possible to link all of the above effects - a change in permeability, the accumulation of Ca2+ in the cell and the activation of ATPases.We believe that permeability changes primarily.The increased cation fluxes are partially compensated by the work of activated ATPases.But despite this, the concentration of ions can change and, in particular, the level of intracellular calcium may increase.

The changes in membrane transport of ions found in our work (under the action of a polyanion) and in the work of other researchers (under the influence of lectins) in itself do not prove the key role of this change in the activation of the cell response to external influence. We paid special attention to this question in our experimental work. It turned out that the modification of the ion-transporting systems of the membrane takes place only in those cells (B-lymphocyte) that react to the polyanion by activation of division. If the cells did not respond to the polyanion by dividing, as was the case with mature T-lymphocytes, then we did not observe changes in the membrane transport of ions (Fig. 15). Such linkage between early membrane rearrangements and later physiological response of cells indirectly indicates the importance of the increase in ion permeability in the mechanism of triggering the response of lymphocytes and, consequently, in the mechanism of stimulation of the immune response by the polyanion.

In favor of such a conclusion, but not indirectly, but directly, the results of testing the immunostimulating properties of non-polymeric membrane-active substances (Chapter 6) testify. Our experiments have shown that with the help of substances that increase the permeability of the cell membrane for 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 isns with the help of these substances induced polyanion-like activation of the initial phase of lymphocyte division in vitro (Fig. 16). Membrane-active substances are especially effective not only as mitogens, but as stimulators of antibody synthesis in vivo (Fig. 18, 21). Co-administration of gramicidin or levorin with the antigen resulted in a strong stimulation of antibody genesis. The production of antigen-specific antibody-producers increased on average from 3 to 30 times. The level of stimulation depended on the level of stimulated response (Fig. 20). A weak immune response with the introduction of threshold doses of the antigen was stimulated by membranotropic agents by 20-30 times. Initially high reaction could be increased no more than 2-3 times. The inverse dependence of the stimulation effect on the level of the stimulated response is shown in Figs. 25.

In our work, the parameters of stimulation of antibody synthesis using one of the agents used, gramicidin S, were characterized in detail. It was shown that the dynamics of stimulated synthesis of IgM- and IgG-antibodies essentially did not differ from the control (Fig. 22). This, apparently, indicates that the immune response 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 administration of the antigen alone.



Rice. 25. Dependence of the immunoadjuvant action of gramicidin S on the level of the immune response being stimulated. The abscissa shows the coefficient of stimulation of the immune response with the introduction of 10 μg of gramicidin S together with different doses of the antigen (BE). The stimulation coefficient was determined as the ratio of the number of antibody-secreting cells (AFC) 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 shows the number of AFCs in the control after administration of different doses of BE.

Apparently, the increase in membrane permeability for ions effectively serves as a "second signal" for cells that recognize the antigen. However, a rigorous study of the mechanisms of enhancement of the immune response by membrane-active substances, in our opinion, remains to be done. So far, it can only be stated that the membrane-active agents used are strong immunostimulants. It is important to note that they can most effectively enhance a weak response to threshold antigen doses. In addition, it is better to use not soluble, but corpuscular variants of antigens. 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 corpuscular antigens are introduced. It is also important that with the help of the membrane-active gramicidin S, the secondary immune reaction is very strongly activated (Table 2, Fig. 23).

The discovery of the immunostimulatory properties of membrane active substances, in our opinion, is of great importance for laboratory and, possibly, veterinary and medical practice. The range of immunostimulants for today is still extremely insufficient. We hope that membrane-active substances will be widely used as immunoadjuvants. The method consists in the fact that one or several groups of membrane-active gramicidin S are introduced into the structure of a non-toxic, inert with respect to the cell membrane, inactive as an immunoadjuvant water-soluble polymer using a covalent bond. Synthesis products are neutral polymers containing membrane-active gramicidin S - have good solubility in aqueous media, extremely low toxicity and very high immunostimulatory activity. In particular, a conjugate of neutral dextran and gramicidin S (called "gradex") has been synthesized and is being actively studied [9]. Gradex provides 10-20-fold stimulation of a weak immune response, degrades in vivo, is non-toxic. Moreover, it is effectively used as an immunostimulatory carrier for antigens. At present, a significant increase in the immune response to hapten, serum proteins, viral and microbial antigens has been shown if they were sutured to Gradex. Thus, as follows from the presented data, we

have discovered a new class of immunostimulatory substances in the form of membrane-active agents. From the point of view of the tasks formulated at the beginning of the thesis (section "Introduction"), it is important that in order to trigger the response of lymphoid cells in vitro and to enhance the immune response in vivo, it is quite enough to increase the permeability of the cell membrane for ions. The facts obtained by us prove that the increase in the permeability of the cell membrane induced by the polyanion 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 at the level of the membrane of lymphoid cells found in our work 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 in which the importance of modifying the membrane transport of ions during the activation of lymphocytes by lectins is of great importance. In the last 2–3 years, reports have appeared proving the importance of membrane ion permeability 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 idea is currently being formed that the activation of a lymphoid cell by various ligands begins with a modification of the permeability of its outer membrane for ions.

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

Other researchers [55] prefer the flow of Ca2+ from the extracellular medium 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 inclined towards just such a 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 junction.

A similar hypothetical scheme was proposed by Norcross [61] as applied to the activation mechanism of the T-killer precursor. As is known, the 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 has not been understood. Norcross suggested that the meaning of double recognition is to stabilize the connection between the receptor and the recognizable structure. The prolongation of the lifetime of the T-receptor complex with an associate (MHC + antigen) contributes to 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 "recognising" and "recognizable" molecules, which belong respectively to the T-cell and the antigen-presenting cell. There is a kind of aggregation of the T-cell receptor in the plasma membrane, and, as Norcross suggests, membrane domains (T3M) T -cell receptors, clustering, form ion-conducting channels.It is the appearance of channels and a weaker increase in membrane permeability that marks the beginning of the restructuring of cellular metabolism.

Apparently passive flows of ions are quickly compensated by an increase in the active transfer of the same ions with the help of ATPases. However, the mere fact of

imbalance is already sufficient for signaling about the ligand and for the cell to start a response.

R.V. Petrov and co-authors believe that the perturbation 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 some proteins on the cell surface is enhanced. For example, the content of Ia - molecules on the cell membrane increases, special proteins appear - receptors for growth and differentiation factors. Acceptance of growth factors triggers DNA synthesis and replication.

On the whole, apparently, the mechanisms of initiation of the cell response to exogenous influence are much more complex than it is presented in any of the above mentioned hypotheses. For example, we did not at all consider the role of such important processes as the activation of membrane cyclase enzymes and protein kinases, the 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 events mentioned. Most likely, they are different links in the complex biochemical pathways triggered by the ligand. Nevertheless, there is no doubt that the change in ion transport at the level of the plasma membrane is one of the key events when the reaction of lymphocytes is triggered by various ligands, including polyanions.

CONCLUSION

Our work, in general, made it possible 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 are of independent scientific and practical importance. 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 paper presents detailed data on the dose dependences of the immunoadjuvant action 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 the secondary production of antibodies, on the dependence of the severity of the immunoadjuvant effect on the level of the modified reaction (from the dose of the immunogen) and on the structural features of the studied antigens (soluble or corpuscular). All this is information that may 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 Salmonella antigens and the anthrax pathogen, together with a 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, the same polyanions induce neither changes in the membrane transport of ions, nor activation of cell divisions.

2. Membrane-active compounds - gramicidin S, nystatin and levorin - effectively model the immunostimulatory effect of polyanions. In cell culture in vitro, these substances activate the division of lymphocytes. With the joint introduction of

membrane-active compounds with an antigen in vivo, multiple stimulation of the synthesis of specific antibodies occurs.

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

4. Gramicidin S can stimulate both primary and secondary antibody synthesis. At the same time, the production of IgM- and IgG-antibodies increases equally. The dynamics of the stimulated synthesis of antibodies does not differ from the dynamics of the control response to the antigen (without harmicidin 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, and soluble protein antigen (p90) from the causative agent of Siberian ulcers.

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