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From synthetic polyelectrolytes to polymer-subunit vaccines*

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Abstract: The results of many years of collaborative research by chemists and immunologists in the area of application of synthetic polyelectrolytes in immunology are reviewed. Linear synthetic polyelectrolytes with diverse structures (which are not structural analogs of biopolymers and, hence, not antigenic because they are unknown to the immune system), when introduced into the organism, noticeably intensify the formation, migration, and dissemination of stem cells, which are precursors of all specialized cells including functional immune cells. In addition, synthetic polyelectrolytes, when introduced in mixtures with typical antigens (proteins, natural microbial polysaccharides, and their synthetic analogs), serve as immunostimulants enhancing immune response by several times. Moreover, individual bacterial or viral antigens, not sufficiently active by themselves, induce specific immune response enhanced by orders of magnitude if chemically bound to synthetic polyelectrolytes. Such conjugates being preliminarily administrated, protect organisms from absolutely mortal doses of the corresponding bacteria or viruses. The nontoxic immunostimulant was developed: the ternary copolymer of 1,4-ethylenepiperazine, 1,4-ethylenepiperazine-*N*-oxide, and (*N*-carboxymethylene)-1,4-ethylenepiperazinium bromide (brand name "polyoxidonium"™) permitted for human administration. The conjugate of polyoxidonium with hemagglutinin and neuraminidase, protein subunits of influenza viruses, has appeared as the first non-Pasteurian vaccine, which now is successfully used in Russia (about 50 million immunized people for the last 7 years). The physicochemical mechanisms of the biological effect of these compounds and challenges of the further use of the approach developed are considered in the review.

INTRODUCTION

It is well known that foreign natural polyelectrolytes (proteins, polysaccharides, nucleic acids) and their structural analogs (polypeptides, polynucleotides) in the organism manifest the properties of antigens. This implies that the immune cells recognize their specific fragments (antigenic determinants) and, as a response, produce structurally complementary proteins, viz., antibodies that block these antigens. In addition, natural polyelectrolytes (polysaccharides, native nucleic acids, double-strand synthetic polynucleotides) are known to activate the immune system toward other antigens, that is, serve as immunostimulants [1–3]. It was of interest to understand how the system reacts to unknown synthetic polyelectrolytes (SPEs), whose chemical structures do not resemble biopolymers.

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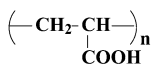
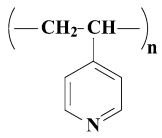
ALTERNATIVE MECHANISM OF ACTIVATION OF IMMUNE CELLS

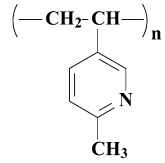
We started with two simple SPEs of the vinyl series: polyacrylic acid (PAA) and poly-4-vinylpyridine (PVP). The first polymer can dissociate in an aqueous medium to form the polyanions, and the second one can add protons to form the corresponding polycations.The introduction of aqueous solutions of the above-mentioned polymers into blood noticeably intensified the formation in the marrow of the so-called stem cells (precursors of all functional cells of the immune system) and their migration and dissemination in the organism [4]. In addition, it turned out that both PAA and PVP, which are not antigens, being introduced in combination with sheep erythrocytes, by several times enhance the immune response (i.e., production of antibody-forming cells, AFCs) to this typical testing antigen, that is, they serve as immunostimulants [5]. In fact, the adjuvant effect of PAA in vivo has been found earlier by Diamanstein et al., who tested PAA along with numerous natural polyanions [6]. However, this fact did not deserve the authors' special attention. When analyzing our data, we noticed a surprising fact that PAA and PVP anions, whose chains are formed of monomeric units with different chemical structures and which even differ in sign of the charge, stimulate the immune response to approximately equal extents. The extension of the scope of SPE convinced us that many other chemical structures act similarly to PVP and PAA. The formulas of some of them are presented below.



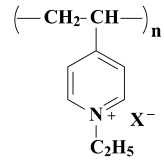
Polyacrylic acid



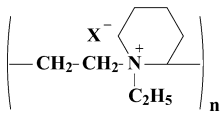
Poly-4-vinylpyridine



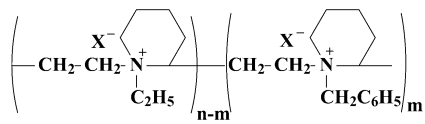
Poly-2-methyl-5-vinyl-pyridine



Poly-4-vinylpyridine quaternary salt



Pcat-1



Pcat-2

Polyconidinium quaternary salts

This unexpected result suggested that different SPEs affect the immune system according to a general mechanism related to their polymeric nature. Figure 1 shows that the low-molecular analogs of typical SPE stimulants exhibit no activity in tests in vivo. The effect appears only when some sufficiently high degrees of polymerization are achieved [7–9].

It is known that rather complicated specific interactions (cooperation) of several varieties of immune cells are needed to start the natural process of antibody production in the organism [10,11]. The main participants of this cooperation are T-lymphocytes (helpers, Th), antigen-presenting cells (APC, microphages are one of their varieties), and B-lymphocytes (cells producing antibodies). However, receptors of B- and Th-cells differ substantially. The first receptor itself recognizes the structurally complementary antigen determinant. The second receptor needs a coparticipant for recognition: a peptide with the rigidly certain structure (double recognition). This structure is programmed in immune response genes (Ir-genes), which are in the composition of the main complex of histocompatibility.

One more fact revealed in experiments in vitro seemed to be especially surprising. Minor amounts of SPE added to a suspension of isolated B-lymphocytes activated the DNA synthesis and induced the

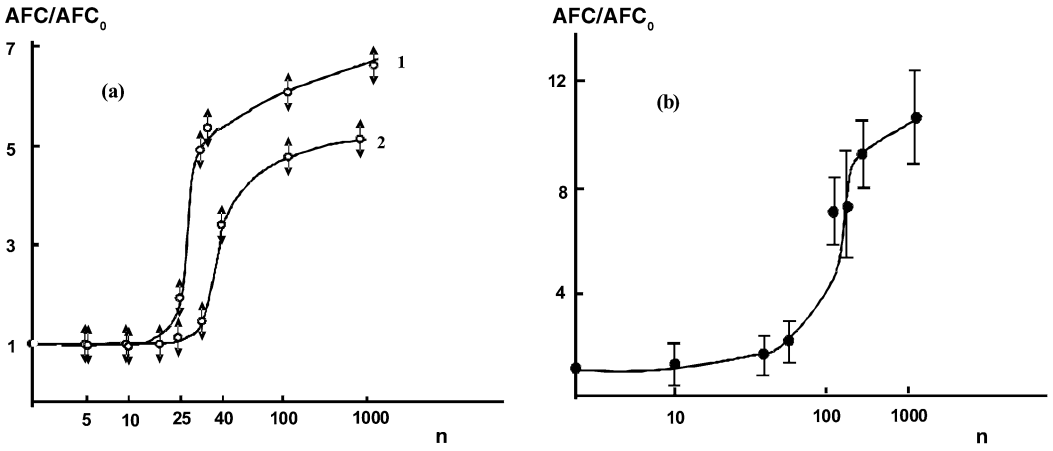


Fig. 1 Relative number of AFCs in the mice spleen as a function of the degree of polymerization of Pcat-1 (1), Pcat-2 (a), and PAA (b). Antigen dose (sheep erythrocytes) $5 \cdot 10^6$, adjuvant dose 50 mg/kg.

cell fission and, in the presence of antigens, antigen-independent differentiation of cells, i.e., the immune response was started directly in a tube without assistance of other cells of the immune system [8,9].

The possible explanation was found by studying the physicochemical and biochemical consequences of treatment of B-lymphocytes with the SPE. It turned out that the addition of an aqueous solution of PAA to a suspension of B-lymphocytes increased sharply the ion permeability of the external cellular membrane. In particular, the potassium ion flow rushes from the cell into the surrounding solution, where the potassium ion concentration is lower than that in the intracellular space (Fig. 2). Other water-soluble SPEs act similarly. Unlike them, water-soluble electroneutral polymers exhibit no activity.

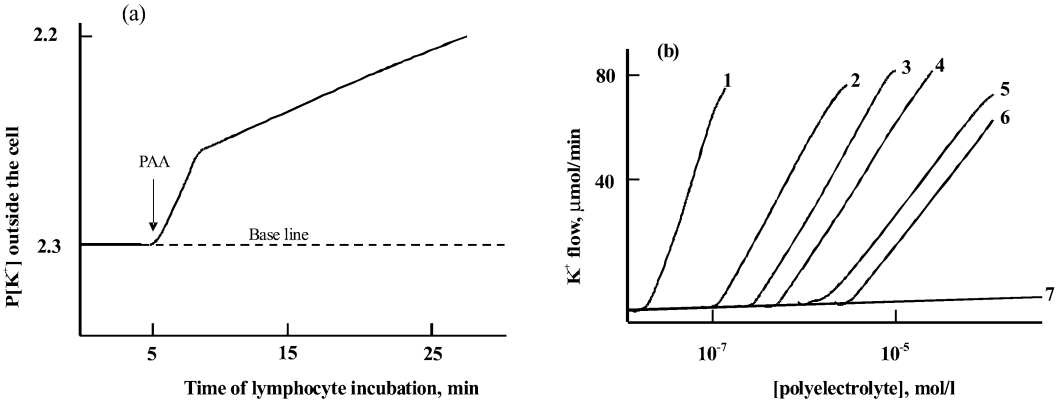
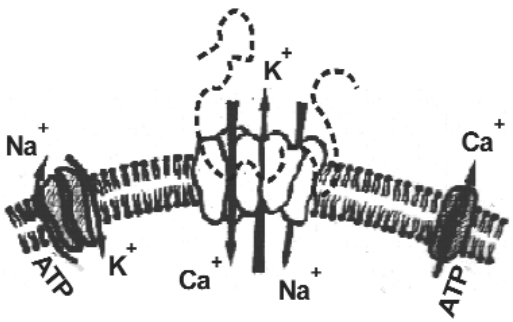


Fig. 2 Influence of polyelectrolytes on the permeability of B-lymphocyte membranes for K^+ ions in vitro. (a) Kinetics of establishment of the enhanced stationary flow upon the addition of a PAA solution to the cell culture; (b) dependence of the stationary K^+ ion flows on the concentrations of different polyelectrolytes: 1, PEP; 2, PEP containing 3 mol% $C_{16}H_{33}$ substituents; 3, poly-L-lysine; 4, PAA; 5, dimethylaminoethyl methacrylate; 6, dextransulfate; 7, poly-4-vinylpyridinium-*N*-oxide (nonionogenic polymer presented for comparison).

Calcium ions, whose concentration is higher in the surrounding solution, on the contrary, rush inside the cell.

In other words, SPEs induce the formation of nanopores in the cellular membrane, and ions begin to diffuse through the pores along the concentration gradient. The electronic microphotographs of the

longitudinal chips of the membrane show that the treatment of individual B-cells with SPE actually results in the aggregation of the membrane proteins [8,9]. The phenomenon of aggregation of the protein globules upon their binding with linear polyelectrolytes in aqueous solutions is known and well studied [12–14]. The binding sites are, first of all, ion groups on the globule surface, whose charge is opposite to the SPE charge. In addition, depending on the chemical structure of the polyion and protein, binding can occur due to the donor–acceptor or hydrophobic interaction. Therefore, it seemed natural to assume that they serve as the sites of SPE sorption on the external membrane of the cell, and the membrane proteins interact with the polyion to form two-dimensional clusters via the mechanism similar to that occurring in aqueous solutions. Then gaps between the aggregated protein globules probably act as pores (Scheme 1).



Scheme 1

It is known that the energy necessary for maintaining the living activity is obtained by any cell from the same universal source: oxidation of adenosinetriphosphate (ATP), and the natural ion balance in the cell is maintained by the membrane enzymes: (K^+,Na^+)- and Ca^{2+}-ATPases. The partial consumption of ATP by each kind of ATPase can be estimated by the addition of selectively acting inhibitors to the cell culture: ouabain (strophantin) for (K^+,Na^+)- and $LaCl_3$ for Ca^{2+}-ATPase.

Introduction of PAA into an aqueous suspension of B-lymphocytes increased sharply the relative consumption of ATP by the cells, and inhibitory analysis showed that this increase caused additional activation of the ATPases of both kinds [15,16]. In other words, distortion of the natural state of the cell because of the potassium ion drain and the inlet of an additional amount of calcium ions results in the compensatory switching of molecular pumps, and this serves as the signal for launching of other intracellular systems. B-lymphocytes begin to divide and differentiate, synthesizing receptors for the recognition of antigens, and thus they are going to produce complementary antibodies.

It is characteristic that cyclase enzymes, which are necessary participants of the reaction of immune cells during the standard mechanism of immune system launching, are not virtually activated in this case.

The above-presented experimental facts allowed us to make an important conclusion: on contact with the immune cell, SPE acts as a trigger factor unknown to this cell, and its action is directed against genetic control of the strength of the immune response. Indeed, Th-lymphocytes responsible for expression of Ir-genes were absent in the above-described systems in vitro. This conclusion agrees completely with the results obtained in in vivo experiments with the use of mice devoid of T-cells. Such mice do not response to administration of ordinary antigens. At the same time, B-lymphocytes potentially able to ripen and differentiate still remain in these mice. When the same antigens are administrated in a mixture with SPE, B-lymphocytes are activated and then produce antibodies in the spleen exhibiting almost the same strong immune response as that characteristic of normal mice.

Thus, SPEs stimulate the development of the immune reaction via the alternative mechanism bypassing the Ir-genes controlled not only in vitro but also in vivo. Meanwile, this is also indicated by the data in Fig. 3, which demonstrate the strikingly parallel character of the dependences of the intensity of

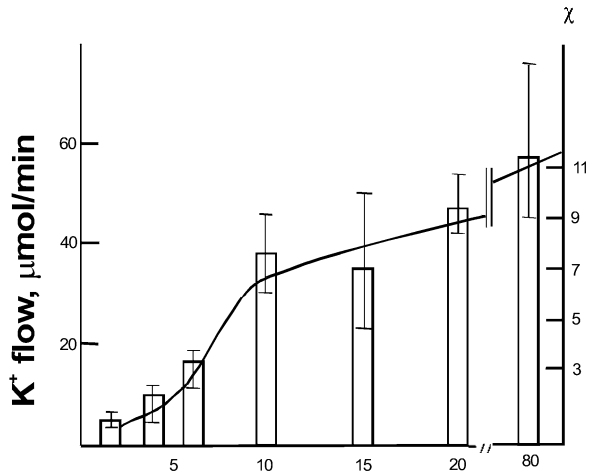
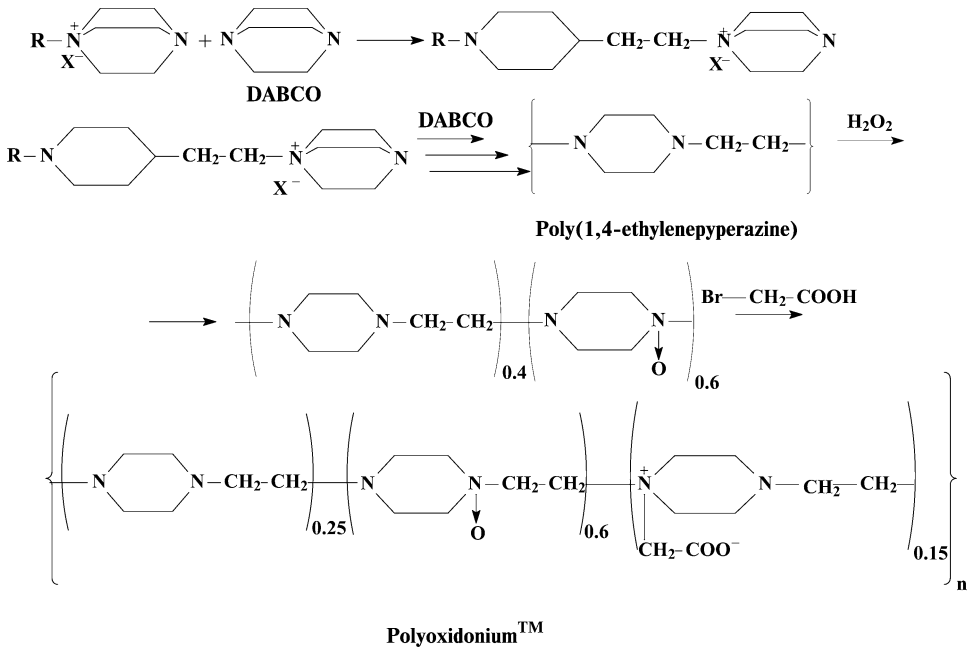


Fig. 3 Dependence of the K^+ ion flow through the B-lymphocyte membranes in vitro and enhancement of the immune response in vivo on the molecular weight of PAA. χ is the maximum stimulation coefficient.

the transmembrane ion flow in vitro and the coefficient of immune response enhancement in vivo on the degree of polymerization of SPEs.

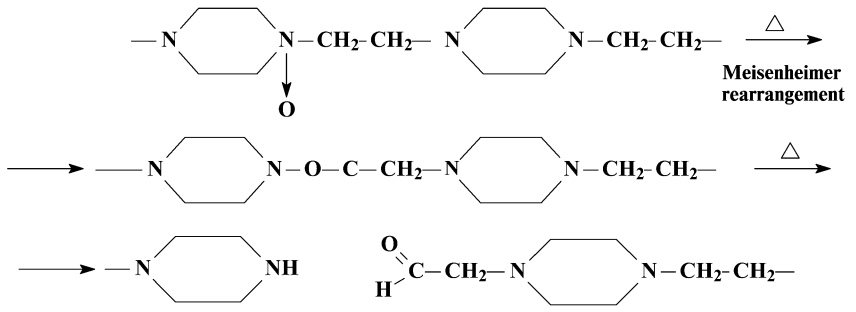
POLYELECTROLYTE IMMUNOSTIMULANT FOR HUMANS

The practical use of SPEs as immunostimulants seemed very attractive because, first, SPEs themselves are not immunogenic. In addition, the established fact of immune system launching without T-cell-helpers provides the compensation of immunodeficiency for individuals, which are weakly protected from the microbes bearing the specific antigen. Naturally, the main purpose was the synthesis of SPEs appropriate for human administration without ill side effects. The previously discovered and studied in detail polymerization of sterically strained bicyclic amines with one ring opening was used for the synthesis of the starting macromolecules [17–19]. 1,4-diaminobicyclo(2,2,2)octane (DABCO) (triethylenediamine) was chosen as the starting monomer for several reasons (including technical economical parameters). Its polymerization via the "living" chain mechanism leads to the formation of poly-1,4-ethylenepiperazine with a very narrow molecular mass distribution. The nontoxic polycationic immunostimulant "polyoxidonium" was synthesized from this polymer by the oxidation of a certain fraction of its units with hydrogen peroxide to *N*-oxide followed by partial quaternization of nonoxidized amine groups with bromoacetic acid [20,21]. The scheme of the synthesis and structural formula of polyoxidonium, which in fact is a water-soluble ternary copolymer of 1,4-ethylenepiperazine, 1,4-ethylenepiperazine-*N*-oxide, and (*N*-carboxymethylene)-1,4-ethylenepiperazinium bromide, are presented below (Scheme 2).



Scheme 2 Synthesis of polyoxidonium™.

Here, the *N*-oxide groups play the key role in the dramatic decrease in the high toxicity usually inherent in polyamines, because they decrease the linear density of positive charges. However, the copolymer does not lose solubility in water, because the electroneutral *N*-oxide groups are hydrophilic dipoles. During the development of polyoxidonium, the optimum ratio of the amino to *N*-oxide groups was experimentally found at which the toxicity of the copolymer has already decreased to a quite appropriate level, but its capability of interacting with the membrane and activating immune cells has not been lost yet. In addition, the *N*-oxide units included in the main polyoxidonium chain are rearranged to form oximes (Meisenheimer rearrangement, Scheme 3), which then are decomposed at the N–C bond to form the amine and aldehyde groups. As a result, the copolymer chains are cleaved to relatively short fragments, which then release from the organism. [22].



Scheme 3 Cleavage of polyoxidonium by Meisenheimer rearrangement.

Under the organism conditions, these reactions are rather slow, so that the immunostimulant administrated has enough time to perform its function. It is shown that its almost complete removal from the organism takes about a fortnight, and at most 2 h are required for the activation of the immune system. Further, the triggered immune reaction is developed without SPE.

The molecular mechanism of cell activation under the SPE effect established for isolated B-lymphocytes in experiments in vitro is not specific. SPEs can interact in the same manner with other membranes to activate different kinds of immune cells: such a breadth is an undoubted advantage for the immunostimulant. However, the nonspecific stimulation is insufficient to induce the strong target immune response in the organism to a particular antigen or a particular group of antigens bypassing Ir-gene control.

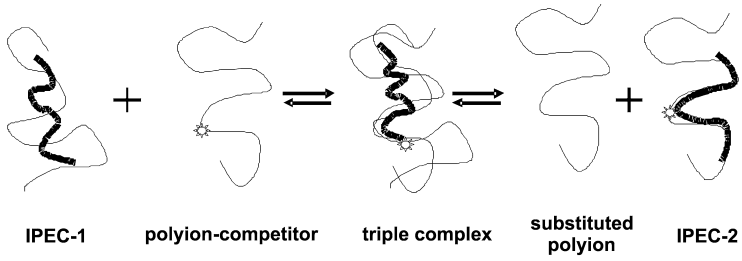
MOLECULAR RECOGNITION

To achieve the strong target immune response, the SPE action should be at least focused, providing its "address" delivery and selective sorption on the surface of the corresponding B-lymphocytes. The necessary "vector" (antigen or antigenic determinant) as an "address" can easily be attached chemically to the polymeric chain. Then, if the resulting conjugate, traveling between different cells in the organism, accidentally reaches the cell containing receptors complementary to this antigen on the surface, its determinant becomes capable of binding with the receptor.

The possibility of free traveling of the antigen–SPE conjugate introduced into the organism and searching for the cell-addressee did not seem obvious. It is known that long-chain polymers are usually irreversibly sorbed on surfaces and, for cooperative interaction reasons, do not leave the sorbent surface even at a very great (infinite in the limit) dilution of the solution. The cellular membrane is a good sorbent for most polyelectrolytes, especially for polycations. Therefore, we apprehended, naturally, that the conjugates addressed to a specific clone of B-lymphocytes adhere to other cells, which are present in a great excess, and thus cannot reach the addressee.

At the same time, when stating this problem, we already had indirect data for the existence of the mechanism of searching for necessary cells by the conjugate. These data were obtained by studying the behavior of the complexes formed from two oppositely charged polyelectrolytes in aqueous solutions.

In this interpolyelectrolyte complex (IPEC), the polycation and polyanion are bound to each other through numerous salt bonds, which can dissociate only cooperatively. Therefore, in some pH and ionic strength intervals, IPECs are absolutely stable and not cleaved to the starting components upon dilution. Nevertheless, one of the fundamental properties of IPECs is their ability to enter into competitive exchange and substitution reactions with other polyelectrolytes [23,24]. These reactions do not need the preliminary dissociation of IPECs to the starting components. They proceed through the formation of intermediate triple complexes as shown in the Scheme 4.



Scheme 4

This transfer within the triple complex proceeds, with a certain probability, by the fluctuation rearrangement of ion pairs connecting the oppositely charged polyions. Moreover, if the asterisk that marks one of such "thin" polyions denotes a functional group capable of additional stabilizing IPECs, then the "thick" polyion is fixed in the IPEC-2 composition. This takes place even when unmarked "thin" polyions are present in the system in a great excess. This process does not require many stabilizing groups, and the additional affinity sufficient for recognition is provided by the bond energy ex-

ceeding the energy of thermal motion only by several times. For example, the poly(*N*-ethyl-4-vinylpyridinium) cations (PEPs) in an aqueous solution recognize among the polymethacrylate anions those marked by one pyrenyl group per 1000–1500 monomeric units [25]. In this case, the additional affinity is provided by the hydrophobic interaction of the pyrenyl group with the hydrocarbon fragments of the PEP chain.

However, it was far from evident that the mechanism of molecular recognition similar to that experimentally established for the oppositely charged polymeric chains can also act in systems of the polyion–cell type, where the linear sizes of the cell considerably exceed the contour length of the polyion interacting with the cell.

Relatively large particles of polystyrene latex (5 μm in diameter) were used as a rough model of a particular cell. The surface of each particle was covered by the sulfo groups chemically bound to the surface and, hence, the particle was negatively charged similarly to external membranes of the most cells.

The first task was to elucidate whether the polycations irreversibly adsorbed on the negatively charged surface can migrate freely in the adsorption layer or not. For this purpose, the latex mentioned above was mixed with an aqueous solution of poly-L-lysine modified by a minor amount of fluorescein-isothiocyanyl (FITC) groups. As a result, the fluorescein-labeled polycations were adsorbed on the surface of the latex particles. Then one of these particles was chosen and irradiated in an aqueous medium at the wavelength in the region of absorption of FITC groups. The characteristic green fluorescence was observed by an optical microscope. In the initial state, the green light was uniformly emitted by the whole particle surface. Being convinced in the latter, we directed a powerful laser beam with a thickness (2.5 μm) somewhat smaller than the particle diameter to the center of the fluorescing surface. The FITC groups were partially photodecomposed in the zone of beam action, and a spot with somewhat weakened fluorescence appeared on the surface. It turned out that after the end of laser beam irradiation the fluorescence of the spot was gradually recovered and, after 15–20 min, the whole particle surface begin again to emit uniformly. This result proved unambiguously that the polycations adsorbed in the extended negatively charged surface contacting with water can migrate in the adsorption layer rather rapidly. The coefficient of two-dimensional self-diffusion of poly-L-lysine in the adsorption layer [$D = (2 - 6)\cdot 10^{-8}$ cm^2/s] was estimated from the rate of fluorescence recovery [26].

Monodispersed PS-latex with the particle diameter about 0.5 μm served as a rough model of the totality of cells. In this case, the surface was covered by the chemically bound carboxyl groups (on the average, 1 group per 25 Å^2) and, hence, the surface was negatively charged in the neutral and basic media [27,28]. This model was chosen to reveal whether the adsorbed cations can transit from the surface of one large negatively charged particle to another or not [27,29]. PEP with a degree of polymerization of ~10^3 was used as a polycation. An aqueous solution of PEP was added to the dilute latex varying the number of the positively charged pyridinium units per negative charge of the surface. For each charge ratio, the electrophoretic mobility of latex particles, which characterizes the value and sign of their charge, was measured in one series of experiments. In another parallel experimental series, we measured the amount of PEP remaining in a solution after the latex was precipitated by a preparative centrifuge. The data obtained are presented in Fig. 4. It is seen that the initial negative charge decreases with an increase in the PEP content. Then the overcharge occurs in a PEP excess: the electrophoretic mobility values become positive (Fig. 4a). It is substantial that the size of the resulting positively charged particles is the same as that of the initial particles. It is also important that the run of the dependence of the electrophoretic mobility on the charge ratio remained unchanged with variation of the initial partial concentration of the latex within the range exceeding two orders of magnitude. The latter implies that in the region of charge ratios studied, all added polycations are strongly adsorbed on the latex surface and are not desorbed upon the dilution of the system. The irreversible character of adsorption is also indicated by the data in Fig. 4b, which characterizes the influence of the initial charge ratio on the absorbance of the supernatant measured after the latex was precipitated.

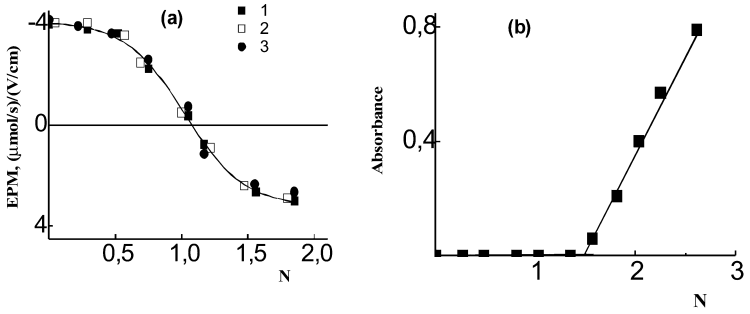


Fig. 4 Dependences of the electrophoretic mobility (EPM) of latex particles (a) and PEP absorbance in the supernatant (b) on the ratio of the number of PEP units to the number of carboxyl groups on the latex particle surface. Latex concentration $9 \cdot 10^9$ (1), $1.8 \cdot 10^{11}$ (2), and $1.8 \cdot 10^{12}$ particle/l (3).

Nevertheless, the next two series of experiments showed unambiguously the existence of the efficient mechanism of migration of polycations from the surface of one latex particle to another despite the irreversibility of adsorption. A suspension of the starting negatively charged particles was mixed with a suspension of those that gained a positive charge due to the polycation adsorption. The data on the electrophoretic mobility obtained 5 min after mixing (Fig. 5) indicated cardinal changes in the reaction system during this time. Along with the particles characterized by the electrophoretic mobility close to the initial values, other particles appeared, whose electrophoretic mobility was intermediate. At the same time, the average size increased by at least an order of magnitude, indicating the aggregation of the initial particles. The measurements performed after 40 min showed that the particle diameter returned to the initial one, and the electrophoretic mobility of all particles took an intermediate value. This experiment proved unambiguously that the polycations can migrate in the adsorption layer of each particular particle and also can transit from the surface of one particle to another. Immediately after mixing, the initial negatively charged latex particles meet with the positively charged particles and bind with each other due to the electrostatic attraction to form aggregates. When the polycations are uniformly distributed inside the aggregate, the charge of the latex particles is equalized and, hence, their mutual electrostatic attraction is eliminated. Then, the aggregates are dispersed to particles with the initial size under the action of thermal motion.

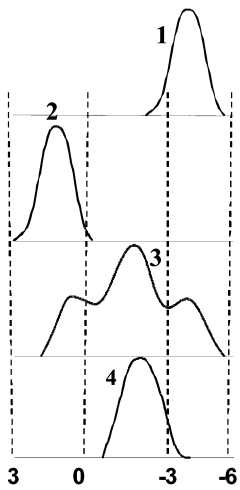
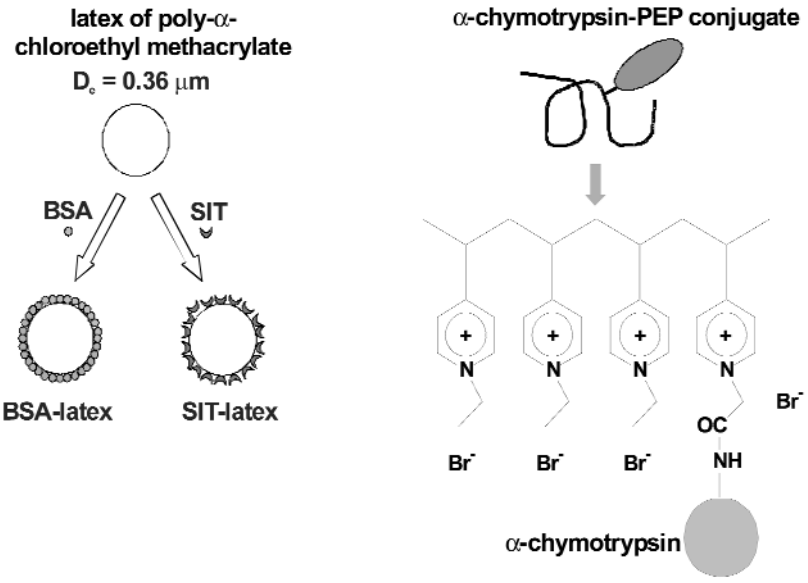


Fig. 5 Experimental proof for the migration of adsorbed PEP between latex particles. Electrophoretic mobility of latex particles: 1, initial; 2, recharged by adsorbed PEP; 3 and 4, 5 and 40 min after mixing of 1 and 2, respectively.

Thus, the experiments show that it is not forbidden, in fact, for a polycation to search for one particle-addressee among numerous negatively charged particles using the trial-and-error method. Then, a molecule attached to the polycation capable of recognizing the structurally complementary receptor attached to the surface of the particle-addressee may serve as the address.

The α-chymotrypsin (ChT) enzyme and another protein, viz., soybean inhibitor of trypsin (SIT), were chosen as examples of such partners [29,30]. The elements of the system used for the simulation of cell recognition by the polycation–antigen conjugate are presented in the following scheme (Scheme 5):



Scheme 5

Two varieties of modified poly-α-chloroacrylate latex particles (0.36 μm in diameter) served as models of cells. SIT was linked to the surface of one type of particles by covalent bonds, and the surface of the other particles was modified by bovine serum albumin (BSA). These proteins have close molecular weights. Their isoelectric points are arranged in the acidic pH region. Therefore, both of them are negatively charged in the neutral medium and, hence, can serve as centers of strong electrostatic binding of polycations to the latex particle surface. To prepare the polycation–antigen conjugate model, ChT as a vector was chemically linked to PEP in a ratio of 1 protein molecule per 1000 monomeric units. Thus, latex particles covered by SIT (SIT-latex) should serve as potential targets for the conjugate, and their competitors were latex particles covered by BSA (BSA-latex). The process of ChT-vector binding to the SIT-receptor was monitored by enzimatic hydrolysis of nitrophenyl acetate introduced into the system. This reaction rate can easily be measured spectrophotometrically by the formation of nitrophenol. The main results of experiments with the model system described above are presented in Fig. 6. As should be expected, for separate mixing, the ChT–PEP conjugate dissolved in water was quantitatively adsorbed on both latexes up to the complete saturation of the latex particle surface with the conjugate (Fig. 6a). In both cases, the adsorption pattern coincided with that observed for the interaction of PEP with the latex particles covered by carboxyl groups (compare Figs. 4b and 6a). At the same time, it follows from the data in Fig. 6b that the BSA-latex particles exerted almost no effect on the enzymatic activity of the ChT–PEP conjugate adsorbed on their surface, whereas its adsorption on the SIT-latex particle surface was accompanied by a linear decrease in the enzymatic reaction rate and, finally, by the almost complete inhibition of the enzyme. Therefore, during the ad-

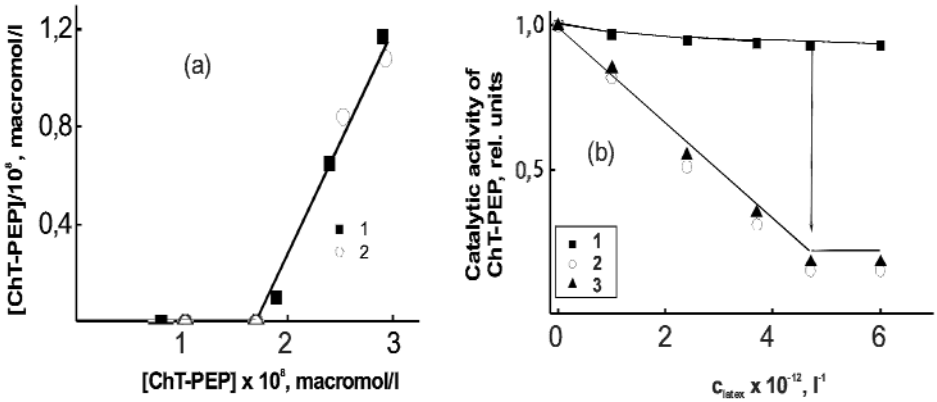
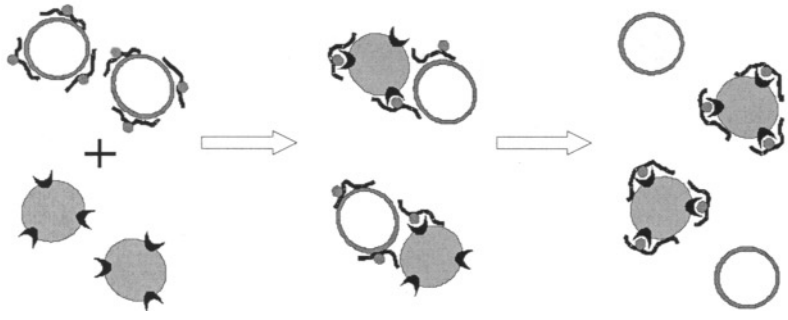


Fig. 6 Interaction of the ChT–conjugate with latexes covered by BSA (L-BSA) and SIT (L-SIT). (a) Dependence of the residual concentration of the conjugate in the above-precipitate liquid on the amount of the added conjugate: L-BSA (1) and L-SIT (2). (b) Change in the catalytic activity of the ChT–conjugate upon the interaction with L-BSA (1), L-SIT (2), and their mixture (3).

sorption of the ChT–PEP conjugate, the enzyme molecules additionally interacted with the complementary SIT molecules.

However, the key question was the following. Can the ChT–PEP conjugate recognize the SIT-latex particles against the background of the BSA-latex particles, because the latter are also capable of strong adsorption of PEP? It can, as follows from the data presented in Fig. 6b. It is seen that the efficiency of the inhibition of the conjugate initially adsorbed in the BSA-latex by the SIT-latex added to the preliminary prepared mixture of the ChT–PEP conjugate with the BSA-latex is the same as that in the absence of a latex-competitor. The catalytic activity in the ternary mixture was measured each 5 min after the addition of the SIT-latex. In other words, the conjugate quantitatively transferred from the BSA-covered latex particles to the SIT-covered latex particles for the time not longer than 5 min. In other words, at the contact between the latex particles the conjugate can transfer from the surface of one particle to another and finally be fixed due to additional free-energy gain brought by vector–receptor interaction [30]. The process of specific recognition by the conjugate of particle-targets among other particles (on which the conjugate is adsorbed, but in the nonspecific manner) can be presented as follows (Scheme 6):



Scheme 6

The above-described rather simple models served as the physicochemical substantiation for the unexpected phenomena observed by immunologists when antigens conjugated with SPE were introduced into the organism.

POLYMER-SUBUNIT IMMUNOSTIMULANTS

The simplest example is the copolymer of acrylic acid (AA) with *N*-vinylpyrrolidone (VP) carrying trinitrophenyl (TNP) groups attached by covalent bonds (TNP–AA–VP) [8,9,31]. The low-molecular TNP compounds themselves are not immunogenic. However, the data presented in Fig. 7 show that the TNP–AA–VP conjugate, being primarily introduced, induces the formation of the great number of TNP-specific antibody-forming cells (APCs) in the animal spleen. The number of these cells, as well as the titer of the antibodies themselves, serves as a measure of the efficiency of the immune response of the organism to the antigen introduced. The repeated conjugate administration was accompanied by a great enhancement of the immune reaction. The immune system produced no other antibodies except those complementary to TNP.

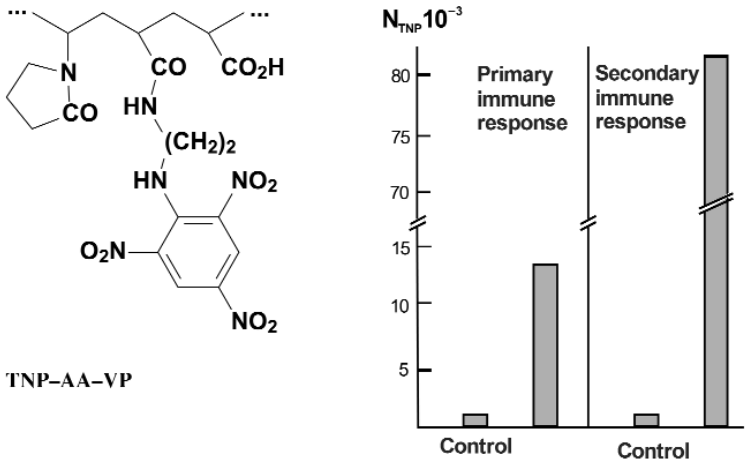
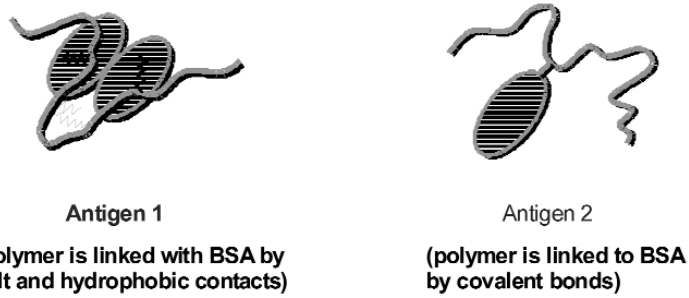


Fig. 7 Immune response of mice to the administration of TNP conjugated with the AA–VP copolymer. N_{TNP} is the number of TNP-specific AFCs in the spleen.

At the next stage, we added the real protein antigen BSA to SPE. Two modifications of PEP chains were used as SPE. One chain contained several molar percentage of hydrophobic cetyl groups, and another chain contained several molar percentage of carboxymethelene groups [8,9,32,33]. The globule of serum albumins is known to contain the hydrophobic "pocket" capable of sorbing aliphatic fragments of molecules. Due to this, the BSA globules in an aqueous solution were attached to the polycations modified by hydrophobic groups. In the second variant, they were attached by covalent bonds, condensing the carboxyl groups of SPE with the amino groups of the protein.



The BSA itself, as other serum albumins, is a very weak antigen. However, being linked to SPE, it induced a much stronger immune reaction in mice (Fig. 8). In the case of the covalent conjugate, the enhancement reached two orders of magnitude [32,33]. In this example, as in the previous one, AFCs accumulated in the spleen were rigidly specific toward BSA.

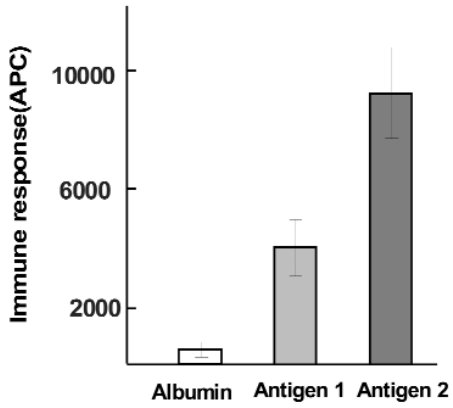


Fig. 8 Immune response of mice to the administration of BSA and its complex (antigen 1) and conjugate (antigen 2) with PEP.

In light of the data obtained, one can assume that the biochemical mechanism of launching the specific immune reaction by the antigen–SPE conjugate is analogous to that considered above for the SPE immunostimulants free of genetic control. However, unlike the nonselective SPE polyions, the action of the conjugates is focused on the cells bearing receptors, which are complementary to the antigens attached to the SPE. This was also confirmed seriously, although indirectly, by experiments with mice of two genetic lines [8,9,34]. One of these lines, "nude" (nu/nu), was artificially derived specially for immunological studies, genetically devoid of the immune protection. Correspondingly, nude mice are not able to produce antibodies as a response to the introduction of any antigen. The mice of another line (nu/+) noticeably react to BSA (as it has already been mentioned, it is a relatively weak antigen), and their reaction to bovine gammaglobulin (BGG) is much stronger. This known fact was naturally confirmed in blank experiments (Fig. 9). Based on the already obtained data on the enhancement of the specific immune response in outbred mice to the TNP–SPE and BSA–SPE conjugates, we could expect

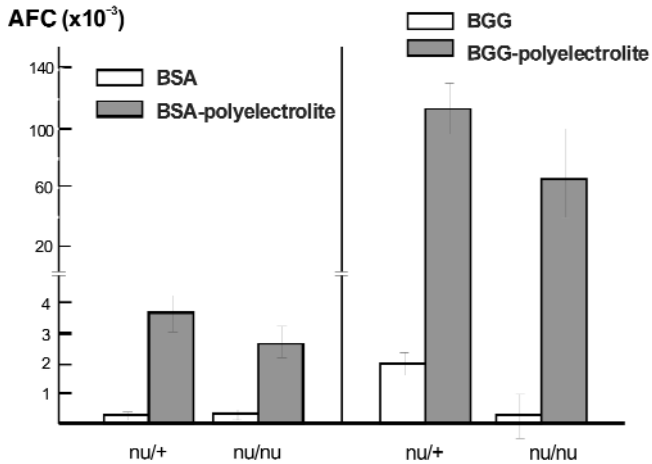


Fig. 9 Immune response strength to BSA, BGG, and their conjugates with SPE. For clarification, see text.

the experimentally observed enhanced reaction of the (nu/+) mice to the administration of both BSA and BGG conjugated with AA–VP. In the light of our logical conclusions, we were glad, but not surprised to obtain another result presented in Fig. 9. The genetically unprotected nude mice as a response to the conjugate administration produced almost the same amount of APC as the genetically protected mice did. Thus, the phenotypic correction of the immune response was performed by the polymer-subunit immunogens.

The last point in the system of proofs was obtained in experiments with the conjugate in which a special synthetic polypeptide, which is known in immunology as (T,G)-A-L antigen, was used as the conjugate vector, which in fact is a comb-like copolymer consisting of poly-L-lysine main chain and tyr-glu-$(ala)_3$-penthapeptide branches [8,9]. This antigen is famous owing to the fact that its very high immunogenic specificity in due time has served as one of the key substances in the discovery of the existence of Ir-genes [35,36]. In our experiments, we prepared the conjugate by the attachment of the (T,G)-A-L antigen to the AA–VP copolymer. The conjugate was administrated in mice of two genetic lines (CBA and C57BL), and its effect was compared to that of the free (T,G)-A-L antigen. The results are presented in Fig. 10. The CBA mice contain no IR-genes with the programmed immune response to the (T,G)-A-L sequence. Correspondingly, the immune system of these mice does not react to the free (T,G)-A-L antigen. The opposite situation with (T,G)-A-L is typical of the C57BL mice. This line produce a sufficiently great number of highly specific AFC as a response to the administration of the free (T,G)-A-L antigen. The administration of the (T,G)-A-L antigen conjugated with the AA–VP copolymer to the C57BL mice, as in the case of other conjugated antigens, resulted in the additional manifold enhancement of their specific immune reaction. It is remarkable, however, that the reaction to the conjugate of the CBA mice, which did not react at all to the free polypeptide, was as strong as the reaction of their genetically "successful" colleagues [8,9]. The last fact proves rigidly that the addition of the antigen to SPE provides immunogens, whose immunization reaches, in fact, the phenotypic correlation of the immune response.

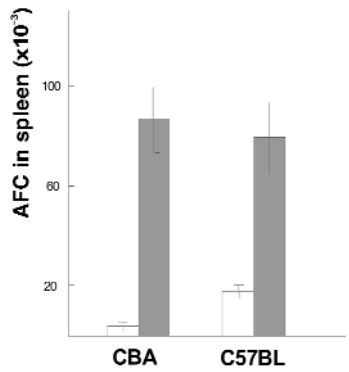


Fig. 10 Phenotypic correction of the immune response to (T,G)-A-L (open rectangles) and (T,G)-A-L-AA-VP conjugates (filled rectangles). For clarification, see text.

EXPERIMENTAL POLYMER-SUBUNIT VACCINES

At the next stage of studies, it was necessary to answer a significant question about the appearance of protective properties of the antigen–SPE conjugates when preisolated and purified microbial proteins or polysaccharides are linked as antigens to SPE immunostimulant chains.

Salmonollosis was the first of tested infections. Two varieties of conjugates were prepared and administrated to laboratory mice (Fig. 11). In one case, it was polysaccharide antigen (PS) isolated from bacteria bound to the AA–VP copolymer (conjugate 1). In the second case, PS and also flagellin (protein from bacterial flagellants) both were conjugated with the same AA–VP copolymer (conjugate 2).

After a certain time period (from 14 to 30 days), the immunized mice were infected by the absolutely lethal bacterial dose in parallel with the control group. Then, all animals of the control group naturally died. Unlike these, all preliminarily immunized animals recuperated, although, in the case of immunogen 1 containing only one polysaccharide antigen, the immunization by a tenfold higher dose than that in the case of the conjugate 2 was required to achieve the 100 % survival (Fig. 11, curves 1 and 2). The immunization by the free polysaccharide antigen in the region of reasonable doses did not virtually protect the animals (curve 3). Thus, it was shown for the first time that the antigen–SPE conjugates can serve, in fact, as antibacterial vaccines.

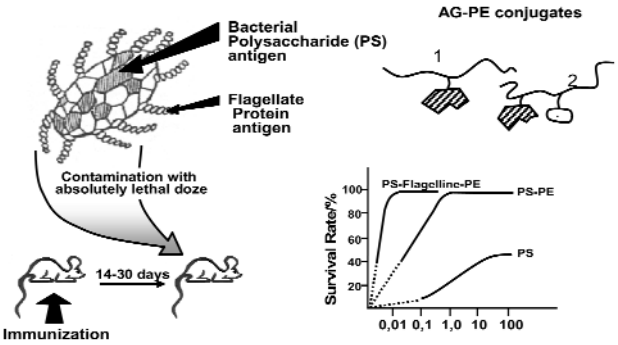


Fig. 11 Protective effect of the conjugates of salmonolla antigens with synthetic polyelectrolyte (see text).

It is known that the protection from viral infections is a more complicated task than bacterial infections. In order to clear up whether the above approach is applicable to solve this problem, two more conjugates containing influenza virus antigens were synthesized by chemical binding of hemagglutinin or both hemagglutinin and neuraminidase to the AA–VP copolymer (conjugates 1 and 2, correspondingly). Both resulting conjugates forced the immune system of laboratory mice to produce a very great number of AFC compared to the negligible number in the blank experiments (Fig. 12a). As in the case of bacterial infection, the mice were immunized by the conjugates and then infected by the absolutely lethal dose of the virus. The result surpassed all expectations. Both conjugates protected all lethal-infected animals (Fig. 12b) [8,9].

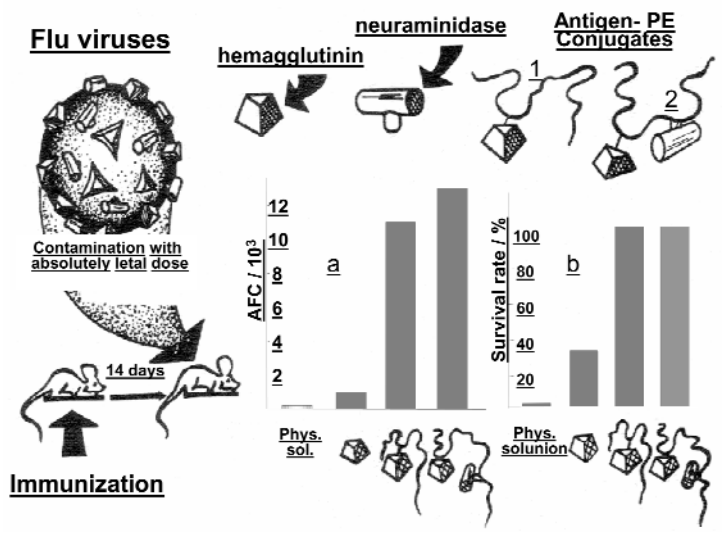


Fig. 12 Protective effect of the conjugates of influenza ("Flu") antigens with the synthetic polyelectrolyte (see text).

Moreover, it turned out that even the antigen–SPE conjugates containing "conservative" M- and NP-proteins nonexposed on the surface, but incorporated in the influenza virions were rather protective. The laboratory mice were immunized by the conjugates carrying these antigens separated from A(H_1N_1) virus strain and then infected by the absolutely lethal dose of viruses of another strain, A(H_3N_2). The data presented in Fig. 13 show that the survival of the immunized mice reaches 40–60 % at the 100 % mortality in control experiments along with the colossal enhancement of the M-specific immune response.

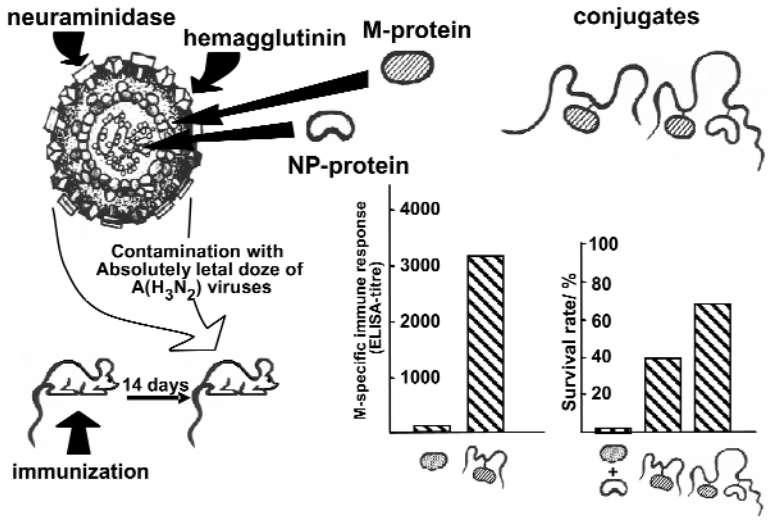


Fig. 13 Protective effect of the conjugated influenza vaccine containing antigens of the virus strain A(H_1N_1) for the virus strain A(H_3N_2) infection (for clarification, see text).

"GRIPPOL" AS THE FIRST HUMAN VACCINE OF THE NEW GENERATION

The results of the above-described studies made it possible to develop the polymer-subunit human vaccines.

The "Grippol" vaccine was obtained by covalent binding of protein antigens of the influenza virus, namely hemagluttinin and neuroaminidase, to polyoxidonium [37]. Now it is produced in the industrial scale and has been used in medicine practice for seven years. During the last 7 years, about 50 million recipients were vaccinated, and extensive statistical data indicating the high efficiency and innocuous character of the preparation were obtained. The plots of the desease rate during the 1987–1998 epidemic for people vaccinated by Grippol and people not vaccined, but living among the vaccined people (internal control) compared to those without any vaccination (external control) are presented in Fig. 14.

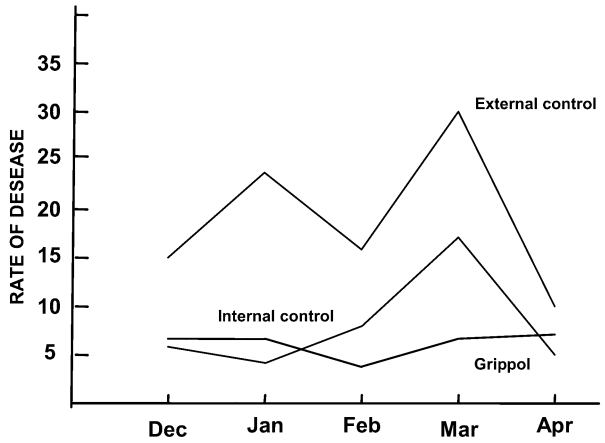


Fig. 14 Rate of desease (number of sick people per 1000) during influenza epidemic of 1997–1998 in Russia among 15 million recipients (official data of Russian Ministry of Health).

CONCLUSION

The mechanism of the stimulation of immune response by SPE manifested as the compensation of the function of T-cell-helpers was discovered. The membrane activity and, correspondingly, immunostimulation were shown to be inherent in SPE with different chemical structures of the repeating units and to depend critically on the SPE degree of polymerization.

These facts served as a basis for the search for a polyelectrolyte structure corresponding to the whole complex of pharmacological requirements to medicines. As a result, the new polyelectrolyte-copolymer of 1,4-ethylenepiperazine, 1,4-ethylenepiperazine-*N*-oxide and (*N*-carboxymethylene)-1,4-ethylenepiperazinium bromide (polyoxidonium) units was synthesized. The immunostimulation activity of this copolymer is combined with the low toxicity and the ability to destroy under the organism conditions and then to be ejected from the organism. Polyoxidonium is permitted and widely used in the Russian Federation as an adjuvant.

The principle of creation of conjugated polymer-subunit immunogens and vaccines by the addition of antigens to SPE-immunostimulants was formulated and experimentally substantiated and confirmed. The immunogenicity and protective properties of antigens covalently bonded to SPE increase by tens and hundreds of times. It is substantial that immunogens and vaccines built according to this principle act bypassing Ir-genes control, thus providing sufficient immunity even for organisms, whose reaction to this antigen is genetically weak.

The use of this principle made it possible to create the polymer-subunit human vaccine first in the world. An example of the Grippol vaccine opened the way for development of vaccines of the new generation against other dangerous infections.

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