POTENTIATION OF THE IMMUNE RESPONSE TO INFLUENZA VIRUS SURFACE ANTIGENS BY THEIR COVALENT BINDING WITH SYNTHETIC CARRIER POLYMER

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The surface antigens of influenza virus, namely hemagglutinin (HA) and neuraminidase (NA), are the principal antigens which induce the formation of protective antibodies in the infected organs [11,14]. However, HA and NA isolated from virus, when present in existing subunit vaccines, are only weakly immunogenic and are incapable of inducing a sufficiently effective immune response. Accordingly the development of ways of enhancing the immunogenicity of these surface antigens is an important step in the creation of new effective influenza vaccines. In recent years research into the creation of artificial antigens of a fundamentally new type, based on obtaining complexes or conjugates of antigenic structures of different nature with synthetic artificial polyelectrolytes of assigned composition and structure, which have a marked immunostimulating action, has made considerable progress [1-6]. Weakly immunogenic antigens of varied origin, in the composition of such complexes, are converted into highly immunogenic preparations, exhibiting some properties of T-independent antigens [1], and inducing an optimal specific immune response irrespective of the animals' genotype.

In the investigation described below the primary immune response was studied, in relation to the number of IgM- and IgG-antibody-forming cells (AFC) and the delayed-type hypersensitivity test (DTHT), in mice immunized with NA alone or with a mixture of HA and NA (HA + NA), covalently bound with a nontoxic carrier polymer, performing the role of inbuilt adjuvant. The covalent conjugate of virus antigens with carrier will be described below as a "virogate."

EXPERIMENTAL METHOD

1. Viruses and Virus Antigen. Surface antigens of influenza virus were isolated from high-yield recombinant strains MRC-11 (containing surface antigens H3 HA and N2 NA of epidemic strain A/Port Chalmers/1/73), and R-5 (containing H3 HA and N2 NA of epidemic strain A/USSR/05/80). The viruses were grown in the allantoic sac of chick embryos. HA was isolated from the purified and concentrated virus suspension by means of bromelin protease [7], and the (HA + NA) mixture was isolated with the aid of the nonpolar detergent β-octylglucoside [9]. The purity of the virus preparation and the isolated virus antigens was verified by electrophoresis in polyacrylamide gel and determination of HA and NA activity.

2. Covalent Binding of HA and (HA + NA) with Polymer: Preparation of Virogates. Isolated virus antigens were conjugated covalently with a synthetic copolymer (CP) of acrylic acid and N-vinylpyrrolidone. CP with an equimolar composition of its components and with a molecular weight of 100,000 daltons was synthesized by themethod described previously [8]. Virogates were synthesized by the carbodiimide method, using p-toluenesulfonate-N-cyclohexyl-N'-[2(4-morpholinium)ethyl] carbodiimide. The virogates were separated from unbound protein and free CP by gel filtration on a column (1.7 × 80 cm) with Sephadex G-150 (from "Pharmacia," Sweden), dialyzed against distilled water, lyophilized, and identified by their electrophoretic mobility, which differed from that of proteins and CP. The quantity of protein conjugated with CP with determined by Lowry's method [12]. Animals were immunized with virogates containing 100-150 μg virus protein/mg virogate.

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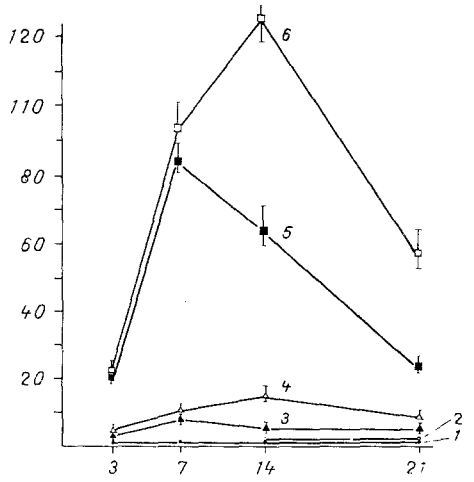

Fig. 1. Time course of anti-HA AFC formation in spleen of mice immunized with pure HA (1 — IgM, 2 — IgG), with a mechanical mixture of HA + CP (3 — IgM, 4 — IgG) and with HA-CP viral conjugate (virogate) (5 — IgM, 6 — IgG). Abscissa, days after immunization; ordinate, number of AFC in spleen ($\times 10^2$).

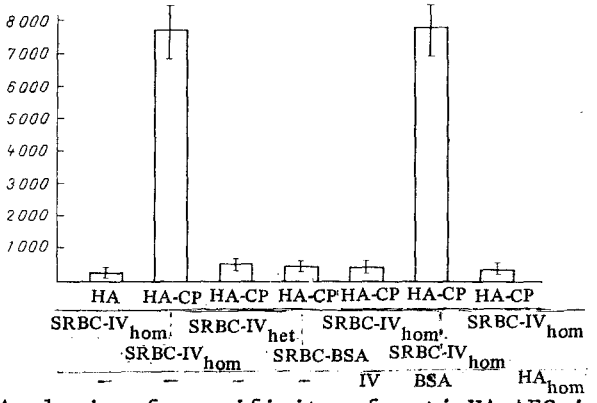

Fig. 2. Analysis of specificity of anti-HA AFC in spleen of mice immunized with HA-CP virogate. Mice immunized 7 days before detection of AFC with HA-CP virogate in a dose of 16 μg virus protein per mouse. Abbreviations below horizontal line (from top to bottom) signify: immunogen, indicator cell, addition of antigen or virus; vertical scale — number of anti-HA AFC in spleen.

3. Assessement of the Primary Immune Response. Experiments were carried out on male CBA mice weighing 22-24 g, obtained from the "Rappolovo" nursery, Academy of Medical Sciences of the USSR. The mice were immunized by a single intraperitoneal injection of isolated protein (0.2 ml), or a mechanical mixture of virus antigens with copolymer [HA + CP and (HA + NA) + CP], or with HA-CP and (HA + NA)-CP virogates in doses of 1-16 μg virus protein per mouse. The number of AFC in the animals' spleen was determined 3, 7, 14, and 21 days after immunization by a modified method [10], using sheep's red blood cells (SRBC), loaded with influenza virus (SRBC-IV) or with HA. As reagent, $CrCl_3 \cdot 6H_2O$ (from "Merck," West Germany) was used. Specificity of the AFC was verified by the use of SRBC loaded with heterologus protein — bovine serum albumin (SRBC-BSA) or heterologous influenza virus A/Singapore/1/57, containing surface antigens H2 HA and N2 NA which differed from those of A/Port Chalmers/1/73 and A/USSR/05/80 viruses. A test of inhibition of AFC formation by preliminary addition of various quantities of IV, HA, or BSA to agarose containing a mixture of spleen cells and SRBC-IV also was used.

DTHT against HA was induced by intraperitoneal immunization of the animals with isolated HA, the HA + CP mixture, or HA-CP virogate. After 7 days the reacting dose of HA (10 μg), in a volume of 50 μl, was injected subcutaneously into the footpad of the right hind limb. Physiological saline, in the same volume (50 μl), was injected into the footpad

of the control left hind limb. The thickness (diameter) of the footpad was measured by means of a micrometer after 6, 24, and 48 h. Results were expressed as percentages of the thickness of footpads of the control limb. The highest intensity of the DTHT was observed 24 h after injection of the reacting dose of HA.

EXPERIMENTAL RESULTS

The time course of formation of anti-HA IgM and IgG-AFC in the spleen of mice immunized with a single dose of HA alone, the HA + CP mixture, or HA-CP virogate, is shown in Fig. 1. On immunization with isolated HA only very few anti-HA IgM-AFC were formed in the animals' spleen throughout the period of observation. The number of these AFC was only 2-2.5 times greater than the number of spontaneous anti-HA AFC (the number of anti-HA IgM-AFC formed in the spleen of unimmunized animals). No anti-HA IgG-AFC were found in this group on the 3rd and 7th days after immunization in the animals' spleen. On the 14th and 21st days of observation only a very few of these AFC could be found. This fact confirms the weak immunogenicity of isolated influenza virus protein noted previously [13]. Injection of the mechanical mixture of HA + CP, not chemically bound with each other, increased the number of anti-HA IgM- and IgG-AFC by about 5 and 7 times respectively compared with their number produced by immunization with the antigens alone. On immunization of the animals both with the mechanical mixture of HA + CP and with HA-CP and (HA + NA)-CP conjugates, the maximal number of anti-HA-IgM- and IgG-AFC was recorded on the 7th and 14th days respectively. Immunization with the HA-CP virogate led to a much greater increase in the number of anti-Ha IgM- and IgG-AFC, up to 10-12 times more than the number formed in response to injection of the HA + CP mixture, and more than 50 and 100 times more respectively than the number of these anti-HA AFC formed in response to immunization with HA alone.

Absolute values of IgM- and IgG-AFC on immunization of mice with solutions of HA, (HA + NA), and their virogates in doses of 1-16 μg virus protein per mouse at the peak of the immune response are given in Table 1. These results are evidence that the (HA + NA)-CP virogate, like HA-CP virogate, induces a marked antiviral immune response. The height of the immune response depends to a certain degree on the dose of virogate.

The results of analysis of specificity of the anti-HA AFC formed after immunization of the mice with HA-CP virogate are given in Fig. 2. The results of analysis indicate strict and marked HA-specificity of AFC. The anti-HA AFC formed on immunization with HA-CP virogate were not found in agarose containing the same indicator cells, but loaded with the heterologous strain of influenza virus A/Singapore/1/57 (SRBC-IV_{het}) or with foreign protein (SRBC-BSA). Preliminary addition of homologous virus or HA to the agarose significantly inhibited the appearance of AFC, whereas after addition of BSA no inhibition took place.

The results given in Fig. 3 show that immunization with HA-CP virogate leads to the development of a stronger DTHT to HA than in mice immunized with the mechanical mixture of

TABLE 1. Level of HA-Specific IgM- and IgG-AFC in Mice Immunized with HA, (HA + NA), or Their Virogates

Antigen injected	Dose of antigen, μg protein/mouse	Number of HA-specific AFC in spleen of mice	
		Ig M	Ig G
HA	16	142±20	184±32
HA-CP	16	8 470±240	12 534±816
(HA + NA)	16	261±64	136±40
(HA + NA)-CP	16	12 284±692	15 416±670
HA	4	79±16	98±14
HA-CP	4	5 216±716	7 156±509
(HA + NA)	4	128±30	108±28
(HA + NA)-CP	4	8 238±489	10 482±892
HA	1	28±7	33±5
HA-CP	1	1 646±318	2 498±206
(HA + NA)	1	34±12	36±8
(HA+ NA)-CP	1	1 916±272	3 874±459

Legend. Statistical analysis of data by Students' test (M ± Ip, P 0.05).

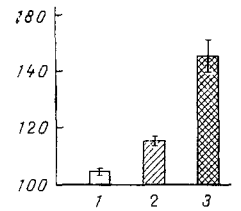

Fig. 3. Intensity of DTHT in mice immunized with HA alone (1), mechanical mixture of HA + CP (2), and with HA-CP virogate (3). Vertical axis, thickness of footpads (% of control).

HA + CP or with HA alone. The intensity of DTHT 24 h after injection of the reacting dose of HA is shown in Fig. 3.

The experimental data thus indicate that weakly immunogenic isolated surface antigens of influenza virus can be converted into highly immunogenic antigens through covalent "cross-linking" with a synthetic carrier polymer, which plays the role of active adjuvant. It must be pointed out that the peak value of the immune response was obtained to injection of conjugates (virogates) or virus antigens with carrier, and not to a mechancial mixture of them. The principle of obtaining highly immunogenic complexes thus developed is promising both for the preparation of high-titer sera against individual virus proteins and for the creation of vaccines of a new type against influenza and other diseases of viral etiology.

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