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Immunology

Anti-influenza response achieved by immunization with a synthetic conjugate

(synthetic vaccine/influenza vaccine/protective immunization/influenza hemagglutinin/hemagglutinin peptide)

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ABSTRACT **The peptide corresponding to sequence 91–108 of the hemagglutinin of type A H3N2 influenza virus has been synthesized by the solid-phase peptide synthesis method and covalently attached to several macromolecular carriers. The conjugate with tetanus toxoid was used for immunization of rabbits and mice. The immunoglobulin fraction of the rabbit antiserum showed the presence of antipeptide antibodies by both agar gel diffusion and radioimmunoassay. In the latter assay, the antibodies showed marked crossreactivity with the intact virus of the A/Texas/77 strain. The antibodies were also capable of inhibiting the hemagglutination of chicken erythrocytes by the virus; the highest hemagglutination inhibition titer (1:32) was achieved with a serum-resistant strain of A/Texas/77. When the *in vitro* virus plaque formation assay was used with monolayers of Madin–Darby canine kidney (MDCK) cells, the number of plaques was reduced on interaction with the immunoglobulin fraction of the antiserum, which was effective up to a dilution of 1:32. Preliminary results indicate that C_3H/DiSn mice immunized with the peptide–tetanus toxoid conjugate are partially protected against a further challenge with A/Texas mouse-adapted influenza virus. The results are thus indicative of the efficacy of the synthetic material in eliciting anti-influenza immune response.**

The idea of using synthetic materials to replace the vaccines currently used against viruses was put forth several years ago (1, 2). According to this approach, the chemically defined vaccine should contain a unique synthetic antigenic determinant(s) capable of provoking antibodies with neutralizing efficacy against the virus. Although this issue, as discussed recently (3), raises several problems, it also offers many potential advantages and its feasibility for a model virus has been proven. Thus, early studies by Anderer (4) have shown that, by immunizing with a conjugate containing the COOH-terminal hexapeptide of tobacco mosaic virus protein, it is possible to obtain antibodies that interact to some extent with the intact virus. In a more recent study (5), an immunologically active synthetic peptide analogous to a fragment of the coat protein of another virus, MS-2 coliphage, was described. A conjugate containing this peptide provoked antibodies capable of efficiently inactivating the native bacteriophage. It seemed of interest to test this chemical approach in an animal virus system.

The influenza virus provides a suitable model for an animal virus for this purpose for the following reasons. (*i*) Detailed information is available on the structure and function of this virus, as well as on its serological specificities and genetic variations. (*ii*) Various reliable assays of the virus are available (6–9) for evaluating the effect of the immune response on the different viral functions. (*iii*) Sufficient information is available on the amino acid sequence of the influenza hemagglutinin (10) and on its immunochemical properties (11) to allow the synthesis of a peptide fragment(s) that might carry some of its immunological activity.

We report here the synthesis of an 18-amino acid peptide analogous to sequence 91–108 of the influenza hemagglutinin and its capacity to elicit an immune response to the intact virus.

MATERIALS AND METHODS

Virus Growth and Purification. Influenza virus (strains A/Texas/77, A/Victoria/75, A/Memphis/72, and A/Hong Kong/77) was grown in embryonated eggs. The virus was purified by centrifugation for 30 min at 40,000 × *g* at 4°C. Aliquots were frozen in liquid nitrogen and thawed only once before testing. Virus resistant to serum inhibitors was obtained by growth of virus in the egg in the presence of a 4-fold (vol/vol) excess of rabbit serum, followed by another passage into the allantoic cavity without serum.

The infectivity titer of the virus was determined by titration in embryonated eggs and expressed as 50% egg-infectious dose.

Animals. Albino rabbits and inbred BALB/c, C3H/DiSn, C57BL/6J, and SJL/J mice were obtained from the Animal Breeding Center of The Weizmann Institute of Science.

Peptide Synthesis. The peptide corresponding to the sequence serine-91 to leucine-108 of the hemagglutinin heavy chain of H3N2 influenza strains (12) was synthesized by the solid-phase method according to Merrifield (13) with slight modifications essentially as described (5). The yield of purified peptide was 400 mg. This peptide was denoted HA1(I).

Preparation of Conjugates. Three carriers were used for conjugation—poly(DL-Ala)poly(L-Lys), poly(L-Pro)poly(L-Lys), and tetanus toxoid (TT) purified on Sephadex G-100. The carrier was dissolved in distilled water or phosphate-buffered saline (P_i/NaCl) and mixed with the desired amount of peptide in dioxane/H_2O (1:1), and a 5-fold (mol/mol) excess of an aqueous solution of 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide hydrochloride was added. After stirring overnight at room temperature, the conjugate was dialyzed against H_2O and lyophilized. The peptide content of the conjugates was determined by amino acid analysis. For the TT conjugate, the results were based on the reported amino acid composition of TT (14). The peptide/carrier (wt/wt) ratio in the different conjugate preparations varied between 9.5% and 50%.

Immunization Procedure. Rabbits were immunized by subcutaneous injections of 1 mg of conjugate dissolved in 0.5 ml of P_i/NaCl and emulsified in 0.5 ml of complete Freund's adjuvant. Three to four boosters with half the amount of conjugate were given at intervals of 3 weeks, of which the first was ad-

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Abbreviations: HA1(I), peptide corresponding to serine-91 to leucine-108 of the hemagglutinin heavy chain of H3N2 influenza virus; TT, tetanus toxoid; HI, hemagglutination inhibition.

ministered in complete adjuvant and the others were administered in incomplete Freund's adjuvant. Inbred 6- to 8-week-old mice were immunized by injection either intraperitoneally with 0.2 ml or into the foot pad with 0.08 ml of 50 μg of conjugate dissolved in P_i/NaCl and emulsified in the same volume of complete adjuvant. The mice were boosted after 4 weeks with the same amount of conjugate in either incomplete adjuvant or P_i/NaCl. Additional boosters were given when needed, using the same procedure. The immunoglobulin fraction of the different antisera was prepared by two precipitations with 40% saturated ammonium sulfate.

Serological Assays. Double gel diffusion was carried out in agarose. Solid-phase radioimmunoassay was carried out in microtiter plates coated with the antigen (100 μl at 50 μg/ml in each well). After incubation with various dilutions of the antibody, ^{125}I-labeled goat anti-rabbit IgG (50 μl containing 75,000 cpm) was added and the mixtures were incubated overnight at 4°C. After extensive washing, the plates were dried and the wells were cut and assayed. Controls consisted of wells previously coated with 1% bovine serum albumin instead of the antigen. When antigen consisted of intact virus, plates were coated with 10 hemagglutination units per well.

Hemagglutination inhibition (HI) tests (7) were carried out in U-bottom microtiter plates by overnight incubation of 2-fold serial dilutions of the antibodies with 0.1% bovine serum albumin in P_i/NaCl with 4 hemagglutination units of the virus followed by addition of chicken erythrocytes.

***In Vitro* Plaque Assay for Influenza Virus.** The assay was a modification of the procedure of Kilbourne (8) using confluent monolayers of Madin–Darby canine kidney (MDCK) cells grown in 35-mm Falcon tissue culture dishes and maintained in minimal essential medium with Hanks' salts/10% fetal calf serum. The washed cells were incubated with 0.6 ml of an appropriate dilution of virus suspension for 1 hr. The virus was aspirated, and the washed plates were overlaid with 2 ml of agar in 0.5% agarose/minimal essential medium with Hanks' salts/0.05% bovine serum albumin/0.005% trypsin and antibiotics and incubated for 2 days at 37°C in 3% CO_2/97% air. The plaques were counted without staining.

Inhibition of plaque formation was assayed by overnight incubation of the same dilution of the virus suspension with several concentrations of antibodies at 4°C, prior to addition to the cell monolayers.

Virus Passages in Mice and Determination of the Infective Dose (9). A/Texas virus was adapted to mice by serial passages in C3H/DiSn mice followed by one passage in eggs. This virus preparation served as stock solution, and its minimal infective dose was determined in groups of three mice. After 3 days, the lungs from each group were homogenized and pooled to give a 10% suspension in P_i/NaCl, which was centrifuged for 15 min at 6000 × g. The lung virus titer was tested by injection of 10-fold serial dilutions into eggs. The minimal infectious dose in mice was the lowest concentration giving rise to a lung virus titer of at least 10^{-2} in the eggs.

In protection experiments, the mice were infected 7 days after the last booster with a virus concentration in the vicinity of the minimal infectious dose.

RESULTS

Synthesis and Characterization of the Hemagglutinin Peptide. The synthetic peptide used in this study corresponds to the region 91–108 in the amino acid sequence of the hemagglutinin heavy chain of H3N2 strains. The only change from the native sequence was the replacement of cysteine-97 by alanine, to avoid the formation of aggregates. The purified peptide was characterized by amino acid analysis. The results show good agreement between the expected and observed values for the amounts of the various amino acid residues (Table 1). The purified peptide served for conjugation to three different carriers (Table 2).

Table 1. Amino acid composition of synthetic peptide HA1(I)

Amino acid	Expected	Found
Lysine	1	1.069
Aspartic acid/ asparagine	3	3.00
Serine	3	2.73
Proline	2	2.11
Alanine	3	3.00
Valine	1	0.995
Leucine	1	0.892
Tyrosine	3	2.662
Phenylalanine	1	0.951

Alanine was used as reference for calculating the ratios between the different amino acid residues.

Antibodies to the Synthetic Peptide. Immunization of rabbits was carried out with all three peptide conjugates. The immunoglobulin fractions of the antisera were evaluated for the presence of antipeptide antibodies by double gel diffusion and by radioimmunoassay. In double gel diffusion, a distinct spur formation, as compared with the carrier control, indicated the presence of antibodies specific to the peptide.

The antipeptide antibodies in the anti-HA1(I)–TT antisera were quantitated by radioimmunoassay with either the free peptide or with the peptide–poly(Pro)poly(Lys) conjugate. The results indicate the presence of antibodies specific to the peptide in this antiserum up to a dilution of at least 1:10,000 (Fig. 1).

Anti-influenza Immune Response Induced by the Synthetic Conjugates. The presence of anti-influenza antibodies in the serum obtained from rabbits after immunization with the HA1(I)–TT conjugate was assessed by the crossreaction with either the viral antigen (denatured virus) or the intact live virus of various strains.

The results obtained in the radioimmunoassay showed a high crossreaction of the antibodies with intact virus of the A/Texas/77 (serum-resistant) strain (Fig. 2). Crossreactivity was also obtained with two other A strains, A/Memphis/72 and A/Victoria/75, which gave higher binding to the immune serum as compared with the preimmune serum (not shown). The B/Victoria strain also showed some crossreaction with the antiserum, but this was less than that of the A strains.

The anticonjugate antisera were also evaluated by the HI assay with influenza virus of various strains. The results showed that hemagglutination by the various H3N2 A strains tested was partially inhibited by the antiserum (Table 3). The HI titer was calculated as the ratio of the titers obtained with immune and preimmune sera of the same rabbit. The specific HI titer of the

Table 2. Properties of the various conjugates of HA1(I)

Carrier	M_r	Peptide/carrier ratio: Used for coupling (wt/wt)	In the conjugate (% wt/wt)	In the conjugate (mol/mol)
Poly(Ala)poly(Lys)	70,000	0.3	9.5–26.2	2.9–8
Poly(Pro)poly(Lys)	110,000	0.8	50	24
TT	150,000	0.8	45–56	29–37

Ranges represent results for different preparations.

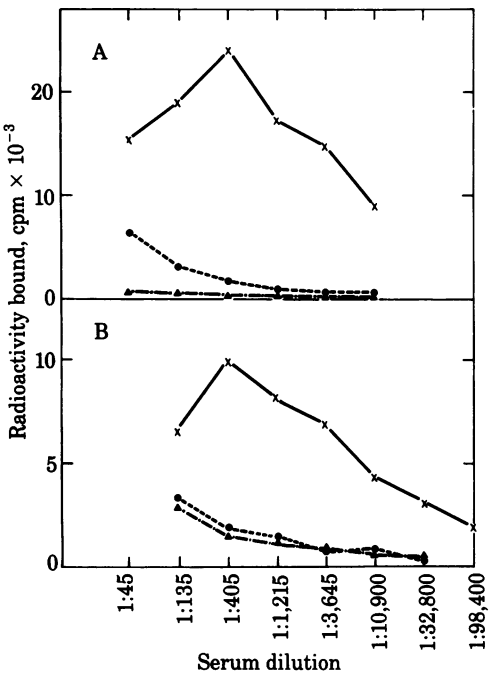

FIG. 1. Radioimmunoassay of anti-HA1(I)–TT serum with HA1(I) (*A*) or HA1(I)–poly(Pro)poly(Lys) (*B*). ×, Reaction with antigen-coated plates; ●, reaction with uncoated plates; ▲, control (preimmune serum).

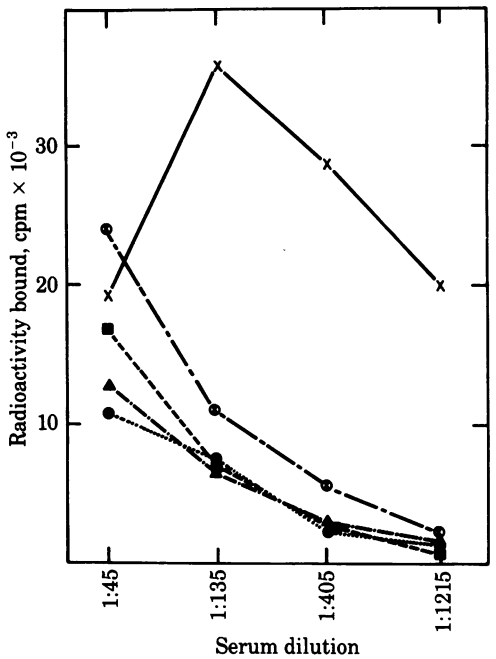

FIG. 2. Radioimmunoassay of rabbit anti-HA1(I)–TT with intact virus. ×, A/Texas/77; ⊗, B/Victoria; ●, no antigen; ▲ and ■, controls (preimmune serum with A/Texas/77 or B/Victoria, respectively).

serum is not high, but the results were very reproducible. The highest titer was obtained for the reaction with the serum-resistant A/Texas strain, in which nonspecific HI by the preimmune serum was markedly reduced.

***In Vitro* Neutralization of Virus by the Antipeptide Serum.** Neutralization of the virus was tested by the capacity of the rabbit anti-HA1(I)–TT immunoglobulin to inhibit virus plaque formation in tissue culture *in vitro*. The virus used was the serum-resistant A/Texas strain. The results of two experiments demonstrating the neutralization of the virus at different dilutions by different concentration of the immunoglobulin fraction of the immune serum are given in Table 4. As the results suggest, at all virus dilutions tested, the antibodies caused inhibition of virus plaque formation that was dependent on the antibody concentration and apparently on virus dilution as well. At a dilution of 3×10^{-7}, the inhibition was statistically significant ($P < 0.01$). With the lowest virus concentration, the extent of inhibition is apparently higher. The antiserum is effective up to a dilution of 1:32, which is consistent with the results of the HI assay. These results were confirmed by two additional experiments that gave very similar data. For each assay, a control experiment with preimmune serum from the same animal was carried out to avoid any artefacts.

Protection of Mice Against Infection. Mice of several strains were immunized with the HA1(I)–TT conjugate, and their immune response was assessed by the HI assay. The results showed that three out of four tested mouse strains, SJL/J, BALB/c, and C3H/DiSn, yielded antibodies that inhibited hemagglutination, whereas antiserum from mice of the C57BL/6J strain did not cause inhibition. Thus, the strain C3H/DiSn was selected for checking the potential of the synthetic conjugate to provide protection against viral challenge.

Preliminary results of such experiments indicate that immunization of the mice with the HA1(I)–TT conjugate, according to the schedule described above, gave partial protection against infection with A/Texas mouse-adapted influenza virus (Table 5). This was determined by evaluating the number of infective virus particles that could be recovered from the lungs of immunized and nonimmunized mice. The protective effect is demonstrated by the difference in the incidence of infection in the mice, as well as by the lower egg-infective dose of the lung homogenates of the immunized mice as compared with control groups, which were either untreated or immunized with

Table 3. HI activity of the antisera

Antiserum specificity	Antigen	Titer: Immune serum	Titer: Preimmune serum	ΔHI titer*
Anti-HA1(I)–poly(Ala)poly(Lys)	A/Victoria/75 denatured	1:64	1:16	1:4
Anti-HA1(I)–poly(Pro)poly(Lys)	A/Victoria/75 denatured	1:64	1:16	1:4
Anti-HA1(I)–TT	A/Victoria/75 denatured	1:64	1:8	1:8
	A/Victoria/75 live virus	1:128	1:32	1:4
	A/HK/1/77 live virus	1:128	1:32	1:4
	A/Texas/1/77 live virus	1:256	1:64	1:4
	A/Texas/1/77 live virus (serum resistant)	1:256	1:8	1:32

The immunoglobulin fraction of various rabbit antisera was used.
*Ratio of HI titers obtained with immune and preimmune sera.

Table 4. *In vitro* neutralization of A/Texas/77 virus by rabbit antiserum (plaque inhibition)

Dilution of allantoic fluid	Plaques, no. per plate									% inhibition of plaque formation			
	No serum	Preimmune serum				Immune serum							
		1:16	1:32	1:64	1:128	1:16	1:32	1:64	1:128	1:16	1:32	1:64	1:128
1×10^{-6}	113	51	116			39	93			24	20		
3×10^{-7}	35	29	36	36	37	13	22	35	37	55	38	3	0
1×10^{-7}	13	8	17			3	5			65	67		

Results represent the average of two experiments, each one carried out in triplicate.

TT alone prior to infection. However, no protection was observed in an experiment in which a higher infective dose (10^{-6} dilution) of the virus was used. The effect of immunization is stronger when the dose of the virus is lower, a phenomenon similar to that observed in the *in vitro* assay.

DISCUSSION

The influenza virus is an appropriate model system for studying the synthetic approach to vaccination, particularly if the synthetic fragment is derived from the hemagglutinin molecule. In this case, the immune response elicited by a fragment of the molecule can be assessed on four different levels: (*i*) the immunochemical reaction—i.e., the capacity of the elicited antibodies to interact with the peptide as such and to crossreact with the intact virus; (*ii*) the interference with biological activity of the hemagglutinin molecule; (*iii*) the *in vitro* neutralization of the virus, as expressed by the reduction of virus plaque formation in tissue-cultured cell monolayers; and (*iv*) the most crucial criterion—the *in vivo* protection of animals as manifested by the decrease in the incidence and severity of infection after active immunization with the synthetic antigen. The results presented in this paper suggest that, indeed, the conjugate we have synthesized proved effective on all four levels.

Particular consideration should be given to the synthetic peptide that was used in this investigation and the rationale for its choice. As reported, a 170-residue CNBr-cleavage fragment of HA1 subunit, denoted CN1, is responsible for the immunological activity of the hemagglutinin, eliciting antibodies that bind to the intact virus and inhibit its hemagglutinin activity (11). Due to its large size, the CN1 fragment as such is not readily amenable to chemical synthesis nor was this fragment available in sufficient quantities to allow the preparation and screening of smaller immunologically active fragment(s). Because, at the time this investigation was initiated, the three-dimensional structure of influenza hemagglutinin was not yet known, the decision on the peptide to be synthesized was based on the available amino acid sequence (12), which was provided to us by C. W. Ward and W. G. Laver prior to publication, and the predicted folded structure of the hemagglutinin molecule. The prediction of this structure was derived by Christian Sander in our institute from a computer analysis (15) based on the amino acid sequence. We tried to choose a peptide with a relatively high probability of being exposed on the molecule surface and hence a better candidate for serving as an antigenic determinant.

Table 5. Protection of mice against infection

Group	Incidence of infection*		EID†
	10^{-1} dilution into eggs	10^{-2} dilution into eggs	
	Virus dilution, 10^{-8}		
Control (untreated)	4/4	3/4	$10^{-3.5}$
Control (toxoid)	4/4	3/4	10^{-4}
Vaccinated	2/4	1/4	$10^{-1.25}$
	Virus dilution, 10^{-7}		
Control (untreated)	3/5	2/5	$10^{-2.2}$
Vaccinated	2/10	1/10	$10^{-0.7}$

C3H/DiSn mice were immunized with HA1(I)–TT conjugate and injected with A/Texas/77 virus.

* Results represent number of mice per group in which virus was present in a 10% lung homogenate.

† Egg-infective dose, lowest dilution of lung homogenate that is still infective in eggs (average values for all the mice in each group).

The synthesized peptide, which according to the predicted structure should have comprised a β-bend-folded region, consisted of residues 91–108 of HA1. This is a region common to at least nine H3N2 strains of the A subtype (12). It contains two proline residues, which are known to play a crucial role in the spatial conformation of proteins (16) and their antigenic specificity (17). It also contains three tyrosines, an amino acid with a putative contribution to the immunogenic capacity (18). This peptide was thus anticipated to be immunogenic and to perhaps have some distinct conformational features of the native protein. It is of interest that Wiley *et al.* (19) have recently reported that this region possesses a folded "corner" in the three-dimensional structure of influenza hemagglutinin. Moreover, the tyrosine-98 is one of the residues forming the tentative receptor site of the molecule (20). Consequently, although not designated by these authors as one of the antigenic sites, this peptide could readily be visualized as an exposed region having an immunological imprint. It is even possible that, in the intact virus, this region forms a hidden determinant that, when present on a synthetic antigen, might lead to the formation of antibodies that can reach the antigenic zone *in situ*.

A characteristic of the influenza system that plays an important role in the immune response to it is the tremendous genetic variation among influenza viruses and its reflection in the antigenic differences among them (10). Of the two main antigenic proteins of influenza, the hemagglutinin is primarily responsible for the serological specificity of the different viral subtypes and strains (21). The sequences of the hemagglutinin from several strains have been completely determined, and particular amino acid exchanges that can be correlated with the gradual antigenic "shifts" and "drifts" have been identified (19, 22). With this information on "variable" and "constant" regions in the hemagglutinin molecule, it should be feasible, by the use of appropriately designed synthetic antigens, to direct the immune response against one or the other of these two molecular entities. As already indicated, the peptide used in this study is a part of a preserved sequence. Hence, on principle, the antibodies it elicits should be equally reactive with the relevant different strains. In the radioimmunoassay and HI assay, crossreaction with some of these strains has indeed been observed. These results might be indicative of the potential of this synthetic vaccine in cross-strain protection.

This study provides evidence for antiviral immunity elicited by a synthetic material. The titers obtained are not very high, but they are apparently sufficient to provide effective protection against infection with relatively low viral challenge. It is

hoped that the protective effect can be augmented by the use of more adequately designed antigenic determinants or by the use of a combination of peptides (of common and variable regions?) attached to the same carrier molecule.

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