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Immunology

Successful immunization with a totally synthetic diphtheria vaccine

(synthetic immunoactivator/protective antigenic structure/macromolecularization)

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ABSTRACT **Three peptides corresponding to fragments of diphtheria toxin have been synthesized. They include the previously described tetradecapeptide and two structural analogs, the hexadecapeptide and the octadecapeptide. Conjugates of these peptides to proteins or a synthetic carrier have induced in guinea pig protection against the dermonecrotic activity of diphtheria toxin. All of the conjugates were immunogenic when administered either in complete Freund's adjuvant or with *N*-acetylmuramyl-L-alanyl-D-isoglutamine in aqueous medium. Positive immune response toward the octadecapeptide was obtained in mice as well. In this case, the immunogenic combinations were conjugates with bovine serum albumin administered either in Freund's adjuvant or with the muramyl dipeptide and a complete synthetic conjugate comprising both the octadecapeptide and the muramyl dipeptide covalently attached to a synthetic carrier, multichain poly(DLAla). This last immunogen, which induced the most effective immune response, is a completely synthetic immunogen with built-in adjuvanticity and induces protective antitoxic immunity when administered in a physiological medium.**

The synthetic approach to vaccination has as its purpose the replacement of classical vaccines or the development of vaccines not yet available. For this purpose, such a vaccine should contain a synthetic specific antigenic determinant as well as adequate adjuvanticity (1, 2). For protein antigens, steric conformation is of utmost importance for their specificity, and we have shown previously that it is possible to synthesize a peptide, denoted "loop," analogous to an immunopotent conformation-dependent region of the hen egg white enzyme lysozyme, attach it to a polymeric synthetic carrier, and provoke conformation-specific antibodies in experimental animals (3). This approach has been extended to other systems, and recently we reported the formation of antibodies toward a synthetic antigen containing a peptide derived from the coat protein of a bacteriophage, MS-2, that were capable of efficiently neutralizing the virus (4).

We have also previously reported that active antitoxic immunization against diphtheria can be achieved with a synthetic peptide covalently attached to a protein carrier (5). This peptide, a tetradecapeptide denoted STDP, consists of residues 188–201 (Fig. 1) in the amino acid sequence of the diphtheria toxin, and it represents a fragment of the loop (16 amino acid residues, 186–201, sustained by two cysteine residues) that comprises the two functional segments of the natural diphtheria toxin molecule (6, 7). In all these studies, immunization was carried out in Freund's complete adjuvant (FCA).

Earlier studies have shown that it is possible to obtain a high level of antibody response to several antigens by using, instead of FCA, Freund's incomplete adjuvant (FIA) mixed with some of the peptidoglycan components of the bacterial cell wall, or subunits of them, including *N*-acetylmuramyl-L-alanyl-D-isoglutamine (8–11). The latter molecule, denoted muramyl dipeptide (MDP), and several of its derivatives, strongly enhanced the humoral antibody response to bovine serum albumin even when administered in saline (12). This adjuvant activity was confirmed by using various bacterial, viral, or parasitic vaccines (13–19). When covalently attached to the macromolecular antigens, the soluble adjuvants show higher activity. This was demonstrated with the MDP conjugate of the synthetic antigen poly(LTyr,LGlu)-poly(DLAla)--poly(LLys) (20) and with the poly(DLAla)--poly(LLys) (A--L) conjugate of the synthetic P_2 peptide of MS-2 bacteriophage, using either the natural peptidoglycan (21) or synthetic MDP (22). All these conjugates possessed built-in adjuvanticity leading to high level antibody production; thus, MDP-P_2-A--L constitutes a completely synthetic material that induces antiviral activity even when administered in saline (22).

Here, we report the use of several analogs of the synthetic diphtheria toxin peptide conjugated to proteins or synthetic carriers and administered with various adjuvants. We show that, in this case as well, a fully synthetic molecule, including a specific determinant and built-in adjuvanticity, can evoke efficient protective immunization against diphtheria when given in aqueous solution.

MATERIALS AND METHODS

Synthetic Compounds. The adjuvant used was MDP. The carrier was A--L. Its average molecular weight was 100,000, and the Lys/Ala ratio was 1:20. There were 64 NH_2 groups per molecule. Synthesis of peptides was achieved by the solid-phase method as described.§ The various peptides are depicted in Fig. 1, in comparison with the structure of the native diphtheria toxin loop.

Natural Proteins. Proteins used as carriers, bovine serum albumin and ovalbumin (Pierce), were of affinity-purified quality. Purified diphtheria toxin and toxoid were gifts from D. Labert (Institut Pasteur Production).

Methods of Coupling and Polymerization. (*i*) Peptides were linked to carriers via their NH_2 groups [except for the octadecapeptide (SODP) which was linked via its SH and COOH groups]. The coupling agent was glutaraldehyde, and the amounts of peptides and carriers were calculated to give ap-

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Abbreviations: MDP, muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine); A--L, multichain poly(DLAla)--poly(LLys); P_i/NaCl, phosphate-buffered saline; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; STDP, SHDP, and SODP, synthetic tetra-, hexa-, and octadecapeptide, respectively; ACM, carboxamidomethyl; MRD, minimum reactive dose.

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§ Rivaille, P., Siffert, O., Delmas, A., Milhaud, G., Audibert, F., Jolivet, M., Chedid, L., Boquet, P. & Alouf, J. (1981) Seventh Symposium on Peptides, May 14–19, 1981 (Univ. of Wisconsin, Madison, WI).

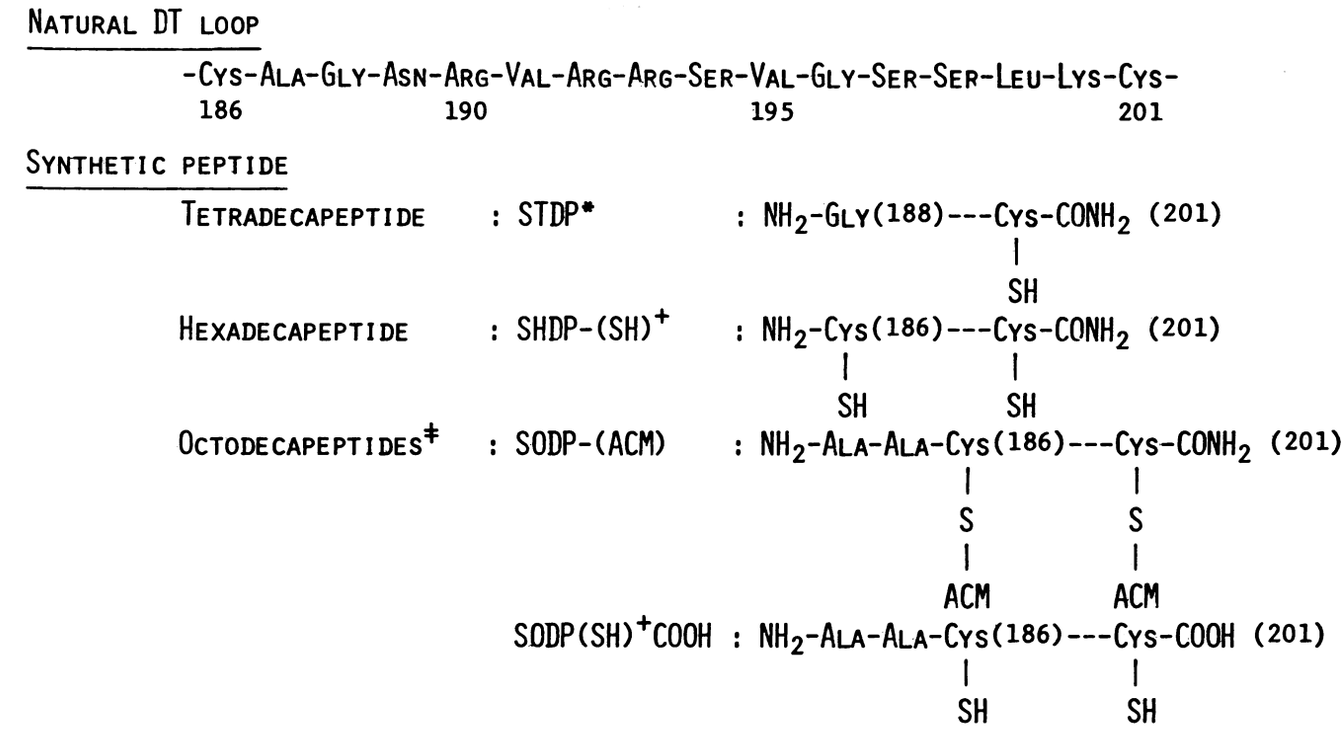

FIG. 1. *, This peptide is likely to be dimeric. †, In these peptides, the sulfhydryl groups are free and it can be assumed that most of the molecules have disulfide bridges. ‡, All three SODPs contained two additional alanines at the NH_2 termini and residue 187 was [^{14}C]alanine to allow monitoring of coupling. In SODP(ACM), sulfhydryl groups were blocked by ACM groups.

proximately 1.2 NH_2 equivalents on the peptide for each amino group on the carrier. The same procedure was applied for coupling to proteins, to A--L, or to MDP-A--L. For example, STDP was linked to bovine serum albumin by the following procedure. Twenty-four milligrams of bovine serum albumin (21 NH_2 microequivalents) in 19 ml of 0.1 M sodium bicarbonate was mixed with 39 mg of STDP (26 microequivalents). After 1 hr, 25% glutaraldehyde (Sigma grade) in H_2O was added to a final concentration of 2.63 mM with continuous stirring at room temperature in the dark (addition time, 5 hr). The resulting mixture was dialyzed exhaustively against 0.01 M sodium phosphate/0.15 M NaCl, pH 7.2 (P_i/NaCl; Mérieux, Lyon, France).

(*ii*) MDP and SODP(SH)COOH were linked to A--L by using soluble carbodiimide, under the conditions described in ref. 23 and following the procedure of Goodfriend *et al.* (24). When MDP-peptide-A--L conjugates were prepared, the first step was always the synthesis of MDP-A--L.

(*iii*) To polymerize peptides, the conditions were as described below for carboxamidomethyl (ACM)-SODP. Fifteen milligrams of SODP in 2.7 ml of 0.1 M sodium bicarbonate was mixed with aqueous glutaraldehyde solution to obtain a final concentration of 0.1%. The mixture was stirred for 3 days at room temperature in the dark. The conjugate was then dialyzed against P_i/NaCl.

When ACM-SODP labeled with ^{14}C was used, it was possible to determine the extent of coupling by using a scintillator counter. It was found that 4.5 mol of peptide was linked to one mole of ovalbumin and 4.6 mol of peptide was linked to 1 mol of A--L (assuming a molecular weight of 100,000).

Immunization. (*i*) Guinea pigs [female Hartley strain, weighing 400 g, purchased from CZC (Romilly/Aigre)] were immunized by the footpad route. They received (except when mentioned) 1 mg of STDP, SODP, or synthetic hexapeptide (SHDP) conjugate or 100 μg of peptide polymer. Conjugates were administered in FCA water-in-oil emulsion or in P_i/NaCl containing 100 μg of MDP. MDP-peptide-A--L conjugates were administered in P_i/NaCl. Control groups received toxoid alone or ovalbumin in FCA or in FIA containing 100 μg of MDP. Animals were boosted 1 month later with the antigen in the absence of adjuvants. Individual sera were collected by cardiac puncture during primary and secondary responses.

(*ii*) Mice (6- to 7-wk-old female BALB/c; Iffa-Credo) were immunized with SODP conjugate or polymer. Groups of eight animals were subcutaneously injected three times (days 1, 3, and 5) with a total dose of 200 μg of antigen in FCA or P_i/NaCl. After 1 month, booster injections were given in FIA or P_i/NaCl. Sera were collected after primary and secondary responses.

Enzyme-Linked Immunosorbent Assay (Indirect ELISA). The general method described by Voller *et al.* (25) was followed. Wells of micro-ELISA trays (microtest immuno Nunc, 96F) were coated by incubation for 2 hr at 37°C with purified diphtheria toxin at 5 μg/ml. The wells were washed exhaustively, and serial twofold dilutions of the sera were distributed to them and left 1 hr at 37°C. The wells were again washed exhaustively, a 1:2,000 dilution of peroxidase-labeled goat anti-guinea pig IgG antibodies (Institut Pasteur Production) was added, and incubation was continued for 1 hr at 37°C. The wells were washed, and the substrate [*O*-phenylenediamine (Sigma), 50 mg/100 ml of 0.05 M citrate/phosphate buffer, pH 5] was added. The reaction was allowed to proceed for 20 min at room temperature and stopped by addition of 50 μl of 12.5% (vol/vol) H_2SO_4. Absorbances were read at 492 nm in an automatic reader (Titertek multiscan). Titers were expressed as the inverse of the maximal dilution giving an absorbance twice that of 1:100 dilution of a pool of 50 normal guinea pig sera. Measurements were generally made at the level OD > 0.15.

Protective Activity. Guinea pig sera were tested for ability to neutralize the dermonecrotic activity of the toxin. Sera and toxin were diluted in 25% calf serum. Control and experimental sera were diluted 1:4, and diphtheria toxin was used at 25 or

Table 1. Antibody responses of guinea pigs

Synthetic peptide	Carrier	Adjuvant	Response	
			Primary	Secondary
STDP	Bovine serum albumin	FCA	(4/5) 7.75 ± 1.5	(5/5) 10.98 ± 1.68
STDP	A--L	FCA	(4/5) 8.25 ± 1.5	(5/5) 10.98 ± 1.49
STDP	A--L	MDP*	(6/9) 7.0 ± 0	(9/9) 9.1 ± 0.78
PolySTDP	—	MDP	(1/6) 7.0	(6/6) 8.88 ± 1.94
SHDP(SH)	Bovine serum albumin	FCA	(5/5) 8.8 ± 1.1	(5/5) 10.03 ± 1.56
SHDP(SH)	A--L	FCA	(9/10) 8.48 ± 1.75	(10/10) 9.03 ± 1.7
SHDP(SH)	A--L	MDP*	(6/8) 8 ± 1.1	(8/8) 8.57 ± 1.13
SODP(ACM)†	Ovalbumin	FCA	(6/10) 7.67 ± 1.03	(9/9) 8.51 ± 2.36
SODP(ACM)‡	A--L	FCA	(8/8) 9.25 ± 1.04	(8/8) 11.15 ± 1.23
SODP(ACM)‡	A--L	MDP*	(7/10) 7.86 ± 1.07	(10/10) 8.5 ± 1.08
Poly[SODP(ACM)]	—	MDP*	(3/6) 8.67 ± 1.53	(6/6) 9.66 ± 1.92
—	Ovalbumin	FCA	(0/12)	(0/12)
	Ovalbumin	FIA/MDP	(0/84)	(0/84)

Groups of guinea pigs were injected in the footpads with 1 mg of conjugate, except that the SODP(ACM)-ovalbumin, poly(STDP), and poly[SODP(ACM)] received 100 μg, in 0.2 ml of water/oil emulsion or P_i/NaCl. Results are mean ± SEM and are given as $\log_2$. Values in parentheses are positive sera/sera tested.
* Administered in saline at 100 μg mixed with the conjugate.
† SODP(ACM)/ovalbumin = 12:88 (wt/wt).
‡ SODP(ACM)/A--L = 84:916 (wt/wt).

50 times the minimum reactive dose (MRD). General conditions were those described in the European Pharmacopea (intradermal injection of 0.2 ml of the mixture of sera and toxin, administered after 30 min of incubation). Horse antidiphtheria toxin and guinea pig antiovalbumin sera were used as positive and negative controls, respectively. Redness and necrosis were checked every day for 4 days.

RESULTS

Antibody Response of Guinea Pigs to Various Synthetic Diphtheria Peptides Conjugated to Proteins or a Synthetic Carrier and Administered with FCA or MDP in P_i/NaCl. STDP and two structural analogs, SHDP and SODP, were synthesized and coupled to proteins or to the synthetic A--L carrier. These conjugates were administered with either FCA or MDP in aqueous medium. STDP and SODP were also polymerized and administered with MDP and without a carrier.

The results are summarized in Table 1.

(*i*) As expected from our previous finding with STDP (5), conjugates of SHDP and SODP and a protein carrier were immunogenic when administered with FCA. (*ii*) All three synthetic antigens (i.e., peptide coupled to synthetic A--L) were also immunogenic when administered with FCA. (*iii*) More interestingly, these synthetic immunogens elicited antibody responses of the same order of magnitude when administered with MDP in aqueous medium. Positive secondary responses were also observed when STDP and SODP were polymerized and administered with MDP in P_i/NaCl. These responses are relatively weak in view of the fact that guinea pigs immunized under the same conditions with 1 μg of diphtheria toxoid have higher antibody titers (primary response, 9.5 ± 1.22; secondary response, 13.1 ± 0.45). The results nevertheless are highly significant, since no anti-diphtheria antibodies were observed in guinea pigs that received a different antigen (ovalbumin) either with FCA or with MDP emulsified in FIA.

The protective activity of the antibodies elicited after immunization by various fragments was tested by measuring neutralization of the dermonecrotic activity of diphtheria toxin by several individual sera. Results can be summarized as follows: (*i*) there was a correlation between the antibody titer and the protective activity of the serum; (*ii*) high titers produced by all synthetic fragments conjugated to protein or synthetic carrier and administered with FCA could neutralize 50 MRD of toxin. Polymerized STDP associated with MDP in saline also neutralized 50 MRD of toxin. Serum of guinea pigs treated by SODP-A--L associated with MDP in saline, which had a lower antibody titer, neutralized only 25 MRD (Table 2).

Antibody Response of Mice to Free or Conjugated SODP Administered with or without Adjuvants. The results are summarized in Table 3. (*i*) When SODP was administered without a carrier in FCA or in P_i/NaCl with or without MDP, no antibodies were detectable. (*ii*) SODP conjugated to A--L but administered without an adjuvant was also devoid of immunogenicity. (*iii*) In contrast, SODP conjugated to bovine serum albumin and administered with FCA or with MDP in saline gave detectable antibodies. Moreover, when both MDP and the antigenic peptide were coupled to a synthetic carrier and administered in aqueous solution, the responses were stronger than those observed with SODP-bovine serum albumin administered with FCA. The immune response was evoked both by conjugates in which the peptide was attached via the amino group and by those in which the linkage was via the carboxyl group.

DISCUSSION

Production of synthetic vaccines is desirable because this approach should lead to safer and more abundant sources of antigen and to less demanding quality control procedures. This is especially true for those viral vaccines (26) for which efficient procedures for *in vitro* culturing and vaccine preparation are not available. For the synthesis of such immunogens, knowledge of the viral structure is required, and this knowledge should also allow a better understanding of the mechanisms in-

Table 2. Protective activity of guinea pig anti-peptide sera against the dermonecrotic effect of diphtheria toxin

Immunizing preparation	Protection
STDP-bovine serum albumin/FCA	++
STDP-A--L/FCA	++
SHDP-bovine serum albumin/FCA	++
SHDP-A--L/FCA	++
SODP(ACM)-A--L/FCA	++
Poly(STDP)/MDP	++
SODP-A--L/MDP	+

Table 3. Antibody responses of mice to SODP

Immunizing preparation	Response	
	Primary	Secondary
SODP(ACM)/FCA	(0/8)	(0/8)
SODP(ACM)	(0/8)	(0/8)
SODP(ACM)-MDP*	(0/8)	(0/8)
SODP(ACM)-A--L†	(1/7) 7	(0/8)
SODP(SH)-bovine serum albumin†/FCA	(3/8) 7	(7/8) 7.28 ± 0.75
SODP(SH)-bovine serum albumin‡/MDP (100 μg)§	(4/8) 7	(8/8) 7.33 ± 0.81
MDP-SODP(SH)-A--L¶	(7/10) 7.57 ± 0.97	(7/8) 7.28 ± 0.75
MDP-SODP(SH)-A--L‖	(6/6) 8.75 ± 1.25	(6/6) 8.8 ± 1.64

Mice (eight per group) were immunized subcutaneously. Results are mean ± SEM and are given as $\log_2$. Values in parentheses are positive sera/sera tested.
* SODP(ACM)/MDP = 81:19 (wt/wt).
† SODP(ACM)/A--L = 3.2:96.8 (wt/wt).
‡ SODP(SH)/bovine serum albumin = 3.5:96.5 (wt/wt).
§ Administered in saline.
¶ MDP/SODP(SH)/A--L = 3.5:20:76.5 (wt/wt); linked via NH_2 groups with glutaraldehyde.
‖ MDP/SODP(SH)/A--L = 4.1:3.5:92 (wt/wt); linked via COOH groups with carbodiimide.

volved in the immunity against immunopotent segments.

In the past, the use of well-defined haptens and antigens has contributed greatly to the advance of various aspects of basic immunology (27). Immunization with artificial antigens and other chemically defined antigens has led to induction of antibodies reactive against intact native proteins. But, until recently, it was not clear whether *in vivo* production against complex bacterial or viral agents could be achieved with such synthetic antigens or whether the use of more complex multideterminant antigenic structures would be required. Early findings by Avery and Goebel (28, 29) showed that immunization with some pneumococcal oligosaccharides can provide protection against pneumonia. These, however, comprised short fragments of repeating sequences of the capsular polysaccharides. Recently, these findings were confirmed and expanded by the use of synthetic peptides. These studies include our report on the use of synthetic peptides that copy a region in diphtheria toxin (5, §) and one describing a study in which streptococcal antigens were used (30). A synthetic conjugate has elicited neutralizing antibodies against a bacteriophage (21). Several synthetic hepatitis B peptides were reported to elicit antibodies that can bind to the infective Dane particles of the virus, but their protective capacity *in vivo* has not yet been demonstrated (31–35). Very recently, we reported the synthesis of a peptide fragment of influenza hemagglutinin, a conjugate of which induces antibodies reactive with the intact virus and inhibits its hemagglutinin activity and plaque formation *in vitro*; moreover, immunization of mice with this conjugate led to their partial protection against challenge infection (36).

All these studies, including our own, were achieved, however, by administering the antigen in FCA and, in most cases, when conjugated to a protein carrier. Only in the case of MS-2 coliphage were neutralizing antibodies induced by immunization in aqueous medium, by using a conjugate containing both the specific antigenic peptide and MDP. To our knowledge, the data reported here provide the first report of effective protective immunization against disease-causing bacteria with a totally synthetic vaccine in the absence of Freund's adjuvants. These results were obtained by attachment of various synthetic diphtheria peptides to a synthetic carrier conjugated to a synthetic adjuvant. In one instance, a polymerized peptide associated with MDP was capable of inducing protection with no requirement for a carrier.

The titers reported here were rather low, but they are highly significant. Studies in which other synthetic vaccines have been used indicate that, in certain cases, much stronger effects can be obtained (unpublished results). Since the immune response reported here was enhanced in aqueous medium and at least one MDP derivative has been shown to be acceptable for human use (37, ¶), vaccination with totally synthetic agents no longer appears to be an impossible goal.

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