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ANTIBODY RESPONSES ELICITED BY A POLYVALENT VACCINE CONTAINING SYNTHETIC DIPHTHERIC, STREPTOCOCCAL AND HEPATITIS PEPTIDES COUPLED TO THE SAME CARRIER

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Three synthetic peptides copying fragments of the diphtheria toxin, the M protein of the streptococcus type 24 and the hepatitis B virus surface antigen (HBs) have been conjugated together to the tetanus toxoid. This polyvalent vaccine has been administered to mice. High antibody titers were obtained against the three antigens. No cross-reactivity could be observed between them as demonstrated by the ability of each peptide to inhibit only the antibodies against the homologous peptide. Log-log plots of the ELISA titers against the natural M protein and the synthetic M protein peptide indicated that the avidity of the antibodies raised against a monovalent streptococcal vaccine were identical to those raised following injection of the polyvalent vaccine. Antibodies raised against the polyvalent streptococcal vaccine were also protective as shown by opsonophagocytic assays.

Antibodies raised against synthetic peptides corresponding to immunopotent conformations of natural proteins have been shown to be capable of recognizing the intact natural structures (1). This observation has led to several attempts to develop artificial vaccines containing chemically synthesized antigens, and a suitable carrier and adjuvant. A model for such vaccines became available when it was shown that antibodies that neutralize virus particles could be elicited by a peptide derived from the coat protein of MS2 bacteriophage covalently linked to a multi-DL-alanyl-poly-L-lysine carrier (2) and to a synthetic adjuvant, N-acetylmuramyl-L-alanyl-D-isoglutamine also

Abbreviations : MDP, muramyl dipeptide (N-acetylmuramyl-L-alanyl-D-isoglutamine); FCA, Freund's complete adjuvant; HBs, hepatitis B virus surface antigen; SODP, diphtheric synthetic octodecapeptide; S-CB7, synthetic cyanogen bromide fragment 7; PBS, phosphate-buffered saline.

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called MDP for muramyl dipeptide (3,4). The first evidence that such artificial immunogens might be capable of eliciting protective antibodies against a pathogen was obtained when it was shown that the administration of a fragment (186-201) of diphtheria toxin coupled to a protein carrier and emulsified in Freund's complete adjuvant (FCA) (5) evoked active-antitoxic immunity. Later, a vaccine containing the three synthetic components (antigen, carrier and MDP) also was shown to afford protective immunity (6).

Following this observation several other synthetic antigens have been shown capable of inducing immune responses against natural structures of bacteria (7-9), viruses (10-18), a parasite (19) and hormones (20-22). In most instances the synthetic fragments (copies of hormones or limited regions of pathogens) have been administered with FCA. However, immune responses have been obtained in the absence of Freund's adjuvant using totally synthetic antigens and adjuvants with carrier (6). Antibodies were also raised against diphtheria toxin (6), streptococcal M protein (23) and the hepatitis B surface antigen (HBs) (24) by injecting polymers of synthetic peptides : a diphtheric octodecapeptide (SODP), a streptococcal type 24 thirty-five peptide (called S-CB7) and a HBs twenty-three peptide (residue 99-121). In all previous studies only monovalent synthetic vaccines have been used.

In the present investigation mice were immunized by a polyvalent vaccine containing the synthetic fragment (99-121) of HBs and two other synthetic antigens : the streptococcal S-CB7 and the diphtheric SODP conjugated to a toxoid carrier. Our results show that all three peptides were immunogenic and that the antibody responses were strictly specific.

MATERIALS AND METHODS

Antigens and adjuvant.

The synthetic HBs peptide (residue 99-121) will be designated as HBs (99-121). Its preparation has been described elsewhere (24). The synthesis of the diphtheria peptide was achieved by Dr. Diaz (Clin-Midy, Montpellier, France). This peptide was called SODP for synthetic octodecapeptide; it represents the residues 186-201 of the toxin to which two alanine residues have been added at the NH_2-terminal end. Its immunogenic properties have been previously described (6). The synthetic streptococcal peptide (S-CB7 for synthetic cyanogen bromide fragment 7) has been described (7). It has been prepared under the conditions given in (23). Tetanus toxoid was generously provided by Dr. D. Labert (Institut Pasteur Production, Paris). The protein

M24 was prepared according to methods previously described (25,26). Freund's complete adjuvant (FCA) was purchased from Difco Laboratories.

Preparation of the polyvalent vaccine.

Peptides were conjugated using glutaraldehyde as follows : HBs (99-121), 11.6 mg corresponding to 5 NH_2 µequivalents; SODP, 9 mg corresponding to 10 NH_2 µequivalents; S-CB7, 5.8 mg corresponding to 10 NH_2 µequivalents. Nine mg of tetanus toxoid were used for the reaction. It can be assumed from previous experiments that 100 µg of the conjugate contained between 10 and 20 µg of each three peptides. The monovalent streptococcal vaccine was prepared under the same conditions as the polyvalent conjugate and 100 µg of the final preparation contained ≅ 50 µg of S-CB7.

Immunization.

Mice (6 to 7 week-old female Swiss or BALB/c; Iffa Credo) were injected subcutaneously with the polymer or the conjugate in saline or FCA. They received a second injection of antigens alone in PBS after 40 days. Sera were collected during primary and secondary responses.

Antibody assays.

ELISA. Anti-peptide or anti-natural structure antibodies were measured by the ELISA technique performed according to the general experimental conditions previously described (23). Antigens were used at 2 µg per well for the peptides and 0.5 µg for the proteins on microtiter plates (Nunc). Readings were performed at 492 nm in a Titerteck multiskan ELISA reader (Flow Laboratories) 10 min after the addition of the substrate. The negative control is a pool of normal mouse sera. Individual titers are expressed as the maximal dilution giving an absorbance 3-fold higher than the negative control diluted 1:100 (generally about 0.3 O.D.). Maximal and minimal titers in each group are given as well as the arithmetic mean. Inhibitory antigens, HBs (99-121), SODP and S-CB7 peptides were incubated with the immune sera at the concentration indicated. After 20 hr at 4°C the sera were tested by the ELISA assay as described.

Opsonophagocytic assays were performed as previously described (25,26). Fresh heparinized (10 U/ml) human blood (0.4 ml) were mixed with 0.05 ml of standard suspension of phagocytosis resistant streptococci and 0.05 ml of test serum appropriately diluted.

RESULTS

Antibody responses after immunization with the polyvalent vaccine containing HBs(99-121), diphtheria SODP and streptococcal S-CB7 coupled to tetanus toxoid.

Swiss mice (8 per group) received subcutaneously 100 µg of a polyvalent conjugate containing HBs (99-121), SODP and S-CB7 in PBS or emulsified in FCA. A control group received a monovalent vaccine (100 µg) containing approximately 50 µg of S-CB7 coupled to the tetanus carrier. A second injection of 100 µg of the same conjugate without adjuvant was given on day 40. Mice were bled on days 21 and 56. All sera were tested for their capacity to recognize the peptides and the corresponding natural protein. As can be seen in Table 1 strong primary and secondary responses were observed against the three peptides

Table 1

Anti-peptide response of Swiss mice immunized with a polyvalent synthetic vaccine*

Mice immunized with :		ELISA titers of sera of mice immunized with synthetic peptides in :			
		PBS		FCA	
		Primary response	Secondary response	Primary response	Secondary response
Polyvalent vaccine	HBs (99-121)	<100-500 (<270)	4,000-50,000 (14,300)	3,000-20,000 (18,750)	30,000-300,000 (160,000)
	SODP	100-1,000 (430)	10,000-20,000 (13,400)	8,000-40,000 (27,120)	20,000-500,000 (250,000)
	S-CB7	100-800 (330)	3,000-30,000 (15,250)	10,000-100,000 (43,750)	10,000-300,000 (101,600)
Monovalent vaccine	S-CB7	1,000-2,000 (1,450)	20,000-80,000 (47,100)	10,000-100,000 (48,750)	50,000-300,000 (130,000)

*Mice (8 per group) received subcutaneously 100 µg of the polyvalent or of the monovalent vaccines. A second injection was given on day 40. Primary response : day 21. Secondary response : day 56. Each serum was titrated individually by ELISA. The maximal and minimal titers in each group are recorded and the arithmetic means are given in parentheses. It must be noted that mice immunized with the polyvalent vaccine developed high titers of antibodies against the tetanus carrier : 116,000 and 983,000 for the secondary response of PBS- and FCA-treated groups respectively.

when the conjugate was injected in FCA. When administered in PBS, only weak primary responses but strong secondary responses were observed. It must be recalled that the monovalent vaccine contained 50 µg of S-CB7 whereas the polyvalent vaccine contained approximately 15 µg of each of three synthetic peptides. Although the amount of S-CB7 in the polyvalent vaccine was one-third that in the monovalent vaccine, the responses were of the same order of magnitude. High titers of antibodies against the tetanus carrier were observed whether conjugates were administered in PBS or in FCA (see footnote of Table 1).

The antibodies evoked by the polyvalent vaccine were highly specific for each of the respective antigens since the antibody reactivity with each of the synthetic peptides could be suppressed only by the homologous peptide (Table 2).

The polyvalent vaccine, especially when administered in FCA, also evoked significant rises in antibody titers measured against the two following proteins : diphtheria toxin and streptococcal protein M24. As can be seen in

Table 2

Absence of cross-reactivity between the secondary response antibodies to HBs (99-121), SODP and S-CB7 conjugated to the same carrier*.

Inhibitors		Percentage of inhibition of the antibody response to :		
		Hbs (99-121)	SODP	S-CB7
HBs (99-121)	100 µg	99	0	0
	10 µg	60	0	0
	1 µg	28	0	0
SODP	100 µg	0	99	0
	10 µg	0	49	0
	1 µg	0	18	0
S-CB7	100 µg	0	0	99
	10 µg	0	0	50
	1 µg	0	0	45

* A pool of sera of the FCA-treated group was diluted 50,000 times to obtain an O.D. between 0.7 and 1.4 when tested against each peptide. Inhibitions were performed as indicated in Materials and Methods.

Table 3 antibodies recognized both natural proteins in the case of the polyvalent vaccine, however titers of anti-diphtheria antibodies were low when compared to those observed vis-à-vis the diphtheria peptide; in contrast, the antibody titers against the streptococcal M protein were comparable to those

Table 3

Recognition of natural proteins by antibodies elicited with a polyvalent synthetic peptides vaccine.

Animals immunized with :		ELISA titers* against : Diphtheria toxin	M24 protein
Polyvalent vaccine in :	PBS	200 - 700 (400)	200 - 5,000 (1,580)
	FCA	500 - 2,000 (980)	5,000 - 100,000 (30,800)
Monovalent vaccine in :	PBS		100 - 4,500 (1,370)
	FCA		10,000 - 100,000 (38,500)

*The range of ELISA titers obtained on individual sera (secondary response) in each group of 8 mice are recorded; the arithmetic average titer for each group is shown in parentheses.

observed vis-à-vis the S-CB7 peptide (see Table 1). Moreover, the response to the natural M protein was of the same order of magnitude as that in mice immunized by the monovalent synthetic vaccine when measured by ELISA (Table 3) and even greater in opsonophagocytic assays using heparinized human blood and intact type 24 streptococci; a pool of the antisera against the monovalent S-CB7 vaccine promoted phagocytosis by 28 % of neutrophils and that against the polyvalent vaccine promoted phagocytosis by 92 % of neutrophils. Control preimmune serum gave a value of only 2 %.

Besides being comparable in titers, the antibodies raised by monovalent or polyvalent vaccines against S-CB7 showed a similar degree of avidity vis-à-vis the peptide and the protein; log-log plots of ELISA titers of pooled sera of mice immunized under the same conditions as those which were titrated separately (see Tables 1 and 3) produced paralell lines against S-CB7 and the parent M24 protein (Fig. 1).

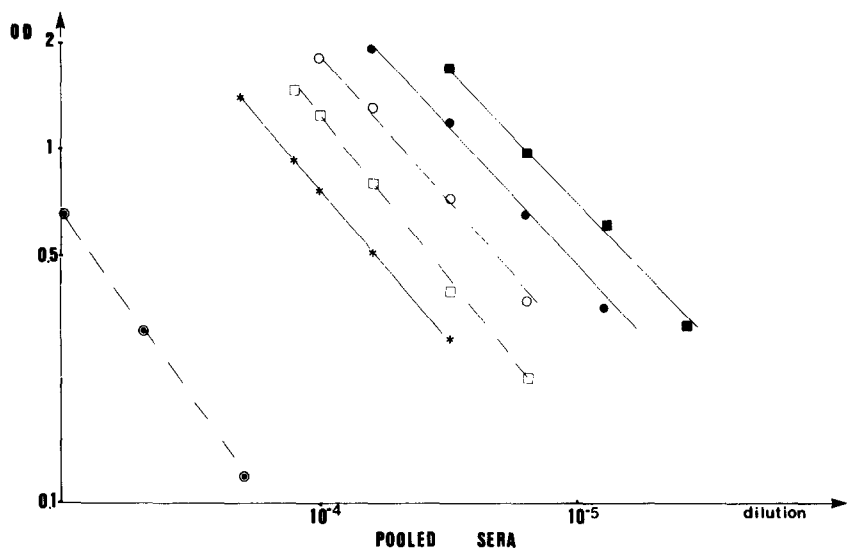

FIGURE 1

Slopes of log-log plots of data obtained with ELISA performed on various dilutions of pooled sera of mice receiving S-CB7 either as a monovalent vaccine or as a polyvalent vaccine. Anti-protein M24 antibodies after immunization by the polyvalent vaccine given in PBS ◎ or in FCA ○ . Anti-protein M24 antibodies after immunization by the monovalent vaccine given in FCA □ . Anti-S-CB7 antibodies after immunization by the polyvalent vaccine given in PBS * , in FCA ● . Anti-S-CB7 antibodies after immunization by the monovalent vaccine given in FCA ■ . Results are plotted as log O.D. 492 (abcissa) vs log serum dilutions (ordinate).

DISCUSSION

Results reported here show that a polyvalent vaccine containing streptococcal, diphtheric and hepatitis synthetic peptides conjugated to a tetanus toxoid carrier produced high anti-peptide antibodies that were strictly specific against each of the synthetic antigens. Although the conjugate presented a high degree of substitution, a marked antibody response was still observed against the carrier (see footnote of Table 1). Immunization by the polyvalent vaccine also produced antibodies capable of recognizing the natural structure of streptococcal protein M24 and of diphtheria toxin. The marked efficacy of anti-S-CB7 antibodies could be explained by the fact that most of the S-CB7 structure is repeated seven times in the M24 protein (26) whereas the diphtheria peptide is not repetitive. A comparison of the responses obtained against a monovalent or against a polyvalent vaccine was made with the S-CB7 peptide. The natural streptococcal M protein was recognized equally well by antisera against the polyvalent or the monovalent vaccine. Moreover, no differences could be detected between the avidity of the anti-peptide and the anti-protein M24 antibodies raised by either vaccine. Finally, immunization by the polyvalent vaccine produced protective antibodies measured by neutralization of diphtheria toxin (data not shown) and by phagocytosis of streptococci.

Although our experiments did not demonstrate that the HBs (99-121) fragment could produce protective antibodies, it is promising to observe that two different synthetic antigens produced such antibodies even when coupled together to tetanus toxoid.

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