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EPITOPE SPECIFIC IMMUNITY ELICITED BY A SYNTHETIC STREPTOCOCCAL ANTIGEN WITHOUT CARRIER OR ADJUVANT

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A polypeptide fragment of type 24 streptococcal M protein (pep M24) has been shown to raise protective anti-streptococcal antibodies in rabbits and humans when administered with adjuvants. More recently, such protective antibodies were shown to be evoked by a synthesized 35-residue sub-peptide fragment (S-CB7 synthetic cyanogen bromide fragment 7) of pep M24. We now show that the weak pep M24 immunogen induces high titers of long lasting antibodies when associated with murabutide, a synthetic derivative of MDP (NAcMur-L-Ala-D-Gln-n-butyl-ester) which is currently undergoing clinical trials. We demonstrate also that the polymerized synthetic S-CB7 administered without adjuvant or carrier evokes a strong epitope specific, protective immune response in mice primed with the parent pep M24. A booster dose of polymerized S-CB7 induced antibodies directed specifically against the S-CB7 structure whereas a booster dose of pep M24 evoked antibodies recognizing additional determinants of the whole pep M24 molecule.

In the past 3 years, attempts have been made to replace natural polypeptide antigens with chemically synthesized peptide fragments of the native molecules to serve as models of acceptable vaccines. Several synthetic antigens, when coupled with polypeptide carriers and generally administered in Freund's complete adjuvant (FCA), were shown to be capable of inducing immune responses (1,2) and even protective immunity (3-6) against viral or bacterial pathogens. Furthermore, immune responses have been obtained using totally synthetic vaccines consisting of synthetic bacterial (7) or hormonal (8) antigens administered with or without a synthetic carrier in conjunction with a synthetic

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Abbreviations : Pep M24, type 24 streptococcal M protein; S-CB7, synthetic cyanogen bromide fragment 7; MDP, muramyl dipeptide (N-acetylmuramyl-L-alanyl-D-isoglutamine); PBS, phosphate-buffered saline.

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adjuvant MDP (NAcMur-L-Ala-D-isoGln) (9). MDP previously had been shown to be adjuvant-active with various natural vaccines (10,11). Despite these findings, it is generally assumed that in most cases artificial vaccines will require the use of protein carriers such as tetanus toxoid. The repeated use of such carriers is difficult, however, because they are often themselves potent immunogens and in some instances may even irreversibily suppress the immune response (12).

In this paper we report data concerning the obtention of antibodies capable of protecting against streptococcus type 24 using a polymerized synthetic peptide. Previous studies had shown that a limited peptic extract of type 24 streptococcal M protein (pep M24) administered with an adjuvant raised protective anti-streptococcal antibodies in rabbits (13) and humans (14). Our results demonstrate that anti-pep M24 antibodies can be induced using a synthetic adjuvant, derivative of MDP, murabutide (NAcMur-L-Ala-D-Gln-α-n-butyl-ester) which is currently undergoing clinical trials (15,16). It had been also demonstrated that protective antibodies could also be evoked by a chemically synthesized 35-residue peptide (S-CB7) of pep M24 coupled with a polylysine carrier and administered to rabbits in FCA (4). In the following experiments polymerized S-CB7 was administered alone to mice previously primed with the native pep M24. The specificity of the antibodies evoked by the priming injection of the native protein and by the booster dose of the synthetic peptide polymer was analyzed.

MATERIALS AND METHODS

Immunogens and adjuvants.

Type 24 streptococcal M protein (pep M24) was prepared as previously described (13). Synthetic cyanogen bromide fragment 7 (S-CB7) was prepared by a solid phase method (17) on a benzhydrylamine resin (Beckman). Using this resin, cleavage with hydrofluoric acid (HF) resulted in a carboxamide group at the C-terminus. After gel filtration (Biogel P6) and preparative reversed phase HPLC (Whatman Magnum 9 ODS) the peptide was homogeneous as checked by analytical reversed phase HPLC and thin-layer-chromatography analysis and had the expected amino acid composition. Aliquots of the peptidyl resin were removed at various stages during the synthesis to prepare shorter fragments : S-CB7 (8-35), (13-35), (18-35) and 24-35). Polymerization of S-CB7 was achieved by treatment with glutaraldehyde under conditions previously described (7). Alum was Alugel 50 purchased from Serva. Murabutide, NAcMur-L-Ala-D-Gln-α-n-butyl-ester was synthesized as described (18).

Immunization.

Eight female Swiss mice (Iffa Credo, St-Germain-sur-L'Arbresle, France) per group were immunized subcutaneously. They received 10 µg of pep M24 either alone in phosphate-buffered saline (PBS) or with 40 µg of alum or 100 µg of murabutide. Animals were boosted after 2 months with antigen alone; sera were collected separately at days 42 and 100. On day 115 and 205 the first group received a boost of monomeric or polymeric synthetic S-CB7 under the conditions described in Results.

Titration of antibodies.

Sera were titrated using indirect ELISA according to experimental conditions previously described (7). Plates were coated with pep M24 protein (0.4 µg/well) or S-CB7 peptide (2 µg/well). The enzymatic reaction was allowed to proceed for 10 min with pep M24 and for 7 min with S-CB7. The negative control was a pool of normal mouse sera. Individual titers are expressed as the maximal dilution giving an absorbance twice as high as the negative control. The inhibition studies were performed on sera diluted in BSA 1 % at the level of 1 ± 0.3 O.D. when tested against pep M24 under the same conditions. The inhibiting antigens, pep M24, or monomeric S-CB7, were incubated with the immune sera at the concentration indicated. After 20 hr at 4°C the sera were tested by indirect ELISA.

Opsonophagocytic assays were performed as previously described using mixtures of lightly heparinized (10 U/ml) human blood, diluted suspensions of phagocytosis resistant streptococci and either preimmune or immune test serums (14).

RESULTS

Anti-pep M24 and S-CB7 response of mice treated with pep M24 in PBS, alum or murabutide.

Three groups of mice received two injections of pep M24 with or without adjuvants as indicated in the legend of Table 1. Strong primary and secondary responses were observed in most mice treated with alum or murabutide (NAcMur-L-Ala-D-Gln-α-n-butyl-ester) as compared with their PBS controls. The highest levels were observed in the glycopeptide-treated group on day 42 and especially on day 100. This marked enhancement was confirmed on day 225 when the experiment was terminated; the average titer of antibodies in the murabutide-treated group was 15,500 against pep M24 and 3,500 against S-CB7, whereas in the alum-treated group the antibody titers were respectively 240 against the protein and < 100 against the synthetic peptide (data not shown). It should be noted that in all cases in the pep M24 immunized mice, the S-CB7 antibody titer was far lower than that against pep M24.

Antibody response of pep M24-treated mice boosted by polymerized S-CB7.

The pep M24-PBS-treated mice were divided into two groups : 4 mice received no further treatment; the remaining 4 received an injection of monomeric S-CB7

Table 1. Anti-pep M24 and S-CB7 response of mice treated with pep M24 in PBS, alum or murabutide.

Treatment*	Primary response† (day 42) anti-pep M24	Primary response† (day 42) anti-S-CB7	Secondary response† (day 100) anti-pep M24	Secondary response† (day 100) anti-S-CB7
PBS	< 100-380 (< 135)	< 100-110 (< 100)	< 100-760 (< 225)	< 100-380 (< 135)
$Al(OH)_3$ 40 µg	110-6 800 (1 820)	< 100-820 (< 280)	190-7 300 (2 440)	< 100-3 000 (< 635)
Murabutide 100 µg	300-10 000 (4 075)	150-2 800 (900)	2 950-22 000 (13 640)	500-5 600 (2 900)

*Eight mice per group received subcutaneously in 0.2 ml of PBS 10 µg of pep M24, alone, with alum or with murabutide. Two months later they were boosted with 10 µg of antigen alone. Sera were taken separately on days 42 and 100.

†Antibodies were measured by ELISA titration. The minimal and maximal titers in each group are recorded and the arithmetic means are given in parentheses. On day 42 the pep M24 and S-CB7 antibody responses of the murabutide-treated group were highly significantly different from the PBS controls $P < 0.01$ as calcultated by Student's t test. On day 100 the pep M24 antibody response of the murabutide-treated group was highly significantly different from PBS controls ($P < 0.001$) and from the alum-treated groups ($P < 0.01$). The S-CB7 response of the latter group was significantly different from the PBS group ($P < 0.01$).

on day 115 and of polymerized S-CB7 on day 205. Antibodies were measured on days 125 and 225. On day 125 the antibody titers were similar in the sera of the two groups (average < 160), but on day 225 the sera of the mice which had received the polymerized synthetic antigen had respectively titers of 37,000 - 35,000 - 4,500 and 3,100 of pep M24 antibodies (Table 2) and 45,000 - 40,000 - 8,000 and 4,500 of S-CB7 antibodies. In contrast, the antibody titers of the sera of the 4 control mice receiving only the pep M24 in PBS remained low both against pep M24 and S-CB7 (average < 200).

Pools of the two groups of sera were assayed in opsonophagocytic test. The pooled poly-S-CB7 immune serum showing an anti-pep M24 antibody titer of 12,800 by ELISA, promoted phagocytosis of type 24 streptococci by human neutrophils (50 % of neutrophils associated with streptococci after 30 min of incubation with heparinized human blood) whereas the pep M24 in PBS immune serum showing an anti-pep M24 titer of 200 had no effect (no neutrophils associated with streptococci). The poly-S-CB7 serum also was shown not to react with frozen section

Table 2. Inhibition by pep M24 or monomeric S-CB7 of the anti-pep M24 antibodies in sera of mice non-boosted and boosted with polymerized S-CB7.

Inhibiting antigen		Day 100 after 2 injections of pep M24			Day 225					
					Non-boosted with poly S-CB7		Boosted with poly S-CB7			
		(760)	(240)	(190)	(230)	(110)	(3 100)	(35 000)	(37 000)	(4 500)
pep M24	100	100	100	100	84	72	89	43	65	43
	10	84	76	70	75	41	72	16	27	20
	1	65	48	40	59	11	48	0	0	0
S-CB7	100	48	48	23	0	29	100	100	100	80
	10	0	0	0	0	20	97	45	92	44
	1	0	0	0	0	0	80	15	63	24

Sera were diluted in BSA 1 % at the level of 1 ± 0.3 O.D. when tested against pep M24 under the conditions described in Materials and Methods.

Each column represents an individual serum. Figures between brackets indicate the titer of each of them. Results are given as the percentage of inhibition obtained with the competitor at the concentrations indicated.

of human heart tissue by immunofluorescence tests performed as previously described (13).

Inhibition by pep M24 or monomeric S-CB7 or its sub-fragments of the anti-pep M24 antibodies in sera of mice non-boosted or boosted with polymerized S-CB7.

The antibody specificity of the sera of the pep M24-PBS-treated mice was analyzed before and after recall by the synthetic preparation (Table 2). Sera obtained after administration of pep M24 were completely inhibited by pep M24 but only partially by S-CB7. In contrast, after the administration of the booster dose of polymerized S-CB7 the monomeric S-CB7 was completely inhibitory whereas pep M24 was only partially inhibitory, indicating that the recall was for antibodies directed against specific epitopes in S-CB7 and not against epitopes in the remaining pep M24 molecule not represented in S-CB7. Similar results were obtained in separate experiments. In these assays a S-CB7 specific antibody response was also obtained after administration of polymerized S-CB7 to mice primed by a single administration of pep M24 given with murabutide.

A more precise analysis of specificity was performed on the serum showing the highest antibody titer after the booster dose of polymerized S-CB7. The

monomeric S-CB7 and 4 chemically synthesized sub-fragments consisting of the 12, 18, 24 or 28 COOH-terminal amino acid residues were incubated with the serum. The strongest inhibition was observed with S-CB7 and the 2 longer fragments (Fig. 1). These inhibition assays are not necessarily correlated with the immunogenicity of the shorter peptide fragments (Beachey *et al.*, unpublished results).

DISCUSSION

Our study shows that murabutide (15), an MDP derivative currently undergoing clinical trials (16), has marked adjuvant activity when injected with a weak immunogen such as pep M24. The data confirm earlier findings showing that administration of a synthetic antigen in saline can induce a high level of biologically-active antibody in the absence of a carrier (8). The most intriguing finding was that after a weak primary response elicited by the natural antigen pep M24 a strong secondary response could be elicited with the polymerized synthetic S-CB7 antigen in the absence of either carrier or adjuvant. Furthermore, antibodies obtained during this secondary response were epitope specific : they reacted strongly and completely with the monomeric S-CB7 and more weakly with the uncleaved parent M protein molecule suggesting that certain epitopes exposed in the synthetic subpeptide are inaccessible for antibody

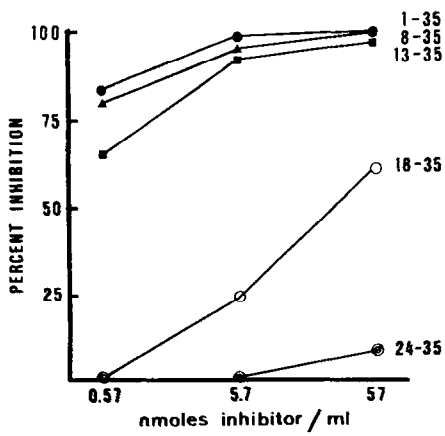

Fig.1 Inhibition of pep M24 antibodies in the serum of a poly-S-CB7-treated mouse by S-CB7 and its subpeptides.

The serum of the highest anti-pep M24 titer was diluted to obtain 0.9 unit of O.D. when tested in the ELISA assay against pep M24 as the solid phase antigen as described in Materials and Methods.

reactivity in the intact parent molecule (19). Nevertheless, they could opsonize homologous M24 streptococci. Many points remain to be investigated to define the optimal procedure to be used to immunize with the polymer of S-CB7 or other synthetic subpeptides. Nevertheless, it must be stressed that our study deals with a highly relevant question because in most cases humans are not immunologically naive subjects; most have had previous exposure to vaccines and infectious agents. Thus, the successful secondary immunization with a polymerized synthetic subpeptide fragment of a virulent protein offers the hope that synthetic vaccines may be administered safely and effectively in humans even without carriers or adjuvants.

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REFERENCES

1. Lerner, R.A., Green, N., Alexander, H., Liu, F.T., Sutcliffe, J.G., and Shinnick, T.M. (1981) Proc. Natl. Acad. Sci. USA 78, 3403-3407.
2. Green, N., Alexander, H., Olson, A., Alexander, S., Shinnick, T.M., Sutcliffe, J.G., and Lerner, R.A. (1982) Cell 28, 477-487.
3. Audibert, F., Jolivet, M., Chedid, L., Alouf, J.E., Boquet, P., Rivaille, P., and Siffert, O. (1981) Nature 289, 593-594.
4. Beachey, E.H., Seyer, J.M., Dale, J.B., Simpson, W.A., and Kang, A.H. (1981) Nature 292, 457-459.
5. Müller, G.M., Shapira, M., and Arnon, R. (1982) Proc. Natl. Acad. Sci. USA 79, 569-573.
6. Bittle, J.J., Houghten, R.A., Alexander, H., Shinnick, T.M., Sutcliffe, J.G., Lerner, R.A., Rowlands, D.J., and Brown, F. (1982) Nature 298, 30-33.
7. Audibert, F., Jolivet, M., Chedid, L., Arnon, R., and Sela, M. (1982) Proc. Natl. Acad. Sci. USA 79, 5042-5046.
8. Carelli, C., Audibert, F., Chedid, L., and Gaillard, J. (1982) Proc. Natl. Acad. Sci. USA 79, 5392-5395.
9. Ellouz, F., Adam, A., Ciorbaru, R., and Lederer, E. (1974) Biochem. Biophys. Res. Com. 59, 1317-1325.
10. Audibert, F., Chedid, L., Lefrancier, P., and Choay, J. (1976) Cell. Immunol. 21, 243-249.
11. Leclerc, C., Morin, A., and Chedid, L. (1983) Recent Advances in Clinical Immunology, pp. 187-204, vol. 3, Thompson R.A. and Rose, N. eds, Churchill Livingstone, Edinburgh.
12. Herzenberg, L.A., Tokuhisa, T., and Herzenberg, L.A. (1980) Nature 285, 664-667.
13. Beachey, E.H., Stollerman, G.H., Chiang, E.Y., Chiang, T.M., Seyer, J.M., and Kang, A.H. (1977) J. Exp. Med. 145, 1469-1483.

14. Beachey, E.H., Stollerman, G.H., Johnson, R.H., Ofek, I., and Bisno, A.L. (1979) J. Exp. Med. 150, 862-877.
15. Chedid, L.A., Parant, M.A., Audibert, F.M., Riveau, G.J., Parant, F.J., Lederer, E., Choay, J.P., and Lefrancier, P.L. (1982) Infect. Immun. 35, 417-424.
16. Oberling, F., Bernard, C., Chedid, L., Choay, J., Giron, C., and Lang, J.M. (1981) Intern. Symp. on Immunomodulation by Microbial Products and Related Synthetic Compounds, Osaka, July 27-29.
17. Merrifield, R.B. (1963) J. Am. Chem. Soc. 85, 2149-2155.
18. Lefrancier, P., Derrien, M., Jamet, X., Choay, J., Lederer, E., Audibert, F., Parant, M., Parant, F., and Chedid, L. (1982) J. Med. Chem. 25, 87-90.
19. Dale, J.B., Ofek, I., and Beachey, E.H. (1980) J. Exp. Med. 151, 1026-1038.
20. Hopp, T.P., and Woods, K.R. (1981) Proc. Natl. Acad. Sci. USA 78, 3824-3838.