Antiviral response elicited by a completely synthetic antigen with built-in adjuvanticity

(synthetic adjuvant/coliphage MS-2/viral coat protein/nonspecific immunity)

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ABSTRACT In a previous study we demonstrated that antiviral response against the coliphage MS-2 can be elicited by immunization with a synthetic antigen consisting of a conjugate (P₂-A - - L) of a synthetic fragment (P₂) of the virus coat protein attached to a synthetic polymeric carrier. The antiviral response was induced when the antigen was administered in complete Freund's adjuvant or when it was administered in incomplete adjuvant, provided that a peptidoglycan was covalently attached to it. In the present study we demonstrate the adjuvant effect of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) in this system. Immunization with a mixture of MDP and P₂-A - - L brought about only slight enhancement in the titer of neutralizing antibodies, as compared to the immunization with P₂-A - - L in saline. The best results were achieved when the MDP was chemically conjugated to P₂-A - - L. This completely synthetic material, when administered in aqueous solution, yielded highly inactivating antiserum with a titer similar to that obtained with complete Freund's adjuvant in the absence of MDP. MDP-P₂-A - - L elicited also a humoral immune response to MDP, but with much lower titer than that induced by complete Freund's adjuvant containing P₂-A - - L only. It was also observed that the capacity of MDP-P₂-A - - L to increase resistance against infection was more than a 100-fold greater than that of unconjugated MDP.

In the last few years progress has been made, and new approaches have been employed, in the field of vaccine preparation. This included the introduction of the concept of genetic engineering for production of viral components (1), in parallel with the chemical synthesis of viral fragments (2), for the purpose of replacing vaccines now used. If found successful, these approaches might show several advantages. (i) The fragments should contain only the unique antigenic determinant(s) required for eliciting neutralizing antibodies, thus eliminating undesired side effects caused by the presence of contaminating components or irrelevant determinants. (ii) The carrier to be used in a synthetic material could be chosen according to the genetic background of the immunized individual, taking advantage of the recent knowledge about the often-occurring correlations between an efficient immune response and the type of histocompatibility antigens of the host (3, 4). (iii) In a synthetic antigen the problem of adjuvanticity and choice of the desirable adjuvant might be solved by introduction into the macromolecule of groups to enhance immunogenicity.

A prerequisite for this approach is the feasibility of synthesis of antigens that contain immunoreactive region(s) of protein molecules, including viral proteins, that will induce immune response toward the intact protein. Indeed, during the last few years this has been demonstrated for several proteins. For ex-

ample, antibodies provoked by a synthetic peptide comprising the "loop" region of the enzyme lysozyme attached to a synthetic carrier were reactive with native lysozyme (5). Similarly, antibodies elicited by a synthetic antigen containing the NH₂-terminal region of the carcinoembryonic antigen of the colon were reactive with the intact protein, and capable of detecting carcinoembryonic antigens in sera of cancer patients (6). We have also shown that this approach could be used for provoking an antiviral response. A synthetic antigen was prepared by attachment of a synthetic fragment (P₂) (residues 89-108) of the coat protein of MS-2 coliphage, which had been previously shown to be involved in the virus neutralization, to a synthetic carrier, multichain poly(DL-alanine) (7). The resultant conjugate (P₂-A - - L), when administered to rabbits in complete Freund's adjuvant, elicited antibodies capable of neutralizing the viability of MS-2 phage (8).

In a recent study we have shown that, when injected in guinea pigs in aqueous solution or in incomplete adjuvant, P₂-A - - L did not elicit any measurable antiphage activity (9). When a water-soluble adjuvant, the peptidoglycan from *Bacillus megaterium* (10), was added to P₂-A - - L, still no significant antiphage activity was induced. However, when the same peptidoglycan was covalently bound to P₂-A - - L it had a marked adjuvant activity, and when injected in incomplete Freund's adjuvant, it elicited an anti-MS-2 effect almost identical to that effected by injection of P₂-A - - L in complete Freund's adjuvant (9).

The minimal adjuvant structure that can substitute for *Mycobacterium* in Freund's adjuvant is synthetic *N*-acetylmuramyl-L-alanyl-D-isoglutamine [or MDP for muramyl dipeptide (11-13)]. Contrary to other mycobacterial fractions, MDP is active as an adjuvant when administered in aqueous medium parenterally or orally (14). It was also shown to augment the biological activity of myelin basic protein in the induction of experimental allergic encephalomyelitis (15), a process that usually occurs only by administration in complete Freund's adjuvant (16). More recently, it was shown that synthetic MDP can exert its adjuvant activity on a synthetic antigen as well. When mixed with, or attached to, the synthetic antigen (T_G)-A - - L (17) it led to an efficient antibody formation (18), thus illustrating the potential of the combined use of synthetic antigens and synthetic adjuvants.

In the present study we demonstrate that the covalent attachment of MDP to the synthetic antigen P₂-A - - L results in built-in adjuvanticity, yielding a completely synthetic antigen capable of evoking antiviral antibodies when administered in aqueous solutions.

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Abbreviations: P₂, synthetic fragment (residues 89-108) of MS-2 coliphage coat protein; P₂-A - - L, P₂ conjugated to multichain poly(DL-alanine); MDP, muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine); P_i/NaCl, phosphate-buffered saline.

MATERIALS AND METHODS

Phage and Reagents. Bacteriophage strain MS-2 was grown on *Escherichia coli* C3000 (19). Tryptone, glucone, yeast extract, agar, and Freund's complete adjuvant were obtained from Difco.

MDP and Derivatives. MDP was prepared as described (20) and coupled to bovine serum albumin or bovine immunoglobulin via carbodiimide as described (21).

Antigen and Conjugate. The P₂ fragment, corresponding to residues 89–108 in the amino acid sequence of the MS-2 coat protein—namely, Glu-Leu-Thr-Ile-Pro-Ile-Phe-Ala-Thr-Asn-Ser-Asp-Cys-Glu-Leu-Ile-Val-Lys-Ala-Met—was synthesized by the Merrifield solid-phase technique (22). This peptide was attached to the multichain synthetic peptide multi-poly(DL-alanyl)-poly(L-lysine) (abbreviated A - - L) to yield the synthetic antigen P₂-A - - L as described (8). Various batches of the conjugate contained between 3.5% and 9.7% P₂ fragment (wt/wt).

MDP-P₂-A - - L conjugate was prepared by adding 22.95 mg (0.135 mmol) of *N*-hydroxybenzotriazole and 57.24 (0.135 mmol) of *N*-ethyl-*N'*-(dimethylaminopropyl)carbodiimide hydrochloride to a solution of 66.5 mg (0.135 mmol) of MDP in 2.5 ml of dimethylformamide. After 1 hr, this organic solution was added to an aqueous solution (5 ml), adjusted to pH 8.5 with 1 M NaHCO₃, of 25 mg of P₂-A - - L (0.0134 meq of free amino groups), which contained 4.5% P₂ on a weight basis. The reaction mixture was stirred for 24 hr at room temperature, diluted with water, and lyophilized. The solution of the resulting powder in water was ultrafiltered through an Amicon membrane PM10 and then lyophilized to give 26 mg of dry material containing 3.4% P₂ and 18% MDP, on a weight basis, as determined by amino acid analysis after acid hydrolysis.

Immunization Procedure. Rabbits were immunized by injecting P₂-A - - L with or without adjuvant or injecting MDP-P₂-A - - L conjugate, subcutaneously at multiple sites. Each group consisted of three or four rabbits (in two different experiments.) A booster injection was given after 2 weeks, and the rabbits were bled weekly from the marginal ear vein, starting 2 weeks after the booster injection. The antisera obtained from the various bleedings were diluted in 0.01 M sodium phosphate/0.15 M sodium chloride, pH 7.0 (P₁/NaCl) for the bacteriophage MS-2 neutralization tests and for determining their titer against MDP by radioimmunoassay.

Antiserum against MDP-albumin was obtained at day 130 after immunization. It reacted in radioimmunoassay with MDP-Ig, giving 60% binding.

Phage Neutralization Test. Response toward MS-2 phage was determined by the capacity of the antisera to neutralize the bacteriophage. The assay was performed essentially according to the small agar-layer method (23). Five milliliters of 1.5% agar in L medium (10 g of tryptone, 8 g of NaCl, and 1 g of yeast extract per liter of H₂O) was poured in petri dishes (9 cm diameter; Nunc, Roskilde, Denmark). The medium for the top layer (L medium and 0.65% agar) was kept at 45°C in 2.5-ml aliquots. Before plating, 0.2 ml of *E. coli* C3000 (OD_{490 nm} = 5) was added to each tube of top-layer agar.

For phage neutralization, approximately 500 plaque-forming units of MS-2 phage in 0.1 ml of P + G medium was incubated with 0.1 ml of appropriately diluted antiserum at 37°C. After 120 min of incubation, 0.2 ml of 1:10 dilution of goat or rabbit anti-guinea pig IgG antiserum in P + G medium was added. After 10 min of incubation, 2.5 ml of top agar containing bacteria was poured into the reaction tube and the mixture was plated. Duplicate tests were performed for each dilution. The assay plates were incubated for 16 hr at 37°C. The plaques were counted on a New Brunswick plaque counter.

Radioimmunoassay. Antibody response toward MDP was determined by a modification of a solid-phase binding assay (24, 25). Flexible plastic microtiter plates were coated with MDP-Ig (100 μ l of 50 μ g/ml solution per well). After 3 hr of incubation at room temperature, plates were washed three times with P₁/NaCl containing 1% bovine serum albumin. The last washing was kept in the wells for 1 hr. Twenty-five microliters of various dilutions of the antisera were then added for a 2-hr incubation. The plates were washed three times with P₁/NaCl containing 0.5% albumin and then incubated with 50 μ l of ¹²⁵I-labeled purified goat anti-rabbit IgG antibodies (containing 25,000 cpm) at 4°C overnight. The plates were washed four times with P₁/NaCl containing 0.5% albumin and dried well, and the wells were cut out of the plates and counted in a gamma counter (Packard).

Bacterial Challenges. The strain of *Klebsiella pneumoniae* (Institut Pasteur, collection no. 7823) of capsular type 2 has been described (26). Mice were infected intravenously with 10⁴ cells of *K. pneumoniae* 1 day after injection of different doses of MDP or MDP-P₂-A - - L. Mortality was recorded for 10 days.

RESULTS

Effect of MDP on the Immune Response Toward P₂-A - - L and MS-2. The criterion used in this study for evaluating the immune response toward P₂-A - - L was the capacity of the antisera to neutralize MS-2 phage—i.e., to decrease its viability.

The four groups of rabbits included in this study were immunized with: (a) P₂-A - - L in P₁/NaCl, each rabbit injected with 1 mg of the antigen; (b) MDP-P₂-A - - L conjugate, 1.2 mg in P₁/NaCl, the conjugate containing 34 μ g of P₂ peptide and 180 μ g of MDP per 1 mg; (c) a mixture of 1 mg of P₂-A - - L and 0.2 mg of MDP, in P₁/NaCl; and (d) P₂-A - - L (1 mg) in complete Freund's adjuvant. The first experiment was carried out with groups of four rabbits each, and the second experiment was with groups of three rabbits.

The results, representing the average for the two groups, are shown in Fig. 1, which depicts the extent of neutralization of the MS-2 bacteriophage by antisera from the different groups at 1:250 dilution. All values were obtained after subtraction of the neutralization by normal rabbit serum at the same dilution. When injected in P₁/NaCl, P₂-A - - L induced a low titer of neutralizing antibodies. The value increased when MDP was attached to the P₂-A - - L. The MDP-P₂-A - - L conjugate elicited a neutralizing capacity close to that found after immunization with P₂-A - - L in complete Freund's adjuvant. When the same amount of MDP was mixed with the P₂-A - - L in aqueous solution instead of being covalently bound, the neutralizing capacity of the resulting antibodies was much lower.

Table 1 demonstrates the neutralizing capacity of the various antisera as reflected in the dilution required for 50% neutralization of the phage. Antiserum raised by a mixture of P₂-A - - L and MDP gave an average titer of 1:48 (range, 1:25 to 1:200), and the MDP-P₂-A - - L conjugate elicited antiserum with a titer of 1:180 (range, 1:50 to 1:1000), close to the titer, 1:265 obtained by immunizing in complete Freund's adjuvant.

Immune Response Toward MDP. Because the MDP group attached to P₂-A - - L could serve as a hapten and elicit a specific immune response, the antisera of the rabbits of the different groups were assayed also for antibody activity against MDP. The tests were performed by a radioimmunoassay on microtiter plates coated with MDP-Ig. The results obtained with the seven individual rabbits in each group were similar (Fig. 2) and show the average values of binding obtained for each

FIG. 1. Adjuvant effect of MDP on capacity of antisera to inactivate P₂-A - - L. Bars indicate the percentage inactivation of MS-2 phage caused by 1:150 dilution of antisera of rabbits immunized with: P₂-A - - L in P_i/NaCl (A), MDP-P₂-A - - L in P_i/NaCl (B), a mixture of P₂-A - - L and MDP in P_i/NaCl (C), or P₂-A - - L in complete Freund's adjuvant (D). Each bar represents the mean for seven rabbits.

group, with a serum dilution of 1:10. Antiserum against MDP-albumin prepared by repeated immunizations in complete Freund's adjuvant showed high binding capacity (43%). Immunization with MDP-P₂-A - - L conjugate also elicited an immune response against MDP, manifested as a binding capacity of 10%. This response, however, was much lower than the anti-MDP response (19.2% binding) obtained in rabbits immunized with P₂-A - - L in complete Freund's adjuvant in the absence of MDP. Antisera of the rabbits immunized with a mixture of P₂-A - - L and MDP or with P₂-A - - L alone gave only ~4% binding, close to the value, 2.8% binding, obtained with normal rabbit serum.

Biological Activities of MDP-P₂-A - - L Conjugate. The evaluation of the biological activities of MDP-P₂-A - - L conjugate concerned mainly the assessment of the effect of macromolecularization on the protective activity of MDP against challenge by *K. pneumoniae*. Adult mice received different concentrations of MDP-P₂-A - - L or MDP 1 day before being infected with 10⁴ *K. pneumoniae*. Mortality was recorded for 10 days (no death was observed after that time). Table 2 represents the results of such an experiment. A dose of 5.5 μg of MDP-P₂-A - - L, which contained 1 μg of MDP, protected 50% of the mice, and a dose containing 10 μg MDP protected 83% of the mice. As reported (14), larger amounts of uncoupled MDP were required to obtain a comparable degree of protection. In the control group, only 3 of 24 mice were alive 10 days after challenge.

Table 1. Neutralization capacity of the various antisera

Immunizing agent	Titer*	Range†
P ₂ -A - - L in P _i /NaCl	<1:10	<1:10
P ₂ -A - - L + MDP	1:48	1:25-1:200
MDP-P ₂ -A - - L	1:180	1:50-1:1000
P ₂ -A - - L in complete Freund's adjuvant	1:265	1:100-1:1500

* Mean dilution required for 50% neutralization.

† Titers for individual sera.

FIG. 2. Radioimmunoassay of anti-MDP response induced by immunization with control, normal serum (A), control P₂-A - - L in P_i/NaCl (B), mixture of P₂-A - - L and MDP in P_i/NaCl (C), MDP-P₂-A - - L conjugate in P_i/NaCl (D), P₂-A - - L in complete Freund's adjuvant (E), or MDP-albumin in complete Freund's adjuvant (F). Bars represent the bound cpm of 25,000 cpm added to each well.

DISCUSSION

The main message of this paper is that it is possible to synthesize a macromolecule that contains an appropriate antigenic determinant and a built-in adjuvant and that, when administered in aqueous solution, may lead to an efficient antiviral immune response. We have shown (8) that a segment of a viral coat protein may be prepared which, when attached to a polymer, produces a conjugate capable of provoking antiviral antibodies; but this took place when the immunization was carried out in complete Freund's adjuvant. Covalent attachment of a peptidoglycan derived from *Bacillus megaterium* to the synthetic conjugate led to a macromolecule that elicited an effective immune response when administered in incomplete Freund's adjuvant—i.e., in the absence of mycobacteria (9). In the present study it is shown that the covalent attachment of MDP converts the synthetic antigen into a conjugate capable of triggering an antiviral immune response in aqueous solution. This seems conceptually of great interest as a new approach to vaccination.

The covalent binding of MDP was much more effective than its mere mixing with the antigen and may serve as another example of the paramount changes in the biological properties of small molecules upon their macromolecularization. We have recently reported (14) that, in the realm of nonspecific immunity, the covalent attachment of MDP to multichain poly(DL-alanine) increases, 100- to 1000-fold, the capacity of MDP to protect mice against death due to infection by *K. pneumoniae*. Moreover, the analog of MDP in which L-alanine is replaced by D-alanine, which by itself is biologically inactive, in this test yields, upon binding to multichain poly(DL-alanine), a preparation almost as active as MDP-A - - L. This exemplifies the new pharmacological possibilities due to macromolecularization of drugs, as well as of inactive small molecules, which might evolve into biologically active materials.

Table 2. Protective activity in mice infected with *K. pneumoniae*

Treatment at day -4	MDP dose, μg	Survivors, no.				Protection, %
		Day 0	Day 3	Day 5	Day 10	
Control	—	24	7	3	3	—
MDP	1	24	15	12	6	21
MDP	100	24	22	17	16	63*
MDP-P ₂ -A - - L	1	24	19	16	15	50*
MDP-P ₂ -A - - L	10	24	24	24	23	83*

* *P* < 0.01.

The same effect of macromolecularization on the biological activity of MDP was observed also in the conjugate MDP-P₂-A - - L used in the present study. The capacity of MDP-P₂-A - - L to protect against infection with *K. pneumoniae* was similar to that of MDP-A - - L—namely, between 100- and 1000-fold higher than that of free MDP (Table 2). Likewise, in parallel experiments MDP-(T,G)-A - - L was 1000-fold more active than MDP whereas administration of the (T,G)-A - - L conjugate was devoid of activity. It thus can be assumed that the effect observed is due to the macromolecularization of the glycopeptide and that the presence of the hapten groups does not have any influence in this process.

There is another potential advantage to the use of MDP as a built-in adjuvant: adjuvants increase the nonspecific immunity—i.e., they promote polyclonal activation. The linkage, within the same molecule, of the adjuvant moiety and the antigenic determinant might decrease such polyclonal activation, because the determinant might steer the adjuvant only to the relevant antigen-specific cells.

Immunization with MDP-P₂-A - - L conjugate in saline provoked an immune response to MDP as well (Fig. 2). This could present a problem in a synthetic vaccine. However, the titer of the anti-MDP antibodies was much lower (10%) than that elicited by immunization with MDP-albumin conjugate administered in complete Freund's adjuvant (43%) and close to the background values observed in the control groups (3–4%). Moreover, it was lower than the titer obtained by immunization with P₂-A - - L in complete Freund's adjuvant in the absence of MDP. Because a synthetic vaccine with MDP groups can be effective in saline, this constitutes an additional advantage.

Recently it was reported (18) that the efficiency of the genetically controlled immune response toward the synthetic antigen (T,G)-A - - L was increased by addition of MDP. The effect of MDP was even stronger when it was covalently attached to the antigen (27). The finding in the present paper corroborates this phenomenon and demonstrates that it extends to the antiviral immune response as well.

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1. Emtage, J. S., Tacon, W. C. A., Catlin, G. H., Jenkins, B., Porter, A. G. & Carey, N. H. (1980) *Nature (London)* **283**, 171–174.
2. Arnon, R. (1978) *Pharm. Ther.* **4**, 68–73.
3. Shearer, G. M., Mozes, E. & Sela, M. (1972) *J. Exp. Med.* **135**, 1009–1027.

4. Mozes, E. & Shearer, G. M. (1972) *Curr. Top. Microbiol. Immunol.* **59**, 167–200.
5. Arnon, R., Maron, E., Sela, M. & Anfinsen, C. B. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 1450–1455.
6. Arnon, R., Bustin, M., Calef, E., Chaitchik, S., Haimovich, J., Novik, N. & Sela, M. (1976) *Proc. Natl. Acad. Sci. USA* **75**, 2123–2127.
7. Katchalski, E., Sela, M. & Gehatia, M. (1955) *J. Am. Chem. Soc.* **77**, 6175–6182.
8. Langbeheim, H., Arnon, R. & Sela, M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4636–4640.
9. Langbeheim, H., Arnon, R. & Sela, M. (1978) *Immunology* **35**, 573–579.
10. Nguyen-Huy, N., Nauciel, C. & Wermuth, C.-G. (1976) *Eur. J. Biochem.* **66**, 79–84.
11. Ellouz, F., Adam, A., Ciorbaru, R. & Lederer, E. (1974) *Biochem. Biophys. Res. Commun.* **59**, 1317–1325.
12. Kotani, S., Watanabe, Y., Kinoshita, F., Shimono, T., Morizaki, C., Shiba, T., Kusumoto, S., Tarumi, Y. & Ikenaka, K. (1975) *Biken J.* **18**, 105–111.
13. Chedid, L., Audibert, F. & Johnson, A. G. (1978) *Progr. Allergy* **25**, 63–105.
14. Chedid, L., Parant, M., Parant, F., Audibert, F., Lefrancier, P., Choay, J. & Sela, M. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 6557–6561.
15. Nagai, Y., Akiyama, K., Suzuki, K., Kotani, S., Watanabe, Y., Shimono, T., Shiba, T., Kusumoto, S., Ikuta, F. & Takeda, S. (1978) *Cell. Immunol.* **35**, 158–167.
16. Paterson, P. Y. (1966) *Adv. Immunol.* **5**, 131–208.
17. Sela, M. & Arnon, R. (1960) *Biochim. Biophys. Acta* **40**, 382–384.
18. Sela, M. & Mozes, E. (1979) *Springer Semin. Immunopathol.* **2**, 119–132.
19. Gesteland, R. & Spahr, P. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1267–1272.
20. Lefrancier, P., Choay, J., Perriem, M. & Lederman, I. (1977) *Int. J. Peptide Protein Res.* **9**, 249–257.
21. Reichert, C. M., Carelli, C., Jolivet, M., Audibert, F., Lefrancier, P. & Chedid, L. (1980) *Mol. Immunol.* **17**, 357–363.
22. Merrifield, B. B. (1965) *Science* **150**, 178–185.
23. Adam, A., Ciorbaru, R., Petit, J. F. & Lederer, E. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 851–854.
24. Kleinman, N. R., Pickard, A. R., Sigal, N. H., Gerhart, P. J., Metcalf, E. S. & Pierce, S. K. (1976) *Ann. Immunol. Paris* **127C**, 489–502.
25. Eshhar, Z., Strassmann, G., Waks, T. & Mozes, E. (1979) *Cell Immunol.* **47**, 378–389.
26. Chedid, L., Parant, M., Parant, F., Lefrancier, P., Choay, J. & Lederer, E. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2089–2093.
27. Mozes, E., Sela, M. & Chedid, L. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4933–4937.