Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 5409–5413, August 1988
Biochemistry

Synthetic peptide vaccine design: Synthesis and properties of a high-density multiple antigenic peptide system

(solid-phase peptide synthesis/peptide antigen/antipeptide antibody)

JAMES P. TAM

The Rockefeller University, 1230 York Avenue, New York, NY 10021

Communicated by Bruce Merrifield, March 31, 1988

ABSTRACT **A convenient and versatile approach to the direct synthesis of a peptide-antigen matrix by the solid-phase method is described. The approach is called the multiple antigen peptide system (MAP) and it utilizes a simple scaffolding of a low number of sequential levels (n) of a trifunctional amino acid as the core matrix and 2^n peptide antigens to form a macromolecule with a high density of peptide antigens of final M_r 10,000. The MAP model chosen for study was an octabranching MAP consisting of a core matrix made up of three levels of lysine and eight amino terminals for anchoring peptide antigens. The MAP, containing both the core matrix and peptides of 9–16 amino acids, was prepared in a single synthesis by the solid-phase method. Six different MAPs elicited specific antibodies in rabbits and mice, of which five produced antibodies that reacted with their corresponding native proteins. In rabbits, the sera had a considerably higher titer of antibodies than sera prepared from the same peptides anchored covalently to keyhole limpet hemocyanin as carrier. Thus, the MAP provided a general, but chemically unambiguous, approach for the preparation of carrier-bound antigens of predetermined and reproducible structure and might be suitable for generating vaccines.**

Recent studies have shown that synthetic peptides can induce antibodies reactive with their cognate sequences in the native proteins (1, 2). Specific antipeptide antibodies are useful laboratory reagents for confirming *de novo* proteins from recombinant DNA, exploring biosynthetic pathways and precursors, and probing structural functions of proteins (2). Synthetic peptide antigens, conveniently available through chemical synthesis, can also be used for producing immunogens and for passive immunoprophylaxis (1–4).

A conventional approach to preparing antipeptide antibodies is conjugation of a peptide to a known protein or synthetic polymer carrier to give a macromolecular structure to the antigen carrier (1–3). Methods designed to avoid the use of carrier by polymerizing synthetic peptide antigens to give peptide polymers have also been reported recently (4). Although such materials are effective in producing animal antibodies, these materials are ambiguous in composition and structure; this shortcoming would be particularly disadvantageous were an antipeptide antibody to be used for a human vaccine. It is obviously preferable to design a chemically unambiguous system for producing antibodies in animals and vaccines for humans. To improve on present procedure, an approach, known as the multiple antigen peptide system (MAP), for the preparation of antipeptide antibodies and synthetic vaccines was developed and is described. This approach uses a small peptidyl core matrix bearing radially branching synthetic peptides as dendritic arms (Fig. 1). One design of a MAP chosen for study consisted of a core matrix with a heptalysine containing eight dendritic arms of peptides 9–16 residues in length. The whole MAP, matrix and peptides, was synthesized by the solid-phase method (5). After a simple purification scheme, the MAP, which usually has a molecular weight of >10,000, was used directly as the immunizing antigen. This design completely eliminates the conventional step of conjugation of peptide to carrier.

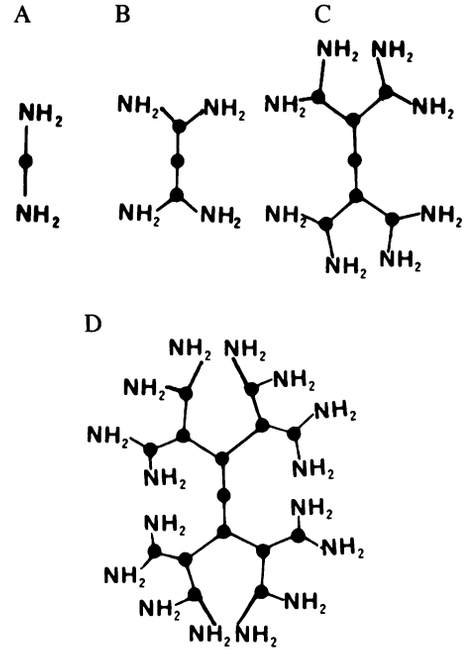

FIG. 1. Schematic representation of the core matrix of the MAP. (*A*) First level, divalent; (*B*) second level, tetravalent; (*C*) third level, octavalent; and (*D*) fourth level, hexadecavalent.

MATERIALS AND METHODS

Synthesis: General Methods. The synthesis of an octabranched matrix core with peptide antigen attached was accomplished manually by a stepwise solid-phase procedure (5) on *t*-butoxycarbonyl (Boc) βAla-OCH_2-Pam resin (6) in which 0.05 mmol of βAla is present in 0.5 g of resin. The synthesis of the first and every subsequent level of the carrier core was achieved using a 4 M excess of preformed symmetrical anhydride of N^{α},N^{ϵ}-Boc-Lys(Boc) (0.2, 0.4, 0.8, and 1.6 mmol consecutively) in dimethylformamide ($HCONMe_2$, 12 ml/g resin) followed by a second coupling via dicyclohexylcarbodiimide alone in CH_2Cl_2 to give, after deprotection, the octa-branched core matrix containing eight func-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Boc, *t*-butoxycarbonyl; $HCONMe_2$, dimethylformamide; KLH, keyhole limpet hemocyanin; MAP, multiple antigen peptide system; Boc-Lys (Boc), N^{α},N^{ϵ}-Boc_2-Lys; TGFα, transforming growth factor type α.

tional amino groups (Fig. 1C). The protecting groups for the synthesis of the peptide antigens were Boc groups for the α-amino termini and benzyl alcohol derivatives for most side-chain amino acids. For all residues except arginine, asparagine, glutamine, and glycine, the first coupling for 1 hr, monitored by a quantitative ninhydrin test (7) was done with the preformed symmetrical anhydride in CH_2Cl_2, a second coupling in $HCONMe_2$, and a third (if needed) in *N*-methylpyrrolidone at 50°C (8). The coupling of Boc-Asn and Boc-Gln was mediated by the preformed 1-hydroxybenzotriazole ester in $HCONMe_2$. Boc-Gly and Boc-Arg were coupled with water-soluble dicyclohexylcarbodiimide alone to avoid, respectively, the risk of formation of dipeptide and lactam. To eliminate the polycationic amino groups, which give highly charged macromolecules, the peptide chains were capped on their α-amino group by acetylation in 3 mM acetic anhydride in $HCONMe_2$ containing 0.3 mmol of *N,N*-dimethylaminopyridine at the completion of the MAP. The deprotection process was initiated by removing the dinitrophenyl protecting group of His(Dnp) with 1 M thiophenol in $HCONMe_2$ for 8 hr (3 times and at 50°C if necessary to complete the reaction). The branched peptide oligolysine was removed from the crosslinked polystyrene resin support with the low–high-HF method or the low–high trifluoromethanesulfonic acid method of cleavage (9) to yield the crude MAP (85–93% cleavage yield). The crude peptide and resin were then washed with cold ether/mercaptoethanol (99:1, vol/vol, 30 ml) to remove *p*-thiocresol and *p*-cresol, and the peptide was extracted with 100 ml of 8 M urea/0.2 M dithiothreitol/0.1 M Tris·HCl buffer, pH 8.0. To remove all remaining aromatic by-products generated in the cleavage step, the peptide was dialyzed in Spectrum Por 6 tubing, 1000 M_r cutoff by equilibration for 24 hr with a deaerated and N_2-purged solution containing 8 M urea, 0.1 M $NH_4HCO_3/(NH_4)_2CO_3$, pH 8.0, with 0.1 M mercaptoethanol at 0°C for 24 hr. The dialysis was then continued in 8 M and then in 2 M urea—all in 0.1 M $NH_4HCO_3/(NH_4)_2CO_3$ buffer, pH 8.0, for 12 hr and then sequentially in H_2O and 1 M HOAc to remove all urea. The MAP was lyophilized and purified batchwise by high-performance gel-permeation or ion-exchange chromatography. All purified materials were analyzed and found to contain the predicted amino acid sequences.

Immunization Procedure. Rabbits (New Zealand White, two for each antigen) were immunized by s.c. injection (0.5 ml) of the MAP (1 mg in 1 ml of phosphate buffered saline) in complete Freund's adjuvant (1:1) on day 0 and in incomplete Freund's adjuvant (1:1) on days 21 and 42 and were bled on day 49. Inbred 6- to 8-week-old mice were immunized in the hind footpad with a total of 80 μg of MAP in complete Freund's adjuvant (1:1) on day 0 and in incomplete Freund's adjuvant at 3-week intervals (total, 2 to 4 times) and bled a week after the last boosting. The antisera were used without purification.

Immunological Assays. An enzyme-linked immunoabsorbent assay (ELISA) was used to test antisera for ability to react with the MAP used for immunization. Peptide antigen (0.5 μg per well) in carbonate/bicarbonate buffer, pH 9.0 was incubated at 4°C overnight in a 96-well microtiter plate before being washed. Rabbit or mouse antiserum (serially diluted in 0.01 M phosphate-buffered saline) was incubated with the microtiter plate-bound antigen for 2 hr at 20°C and then washed with phosphate-buffered saline. Goat anti-rabbit or anti-mouse IgG horseradish peroxidase conjugate was then added and incubated for an additional hour. After washing with phosphate-buffered saline, the bound conjugate was reacted with chromogen (*o*-dianisidine dihydrochloride) at 1 mg/ml in 0.01 M phosphate buffer, pH 5.95, for 0.5 hr, and the absorbance of each well was determined with a micro-ELISA reader.

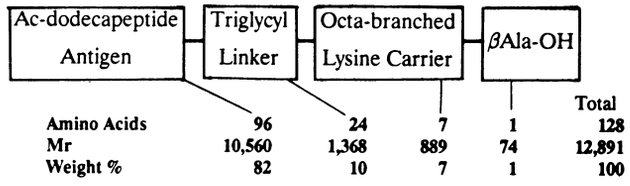

FIG. 2. Structural features and composition of a MAP containing an average peptide of 12 residues.

RESULTS

Concept and Design. The basic idea makes use of a limited sequential propagation of a trifunctional amino acid (or similar homologues) to form a core that serves as a low molecular weight matrix. The trifunctional amino acid, Boc-Lys(Boc), was found to be suitable because both N^{α}- and N^{ϵ}-amino groups are available as reactive ends. As shown in Fig. 1, sequential propagation of Boc-Lys(Boc) will generate 2^n reactive ends. The first level coupling of Boc-Lys(Boc) will produce two reactive amino ends as a bivalent MAP (Fig. 1A). The sequential generation of a second, third, and fourth step with Boc-Lys(Boc) will produce MAP containing four (tetravalent, Fig. 1B), eight (octavalent, Fig. 1C), and sixteen (hexadecavalent, Fig. 1D) reactive amino ends to which peptide antigens are attached. For this study, a design of MAP with an octa-branching core matrix consisting of a heptalysine (Fig. 1C and 2) was synthesized as a prototype. The MAP essentially consists of three structural features (Fig. 2): (*i*) a simple amino acid such as glycine or β-alanine bound as a benzyl ester of benzhydrylamide linkage to the solid-phase polymer to initiate the synthesis and serves as internal standard (one amino acid), (*ii*) an inner core of two to four generating steps of trifunctional amino acids (3–15 amino acids), (*iii*) a surface layer of acetylated synthetic peptide attached to the inner core matrix (36–288 amino acids for a 6- to 15-residue peptide plus a triglycyl extender). Thus, one major characteristic of the MAP is that the core matrix is small, and the bulk is formed by a high density of uniform peptide antigens layered around the core matrix. This design is in strong contrast to the conventional peptide-carrier conjugate that is comprised of a large protein carrier and a low density of peptide antigens randomly distributed along the support in an unidentified form. In an octa-branched MAP, the peptide antigen accounts for >80% of the total weight of the MAP (Fig. 2), the MAP having the molecular weight of a small protein. Furthermore, the MAP is oligomeric and contains noncationic peptidyl and isopeptidyl lysine amide linkages on both the N^{α} and N^{ϵ} termini of lysine. Such a design differs markedly from the conventional polylysyl conjugate that is cationic and polymeric in lysyl residues. The short dendritic peptide chains on the MAP are

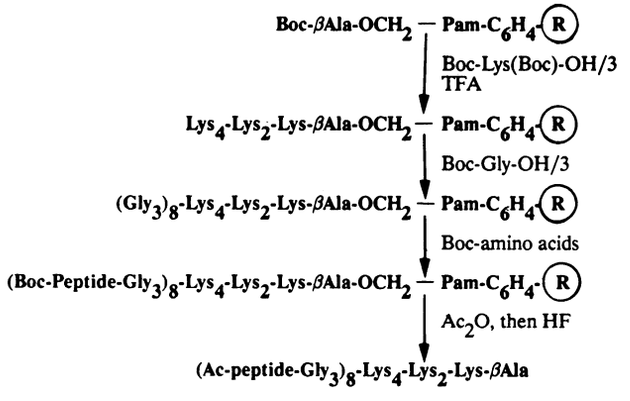

FIG. 3. Steps in synthesis of a MAP by the stepwise solid-phase method (see *Synthesis: General Methods*).

Table 1. Immunological responses of the MAP

Peptide	Sequence	Protein source	Half-maximal response, log 10*			Reactive to native protein†
				Preimmune	Immune	
IG-11	IEDNEYTARQG	$p60^{src}$	rabbit	<0.5	4.6	+ (A)
FA-14	FEPSEAEISHTQKA	T-cell receptor	mouse	<0.5	3.6	+ (A)
YP-13	YIQHKLQEIRHSP	ros	rabbit	<0.5	5.5	− (A)
NP-16	$(NANP)_4$	*P. falciparum*	mouse	<0.5	2.2	+ (B)
DV-9	DGISAAKDV	G_o protein	rabbit	<0.5	4.0	+ (B)
VS-11	VVSHFNDCPDS	human TGFα	rabbit	<0.5	4.1	+ (B)

Single letter code for amino acids is used.
*Half-maximal response of the antiserum in the dilution vs. absorbance curve in ELISA.
†Detection by either immunoprecipitation of the labeled protein and $NaDodSO_4$ gel electrophoresis of the precipitate (A) or by immunoblotting experiment (B).

probably mobile and may contribute to the enhanced immunogenicity of the MAP.

Synthesis and Purification. Several points regarding the unusual properties of the MAP should be emphasized to arrive at a high quality product. (*i*) The synthesis was by the stepwise solid-phase method (5) on a resin synthesized with less than the customary amino acid content because the peptide content increased geometrically with each addition of Boc-Lys(Boc), and the synthesis is difficult if the conventional loading of 0.3–0.8 mmol/g is used (Fig. 3). (*ii*) $HCONMe_2$ was a more suitable coupling solvent than CH_2Cl_2 for avoiding potential aggregation of peptides. (*iii*) The peptide resin should not be dried at any stage of the synthesis because resolvation of dried resin containing MAP is difficult. (*iv*) The efficiency of coupling was closely monitored (10). Synthetic errors such as deletion peptides would be amplified and difficult to correct by conventional purification methods. (*v*) The cleavage of the MAP from the resin was by an improved acid deprotection method with either HF or trifluoromethanesulfonic acid (9) in dimethyl sulfide to avoid side reactions catalyzed by strong acid.

The MAP was found to have an unusual ability to aggregate, and after cleavage from the resin support, the crude MAP was monomeric and was purified by extensive dialysis in a basic and strongly denaturing condition containing 8 M urea and mercaptoethanol to remove the undesirable aromatic additives of the cleavage reactions such as *p*-cresol and thiocresol, which tended to strongly adhere to the peptides. In addition, the base treatment under such conditions would also convert any strong acid catalyzed *O*-acyl rearrangement product of serinyl peptides to the *N*-acyl peptides and convert any residual level of Met(O) to methionine and His(Dnp) to histidine. The crude MAPs could be further purified by high-performance gel-permeation or ion-exchange chromatography. However, in most cases the MAPs could be used for immunization directly, without further purification.

Model Studies. Six model MAPs with synthetic peptides containing 9–16 residues were injected into animals (Table 1). Two MAPs, IG-11 and YP-13, were internal sequences of 11 and 13 residues related respectively to tyrosine protein kinases, $p60^{src}$ (11, 12) and ros (10). Similarly, peptides FA-14, NP-16, DV-9, and VS-11 corresponded respectively, to T-cell receptor (13), circumsporozoite protein of *Plasmodium falciparum* (14), G_o protein (15, 16), and human transforming growth factor type α (TGFα) (17) were synthesized.

Test of the MAP to Elicit Specific Antisera. The titers of the antisera raised by the MAPs in rabbits and mice were evaluated by ELISA with the corresponding MAPs as substrates. All six MAPs were found to be strong immunogens (Table 1). Moreover, five of the six MAPs produced antisera that recognized the cognate sequences of the native proteins. The kinetics of responses of two MAPs, IG-11 and YP-13, are shown in Fig. 4, and an increase of about 10-fold was observed after boostings in either case. In general, good titers of rabbit antibodies were obtained for 10 different MAPs (6 are shown here). However, different strains of inbred mice gave distinctly different responses. MAP NP-16 produced only moderate responses in BALB/c and C57BL/6J mice after the first inoculation. The antibody titer increased ≈10- to 50-fold after boostings in C57BL/6J mice, but BALB/c mice did not respond well to the boostings. On the other hand, MAP FA-14 produced satisfactory antibody titer with BALB/c mice after boostings.

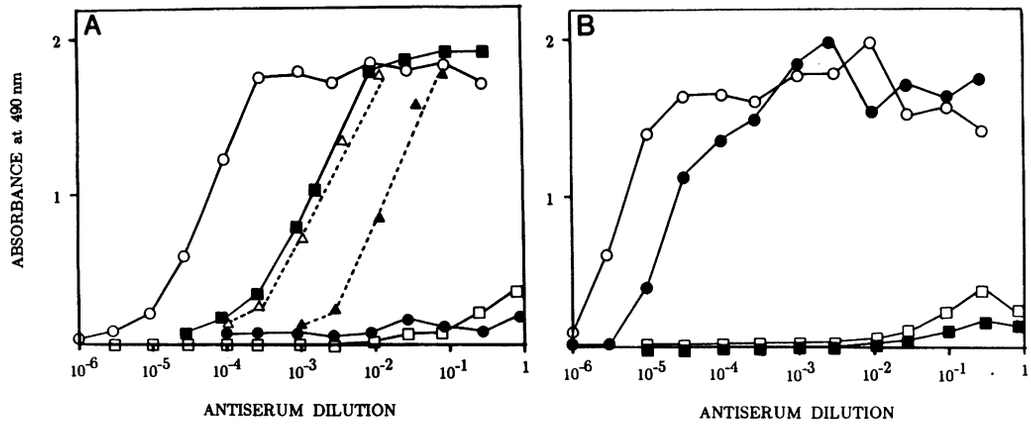

FIG. 4. (*A*) Immunoreactivities by ELISA of antisera derived from the MAP IG-11 (Table 1) and from IG-11 monomer conjugated to KLH. Antisera to MAP IG-11 vs. MAP IG-11: ■, primary immunization; ○, after two boostings. Antisera to IG-11-KLH conjugate vs. IG-11 monomer: ▲, primary immunization; △, after two boostings. Controls: ●, antisera to MAP YP-13 vs. MAP IG-11; □, MAP IG-11 antiserum preincubated with excess IG-11 monomeric peptide. Control preimmune antiserum was negative. (*B*) Antisera raised to the MAP YP-13 (Table 1) studied by ELISA vs. MAP. YP-13: ●, primary immunization; ○, after a single boosting. Controls: □, antiserum to MAP IG-11; ■, preimmune antiserum.

To show that the specificity of the antiserum against the MAP is similar to that obtained from the monomeric peptide, the latter, IG-11, was used in excess to inhibit the binding of IG-11 antisera to the IG-11 MAP in ELISA (Fig. 4A). Furthermore, a greatly diminished titer of IG-11 MAP antiserum was seen when the linear IG-11 peptide was conjugated to carrier proteins through the N terminus by glutaraldehyde to bovine serum albumin or keyhole limpet hemocyanin (KLH), showing that the expected epitope of IG-11 was at or near the N terminus of the IG-11 MAP. In a study of the cross-immunoreactivity of the heptalysyl backbone, which is common to the MAP system, we found no observable cross-reactivity between IG-11 and YP-13 (and other MAPs) in ELISA (Fig. 4). Antibody titers induced by IG-11 conjugated to KLH by the conventional method of glutaraldehyde were compared with those induced by the IG-11 MAP (Fig. 4A); both primary and secondary responses of the antibody titers to IG-11–KLH exhibited lower reactivities than those to the IG-11–MAP.

DISCUSSION

The results demonstrate the usefulness of a new design referred to as MAP in generating antibodies against a synthetic peptide, a system that may prove useful for the design of vaccines. To illustrate the utility of the design, six different peptides with 9–16 amino acids attached to an octa-branched MAP were synthesized and tested in animal models (Table 1). All model MAPs gave high-titer antibody responses, and five of the MAPs provided antisera that recognized the cognate protein from which they were derived. Two such examples, VS-11 and IG-11 MAPs, are shown in Fig. 5. The antisera

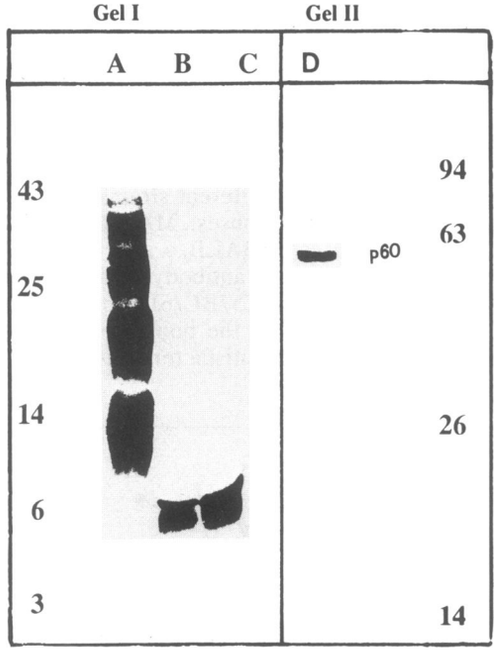

FIG. 5. Specificity of antisera raised to MAP VS-11, TGFα peptide, and IG-11 NaDodSO$_4$/PAGE. Marginal numbers are in kDa. Gel I, lanes A–C: analysis by immunoblotting under limited reducing conditions of anti-VS-11. VS antigens include MAP VS-11 (lane A), rat TGFα (6 kDa, lane B), human TGFα (6 kDa, lane C). Multiple bands in lane A are due to disulfide formation even in mercaptoethanol. Gel II, lane D: immunoprecipitation from ^{3}H-labeled extracts of rat cells transformed by Rous sarcoma virus vs. an antiserum raised to IG-11. The resulting precipitate containing protein A–Sepharose was thoroughly washed, dissociated in NaDodSO$_4$/mercaptoethanol buffer, and the components were separated by gel electrophoresis (14). A single radioactive band (p60^{src}) is indicated at 60 kDa.

induced by VS-11 MAP derived from the N-terminal 11 residues of human TGFα reacted with both human and rat TGFα. Similarly, antisera induced by IG-11 reacted with the intact p60^{src}.

Detailed studies of MAPs for the production of antibodies and vaccines have also been initiated. For example, FA-14, a 14-residue sequence derived from the human T-cell antigen receptor β-chain constant region, when incorporated in MAP, gave rise to both polyclonal and monoclonal antibodies, each reacting with the intact β-chain protein (18). Mapping of the antigenic determinants of FA-14 by these antibodies showed that the majority recognized the N-terminal residues of both the linear monomeric as well as the octameric (MAP) form. More importantly, there were no antibodies to the heptalysyl core of MAP. These results were consistent with those obtained from IG-11 and YP-13 shown in Fig. 4. Although IG-11 and YP-13 used the same heptalysinyl MAP core, antisera raised against IG-11 and YP-13 showed no cross-reactivity. Similarly, antibody induced by DV-9 MAPs (Table 1) was found to be specific to the α_0 subunit of GTP-binding protein but not cross-reactive with α_1 subunit, which has considerable sequence homology to α_0 (19). Thus, the α_0-specific antibody induced by DV-9 MAP was found to be a useful reagent to study the occurrence and distribution of this protein in tissues (19).

The use of a radially branching system to generate a new polymer has been known in and exclusively confined to classical polymer chemistry. Systems such as star polymers, cauliflower polymers, and star-burst polymers, using multifunctional groups to generate controlled sequence propagation to form new dispersed polymers, are used to produce radial branching of 10 or more levels with >100 reactive ends (20).

The present system was designed specifically for a biological application. The combined use of a dispersed system with branching oligolysines as a scaffolding for incorporating a high density of some peptide antigen and synthesis by the solid-phase method will greatly simplify the approach of obtaining a chemically defined peptide-antigen carrier system for a biological purpose. Several important issues concerning the present design remain to be resolved, such as the molecular arrangement—i.e., the size, shape, and levels of branching that will affect the antigenicity. Another issue is its flexibility in allowing multiple antigenic peptides to be attached in a MAP, such as the attachment of both T- and B-cell epitopes. Such a design could potentially lead to improved vaccines.

I thank P. Chang, L. Huang, N. Pileggi, and C. Volin for technical assistance; several titers of the following antisera were kindly supplied to us as indicated: DV-9, (Dr. K Chang), FA-14 (D. Posnett), and NP-16 (V. Nussenzweig). This work was supported, in part, by Public Health Service Grant CA 36544, awarded by the National Cancer Institute, and by the Agency for International Development, Grant AID OGAS122.

1. Sela, M. & Arnon, R. (1980) in *New Developments with Human and Veterinary Vaccines*, eds. Mizrahi, A., Hertman, I., Klingberg, M. A. & Kohn, A. (Liss, New York), pp. 315–323.
2. Lerner, R. A. (1982) *Nature (London)* **299**, 592–596.
3. Bittle, J. L., Houghten, R. A., Alexander, H., Shinnick, T. M., Sutcliffe, J. G., Lerner, R. A., Rowlands, D. J. & Brown, F. (1982) *Nature (London)* **298**, 30–33.
4. DiMarchi, R., Brooke, G., Gale, C., Cracknell, V., Doel, T. & Mowat, N. (1986) *Science* **232**, 639–641.
5. Merrifield, R. B. (1963) *J. Am. Chem. Soc.* **85**, 2149–2154.
6. Mitchell, A. R., Kent, S. B. H., Engelhard, M. & Merrifield, R. B. (1978) *J. Org. Chem.* **43**, 2845–2852.
7. Sarin, V. K., Kent, S. B. H., Tam, J. P. & Merrifield, R. B. (1981) *Anal. Biochem.* **117**, 147–157.
8. Tam, J. P. (1985) in *Proceedings of the Ninth American Peptide Symposium*, eds. Deber, C. M., Kopple, K. D. & Hruby, V. J.

(Pierce Chem., Rockford, IL), pp. 305–308.
9. Tam, J. P., Heath, W. F. & Merrifield, R. B. (1986) *J. Am. Chem. Soc.* **108,** 5242–5251.
10. Neckameyer, W. S. & Wang, L.-H. (1985) *J. Virol.* **53,** 879–884.
11. Czernilofsky, A., Levison, A., Varmus, H., Bishop, J. M., Tisher, E. & Goodman, H. (1980) *Nature (London)* **287,** 198–203.
12. Tanabe, T., Nukada, T., Nishikawa, Y., Sugimoto, K., Suzuki, H., Takahashi, H., Noda, M., Haga, T., Ichiyama, A., Kangawa, K., Minamino, N., Matsuo, H. & Numa, S. (1985) *Nature (London)* **315,** 242–245.
13. Yanagi, Y., Yoshikai, Y., Leggett, K., Clark, S. P., Aleksander, I. & Mak, T. W. (1984) *Nature (London)* **308,** 145–149.
14. Wong, T. Y. & Goldberg, A. R. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2529–2533.
15. Dame, J. B., Williams, J. L., McCutchan, T. F., Weber, J. L., Wirtx, R. A., Hockmeyer, W. T., Maloy, W. L., Haynes, J. D., Schneider, I., Roberts, D., Sanders, G. S., Reddi, E. P., Diggs, C. L. & Miller, L. H. (1984) *Science* **225,** 593–599.
16. Mumby, S. M., Kahn, R. A., Manning, D. R. & Gilman, A. G. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 265–269.
17. Derynck, R., Roberts, A. B., Winkler, M. E., Chen, E. Y. & Goeddel, D. V. (1984) *Cell* **38,** 287–297.
18. Posnett, D. N., McGrath, H. & Tam, J. P. (1988) *J. Biol. Chem.* **263,** 1719–1725.
19. Chang, K. J., Pugh, W., Blanchard, S. G., McDermed, J. & Tam, J. P. (1988) *Proc. Natl. Acad. Sci, USA* **85,** 4929–4933.
20. Tomalia, D. A. (1986) *Macromolecules* **9,** 2466–2468.