Specifities of Rabbit Antisera to Multiple Antigen (MAP) Peptides

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Two multiple antigen peptides consisting of 6 and 7 amino acid residues, respectively, plus a 12-residue fragment, used as a control, all linked to a polylysine core, were used as immunogens in rabbits in order to obtain an immune response.

Rabbit antisera against such polymers were then tested in ELISA against a panel of antigens in order to analyze the specificites of the resulting antibodies. The responses were different for all three immunogens, being partially or totally directed, for two of the three compounds, including the 12-residue control MAP peptide, against the polylysyl core, which is considered as non immunogenic. The third MAP polymer was practically unable to elicit an immune response.

During the last several years, synthetic peptides have been widely used for the generation of anti-peptide antibodies of predetermined specificity. With the exception of large-sized fragments, that can be injected uncoupled into animals (Choppin *et al.,* 1986; Del Giudice *et al.,* 1986), peptides must be linked to a carrier protein, usually BSA or KLH, in order to be sufficiently immunogenic to elicit an appropriate immune response (Green *et al.,* 1982; Bittle *et al.,* 1982).

The conjugation of the peptide to the macromolecule introduces necessarly an alteration of the chemical structure of the fragment. This might lead to a modification of antigenic determinants of the peptide, and to an immune response directed to sites not represented in the original molecule. In addition, the carrier protein, and perhaps adducts of the linking agent with the carrier or the synthetic peptide, may elicit antibodies.

The Multiple Antigen Peptides (MAP) technique appears to solve most of the ambiguities connected with the necessity of preparing suitable immunogens from low-molecular weight peptides. A MAP is made up by a ramificated low molecular weight lysyl core, bound to the resin, that gives then rise to eight copies of the desired peptide. The eight monomers remain linked together through the lysyl alpha and epsylon amino groups after deprotection, and the polymer possesses a sufficient high-molecular weight to be used as immunogen without further chemical modification (Posnett *et al.,* 1988; Tam, 1988).

The second advantage of the MAP system, as compared to conventional KLH-peptide conjugates, is that the former has a very high molar ratio of synthetic peptide to the core carrier, thus providing faster immunological responses. Third, the inner core, containing seven lysines and one or two other aminoacids, one of which is generally a β-alanine functioning as a bridge, accounts for less than 10% of the total molecular weight of the MAP molecule, and is considered unable to give rise to an immune response (Tam, 1989).

This last statement deserves some comments. We have found that under suitable circumstances, probably determined by the length, composition, intrinsic immmunogenicity of the peptide, the core may elicit antibodies. This possibility should not be underextimated when measuring, for instance, the titer of antipeptide antibodies in ELISA using the MAP as antigen, since the binding data would reflect essentially the total response to the immunogen.

Materials and Methods

We examined the immune sera of several rabbits immunized with Multiple Antigen Peptides, and report a partial characterization of the specificites of the antibodies of three of them. Two of the polymeric peptides used as immunogens, M46 and M91, were relatively short, accounting for only 6 and 7 residues , plus core, as compared to the 11 to 21-residue fragments previously investigated by Tam (Tam, 1989). The other peptide,

Abbreviations: Boc, butoxycarbonyl; TFMSA, trifluoromethanesulfonic acid.

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M88, was medium-sized, and was originally chosed as a control.

All three MAPs were synthesized by the solid-phase technique and using N-α-Boc protected amino acids. Protecting groups of side chain functions were Tosyl (Arg), benzyl (Asp, Glu, Ser and Thr), and 2,6 dichlorobenzyl (Tyr). The coupling agent was diisopropylcarbodiimide, and dichloromethane or dimethylformamide the solvents. For all three peptides, the synthesis was initiated by Boc-Gly-PAM resin ester as the C-terminal residue, while β-Ala was added as the second amino acid. The branching core was then Lys_4- Lys_2- Lys-βAla- Gly

In addition, the same monomeric peptides, plus some others used as controls, were prepared by conventional solid-phase synthesis by the Boc/benzyl strategy.

At the end of the syntheses, peptides were cleaved from the resin by aid of TFMSA (Yajima *et al.*, 1973), and then precipitated by cold ether.. MAPs were isolated by column chromatography on Sephadex G25 superfine in 0.02 M ammonia or acetic acid, monomeric peptides by column chromatography on Aca 202 high resolution gel filtration resin and/or reverse-phase HPLC . All peptides were controlled for the expected amino acid composition.

Rabbits were then immunized either with KLH-peptide complexes, prepared by linking the carrier to monomeric peptides by aid of glutaraldehyde, or with the MAP polymers, using similar schedules (Chersi *et al.*, 1986). The blood was collected from the ears of the animals after the fourth injection. Immune sera, at 1:400 or 1:1600 dilution, were then tested with an ELISA (Chersi *et al.*, 1984) precoating the wells of the microtiter plates with a panel of antigens that included the three MAP polymers, the inner core "C", i.e. Lys_4-Lys_2-Lys-βAla-Gly, and the monomeric peptides 46, 47, 88, 91 and X (Table I) .

Results

The binding values, partly reported in Fig. 1, indicate that anti-M88 reacted with all MAP peptides and with the core, while its reactivity on 88 was low. The serum elicited by KLH-88, on the contrary, reacted exclusively with M88 and 88. The second antiserum, anti-M46, exhibited a high reactivity for M46 and partly for peptide 47 (which encompasses the 8-residue fragment 46). It did not bind initially to peptide 46, but essentially because of poor adhesion of the fragment to the plastic surface of the plate. Subsequently, it exhibited a moderate activity when 46 was previously linked to gelatine (G-46) before being coated onto the microtiter plate. The crossreactivity with MAP peptides and with the core was low. The anti-47 antiserum reacted almost equally well with 47 and M46.

Table I. Amino acid sequence of peptides used as immunogens and/or antigens.

a) Monomeric peptides:

X) Asp Ile Met Pro Pro Leu Leu Phe Val
46) Thr Arg Ile Asp Asp Pro Glu Thr
47) Phe Thr Arg Ile Asp Asp Pro Glu Thr Lys Arg Gln Asn Ser Asn Leu
88) Pro Pro Tyr Thr Ile Val Tyr Phe Pro Val Arg Gly
91) Ser Ala Arg Ala Arg Ser

b) Branched polymeric peptides:

M46) Thr Arg Ile Asp Asp Pro Glu Thr-core
M88) Pro Pro Tyr Thr Ile Val Tyr Phe Pro Val Arg Gly-core
M91) Ser Ala Arg Ala Arg Ser-core

X was an unrelated peptide used as a control in binding assays. The core, indicated as "C" in the text, is the nonapeptide Lys_4- Lys_2- Lys- βAla- Gly. The octapeptide 46 (Thr Arg Ile Asp Asp Pro Glu Thr) is the fragment 2–9 of peptide 47. This small peptide scarsely adheres to plastic surfaces, thus the longer peptide 47 was used at his place in ELISA tests. In few experiments, peptide 46 was bound to gelatin in order to increase its adhesion to wells of microtiter plates. This antigen is indicated in the text and in Table II as G46.

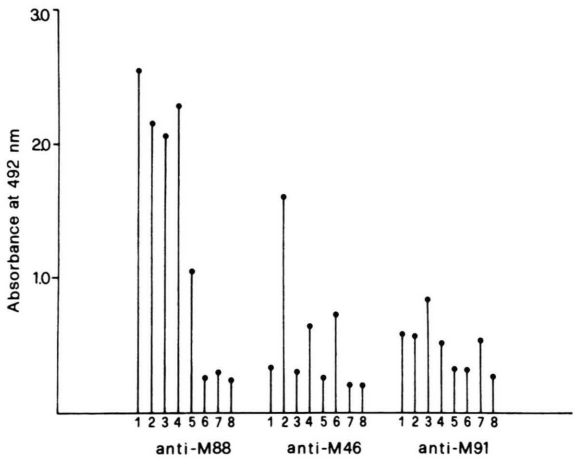

Fig. 1. Binding of anti-MAP antisera anti-M88, anti-M46, anti-M91, at 1:400 dilution, to a panel of antigens, as determined with an ELISA. Wells of microtiter plates were precoated with 0.5 μg of monomeric peptide or 0.05 μg of MAP. No substantial differences were found when the antigens were used at a 10-fold dilution. Pre-immune serum, at the same dilution, was used as a control, and background values were subtracted. The assay was performed in duplicates. The antigens were the followings: 1) M88; 2) M46; 3) M91; 4) core C; 5) 88; 6) 47; 7) 91; 8) X (control).

Finally, antisera against M91 (two rabbits) reacted poorly with M91, 91 and core, this suggesting a weak response to the immunogen. Anti-91 reacted poorly with peptide 91, which apparently did not stick onto the plastic surface, but exhibited remarkably better affinity for M91. A schematic summary of the reactivities of all immune sera is reported in Table II.

The core "C", bound to KLH, elicited in rabbits an immune response which was directed against the carrier KLH as well as against the core, which now acted as the hapten (data not shown).

Comments

The binding data suggest several considerations. First, the immune response of the rabbit immunized with M88 appears to be directed preferentially against the core, since the antiserum reacts with all MAPs and the core. The second antiserum (anti-M46) reacts preferentially with M46, and displays little activity for the MAP peptides , the core, and the monomeric peptides 46 and 47 . This might indicate that the immune response is essentially directed to an epitope within peptide 46 that includes part of the core, likely one or two bi-substituted lysines, or to a determinant made up by fragments of two peptide chains, linked tail-to-tail by a lysine residues . It should be reminded that one of the four anti-MAP antibodies recently described (Briand *et al.,* 1992) reacts exclusively with the MAP , and not with the monomeric peptide. As far as it concerns M91, both anti-M91 antisera had a poor titer of anti-C as well as anti-peptide antibodies. Briand also reports that one of his four MAPs was apparently unable to elicit an immune response (Briand *et al.,* 1992). Finally, the core itself can elicit antibodies, if suitably presented by a carrier protein.

The specificities of the three anti-MAP antisera suggest that, apart from differences that might be ascribed to the individual animals, the core "C", under suitable circumstances, probably dictated by the nature, length, and intrinsic immunogenicity of the bound peptide, may occasionally elicit an immune response, This should not be considered,

Table II. Schematic summary of the reactivities of the three anti-MAP antisera, and of the corresponding sera against KLH-peptide complexes, to the panel of antigens, as determined by ELISA.

Antigens	M88	M46	M91	C	88	47	G46	46	91
anti-M88	+++	+++	+++	+++	+	NT	NT	NT	NT
anti-88	++	−	−	−	++	NT	NT	NT	NT
anti-M46	−	+++	−	+	−	+	+	−	NT
anti-47	NT	++	NT	−	−	+++	++	+	NT
anti-M91	−	−	+	+	NT	−	−	−	+
anti-91	NT	NT	++	−	NT	NT	−	NT	++

The assay was performed as described in the text. NT, not tested. −, Values around background (0.2–0.4); +, binding values below 1.0; ++, binding values 1.0–1.5; +++, binding values 1.5–2.5.

according to our opinion, as a very unlikely event, since βAla, and expecially α,ε-bisubstituted lysines, are highly infrequent compounds in living organisms. In addition, the number of amino acid of the core, and its molecular weight, appears to be sufficient for representing an antigenic site. When bound to a carrier protein, as KLH, the core generates anti-core antibodies, thus acting as the hapten.

As far as it concerns the presentation of the core to the immune system of the injected animal, we suppose that the steric structure of a MAP might not always resemble that of a little sphere, where the core occupies the inner part of it, although this might be the most likely event. The four carbon atom side chains of the lysines, bearing the ε-amino groups, might be indeed relatively free to assume several different steric conformations, this resulting in turns and bends of the attached polypeptide chain, and in a gradual filling of empty spaces around the core. It is not known, however, whether this arrangment would be sufficient for covering the whole core, expecially when, as in two of our three peptides, the number of amino acid residues attached to the last lysines is relatively small. MAPs bearing long or very long peptides, on the contrary, might approximate the condition in which the whole core is covered and masked, and thus not susceptible to an immune response. Surprisingly, the highest titer of anti-core antibodies was detected in the sera of the rabbit immunized with the relatively long peptide M88 (12 residues plus core), this suggesting that the generation of subclasses of antibodies directed against the core might be influenced by more complex factors.

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Bittle J. L., Houghten R. A., Alexander H., Shinnick T. M., Sutcliffe J. G., and Lerner R. A. (1982), Protection against foot-and-mouth disease by immunization with a chemically-synthesized peptide predicted from the viral nucleotide sequence. Nature **298,** 30–33.

Briand J. P., Barin C., Van Regelmortel M. H. V., and Muller S. (1992), Application and limitations of the multiple antigen peptide (MAP) system in the production and evaluationn of anti-peptide and anti-protein antibodies. J. Immunol. Methods **156,** 255–265.

Chersi A., Schulz G., and Houghten R. A. (1984), Immunological and functional characterization of anti-HLA-DR antibodies induced by synthetic peptides. Mol. Immun. **21,** 847–852.

Chersi A., Houghten R. A., Chillemi F., Zito R., and Centis D: (1986), Specificity of rabbit antibodies elicited by related synthetic peptides. Z. Naturforsch. **41c,** 613–617.

Choppin J., Metzger J. J., Bouillot M., Briand J. P., Connan F., Van Regenmortel M. H. V., and Levy .P. (1986), Recognition of HLA class I molecules by antisera directed to synthetic peptides corresponding to different regions of the HLA-B7 heavy chain. J. Immun. **136,** 1738–1744.

DelGiudice G., Cooper J. A., Merino J., Verdhini A. S., Pessi A., Togna A. P., Engers H. D., Corradin G., and Lambert P. H. (1986), The Antibody response in mice to carrier-free synthetic peptides of Plasmodium Falciparum Circumsporozoite repetitive epitope is I-A restricted. Possible implications for Malaria vaccines. J. Immunol. **137,** 2952–2956.

Green N., Alexander H., Olson A., Alexander S., Shinnick T. M., Sutcliffe J. G., and Lerner R. A. (1982), Immunogenic structure of the Influenza Virus Hemagglutinin. Cell 28, 477–487.

Posnett D. N., McGrath H., and Tam J. P. (1988), A novel method for producing antipeptide antibodies. J. Biol. Chem. **263,** 1719–1725.

Tam J. P. (1988), Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigen peptide system. Proc. Natl. Acad. Sci. USA **85,** 5409–5413.

Tam J. P. (1989), Multiple Antigen Peptide System: a novel design for synthetic peptide vaccine and immunoassay. In: Synthetic Peptides: Approaches to Biological Problems (Tam J. P. and Kaiser E. T., eds.). A. R. Liss Inc., New York, pp. 3–18.

Yajima H., Ogawa H., Watanabe H., Fujii N., Kurobe M., and Myiamoto S. (1973), Studies on peptides: application of the trifluoromethanesulfonic acid procedure to the synthesis of Tuftsin. Chem. Pharm. Bull. **23,** 371–373.