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Antibody responses to non-immunogenic synthetic peptides induced by co-immunization with immunogenic peptides

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SUMMARY

Chimeric peptides comprising B- and T-helper cell epitopes from the proteins of infectious agents represent immunogens with potential for use as new vaccines. However, it has become clear that the orientation of the epitopes, the presence of spacer residues and the number of copies of the epitopes influence the specificity, levels and affinity of the antibody produced following immunization with such constructs. Furthermore, the response to peptides is under genetic control leading to major histocompatibility complex (MHC)-linked non-responsiveness. In this study, we have investigated the potential of co-immunization of immunogenic peptides (to provide T-cell help) with non-immunogenic peptides (representing B-cell epitopes) to overcome the non-response to the latter. For this purpose, we have employed peptides representing T- and B-cell epitopes derived from the sequences of the fusion and haemagglutinin glycoproteins of measles virus. The results obtained show that simple co-immunization of a B-cell epitope with a T-cell epitope results in the production of antibody to the B-cell epitope without the requirement for covalent linkage of the two peptides. This approach could thus be used to overcome the problem of poor immunogenicity of peptides and will be of potential value in the design of immunization strategies using synthetic immunogens.

INTRODUCTION

Our current understanding of the induction of an antibody response to a T-cell-dependent antigen suggests the requirement for co-operation and communication between B and Th cells. Early studies using haptenated molecules have shown that for the induction of an immune response to the hapten it had to be covalently linked to a carrier molecule.[1] In addition, it has been shown that immunogens are recognized not only by immunoglobulin receptors on B cells but also by T-cell receptors which bind a processed fragment of the antigen in association with class II major histocompatibility complex (MHC) molecules on antigen-presenting cells.[2] These results suggested that B- and T-cell epitopes need to be linked in order for Th cells to provide cognate help for B-cell activation and antibody production.[3] On the basis of these concepts, there have been numerous reports showing the successful construction of effective synthetic immunogens by the combination of well-defined Th determinants and B-cell sites representing neutralizing or potentially protective epitopes.[4-8] Unlike cognate help, T-cell-dependent B-cell activation can also occur without the requirement for covalent linkage of B- and Th-cell determinants (bystander help). Early studies showed that Th cells primed to a particular carrier, support secondary antibody responses to a hapten linked to a different, non-cross-reactive antigen.[9,10] Jensen and Kapp[11] have shown that co-immunization of non-immunogenic pork insulin with immunogenic sheep insulin (or foreign proteins), overcomes non-responsiveness to the former, in mice of H-2^b and H-2^k haplotypes. In addition, Good *et al.*[12] have shown that the poor immune response to a synthetic peptide from the malaria circumsporozoite protein can be overcome by co-immunization of the peptide with keyhole limpet haemocyanin (KLH).

On the basis of these findings we have investigated the possibility of overcoming non-responsiveness to a synthetic peptide by co-immunization of the peptide with an immunogenic peptide. For this purpose, we used previously studied peptides derived from the fusion (F) and haemagglutinin (H) surface glycoproteins of measles virus.

The results obtained demonstrate that simple co-immunization of peptides representing B-cell epitopes with peptides representing Th determinants results in the production of antibody to the B-cell epitopes without the requirement for the covalent linkage of the B- and Th-cell epitopes. This approach can thus be used to overcome genetically controlled non-responsiveness to synthetic peptides.

MATERIALS AND METHODS

Selection of peptides

Studies on the antigenic structure of measles virus F and H glycoproteins have shown several regions to represent potential

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Table 1. Notation and characteristics of peptides used in this study

Viral protein	Peptide	Immunogenicity in BALB/c and SWR	Antigenic site
Fusion	240-252	–	B
	288-302	+	T/B
	258-277	+	T/B
Haemagglutinin	49-72	+	T/B
	188-199	–	B

B- and Th-cell epitopes (Table 1). Among these, peptide 240-252 was found to represent a B-cell epitope in the F protein and to be immunogenic in TO, CBA and C57BL/6 but not in BALB/c and SWR/J mice.[13] Sequences 288-302 and 258-277 from the F protein represent Th-cell determinants recognized by mice of different H-2 type (ref. 14, C. D. Partidos, O. E. Obeid and M. W. Steward, unpublished results). The sequence 185-195 of the virus H protein has been identified as a B-cell epitope which can induce virus-neutralizing activity[15] and a peptide representing residues 188-199 was therefore synthesized avoiding the glycosylation site in the published sequence. Finally, the sequence 49-72 from the H molecule has been shown to contain both B- and T-cell antigenic sites and was chosen for this study (C. D. Partidos, O. E. Obeid and M. W. Steward, unpublished results).

Peptide synthesis

Peptides representing residues 240-252, 258-277, 288-302 from the F and 188-199, 49-72 from the H glycoproteins of measles virus were synthesized by manual solid-phase synthesis using Fmoc chemistry. In addition a chimeric peptide was produced in which the 240-252 sequence was co-linearly synthesized at the carboxyl terminus of the 288-302 peptide using a proline residue as a spacer (288-P-240). Fmoc-protected amino acids were converted to the hydroxybenzotriazole-activated esters by treatment with hydroxybenzotriazole and N,N-diisopropylcarbodiimide in dimethylformamide (DMF). Subsequent coupling reactions were performed in DMF and the Fmoc groups were removed with 20% piperidine in DMF followed by a series of washes in DMF. After synthesis, side chain-protecting groups were removed and the peptide was cleaved in trifluoroacetic acid in the presence of scavengers. After cleavage, peptides were extracted into diethylether, purified by preparative high-performance liquid chromatography (HPLC) and their purity assessed by analytical HPLC and amino acid analysis. A cysteine residue at the carboxyl-terminus of the 240-252 and 288-P-240 peptides was introduced to increase immunogenicity.[16]

Mice

Mice of the inbred strains BALB/c and SWR/J were purchased from the National Institute of Medical Research (Mill Hill, London, U.K.).

Immunization

Antibody responses to synthetic peptide 240-252 were measured following intraperitoneal injection of 6–8-week-old female mice. One hundred micrograms of the 240-252 peptide was injected as a free peptide or in a chimeric form (288-P-240) or co-immunized with either 100 μg of 288-302 or 100 μg 258-277 peptides emulsified in complete Freund's adjuvant (CFA) (1:1). Eleven weeks after priming, mice were boosted by the same route and with the same dose of peptides in incomplete Freund's adjuvant (IFA). Blood samples were collected every 2 weeks, centrifuged and sera were collected and stored frozen at −20°. For proliferative responses a group of four mice were immunized via the hind footpad with a mixture of 50 μg of the 240-252 peptide and 50 μg of the 258-277 peptide emulsified in CFA.

Antibody assays

Titres of antibodies against the peptides were determined by an enzyme-linked immunosorbent assay (ELISA). Wells of microtitre plates were coated overnight at 4° with 50 μl/well of 5 μg/ml solution of the relevant peptide in carbonate-bicarbonate buffer 0·1 M, pH 9·6. Plates were washed five times with tap water prior to and following blocking with 1% gelatin in phosphate-buffered saline (PBS) for 2 hr at 37°, dried and stored at 4°. Sera were titrated across the plate by serial twofold dilutions in diluent (PBS/0·25% gelatin/0·005% Tween 20) and incubated for 1 hr at 37°. After washing five times with tap water 50 μl of rabbit anti-mouse IgG-peroxidase conjugate (Nordic, Tilburg, The Netherlands) at a dilution of 1/1000 in the PBS/gelatin/Tween diluent was pipetted into each well and the plates incubated as before. After washing five times with tap water to remove unbound conjugate, bound enzyme was detected by adding 50 μl/well of a chromogen solution (0·04% *o*-phenylenediamine + 0·004% hydrogen peroxide in 0·1 M citric acid/phosphate buffer pH 5·0). After 10 min, the colour reaction was stopped with 25 μl/well of 2 M sulphuric acid and the absorbance at 492 nm was measured on a Titertek Multiskan (Flow Laboratories, Irvine, U.K.). Titres are expressed as $\log_{10}$ of the reciprocal of the antibody dilution giving an OD greater than 0·2 at 492 nm.

Lymphocyte stimulation assay

Eight days after immunization, the draining lymph nodes were removed aseptically, pooled and the mononuclear cells collected by centrifugation. Viable, unfractionated lymph node cells (4×10^5) in 0·2 ml RPMI-1640 medium, supplemented with 1% autologous serum, 2 mM L-glutamine, 10 mM HEPES and 100 μg/ml antibiotics, were incubated in the presence of various doses of the peptides or medium alone in humidified 5% CO_2 atmosphere at 37° for 5 days. The cells were pulse-labelled with 1 μCi [^{3}H]thymidine in 10 μl volumes/culture 16–18 hr before harvesting. Thymidine incorporation was assessed by liquid scintillation spectrometry and the results expressed as the mean c.p.m. from triplicate cultures ± SD.

RESULTS

The effect of co-immunization on the genetic control of antibody responses to the 240-252 peptide

Peptide 240-252 is non-immunogenic in H-2^q and H-2^d mice when administered free in Freund's adjuvant. However, immunization with a mixture of 240-252 and the immunogenic 258-277 peptide in CFA resulted in the induction of a primary immune response to both peptides in BALB/c and SWR/J mice (Fig. 1). A booster injection of the same mixture of peptides in IFA 11 weeks after priming resulted in a secondary antibody response to both peptides. Thus the presence of B- and T-cell

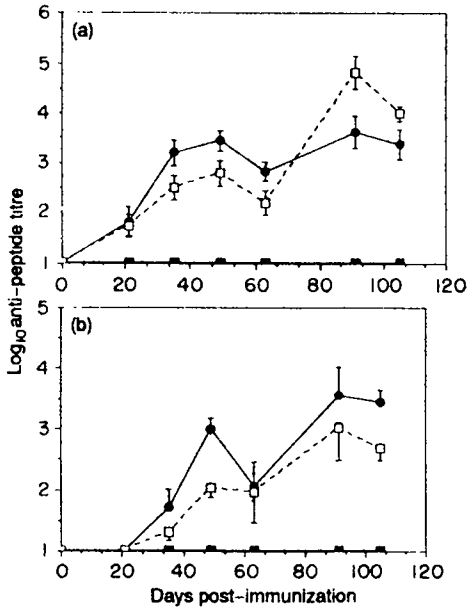

Figure 1. Effect of co-immunization on the immunogenicity of the 240-252 peptide in BALB/c (a) and SWR/J (b) mice. ELISA titres (mean ± SD) in sera from sequential bleeds of groups of four mice injected on Days 0 and 77. Anti-240-252 titres in mice injected with 240-252 alone (■); anti-240-252 titres in mice co-immunized with the 240-252 and 258-277 peptides (□); anti-258-277 titres in mice co-immunized with the 240-252 and 258-277 peptides (●).

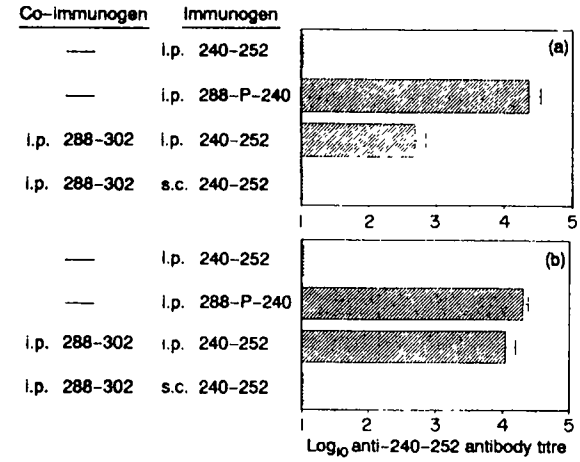

Figure 2. ELISA titres of antibody to the 240-252 peptide in BALB/c (a) and SWR/J (b) mice after co-immunization with the 288-302 peptide at the same or different sites. Results represent mean ± SD of titres of antibody from groups of four mice bled 2 weeks after the booster immunization.

Table 2. Proliferative responses of lymph node cells from BALB/c mice following co-immunization with 258-277 peptide. Lymphocytes were stimulated *in vitro* with varying concentrations of the 258-277 peptide, the 240-252 peptide and with peptide 284-302 as a control. Values represent the mean c.p.m. ± SD of [^{3}H]TdR incorporation of triplicate cultures. Values in bold type represent significant stimulation

μg peptide/well	258-277	240-252	284-302
20	**31,334 ± 2206**	2664 ± 729	6262 ± 253
10	**49,572 ± 2387**	2861 ± 529	6439 ± 391
1	**33,647 ± 5221**	3903 ± 1105	4332 ± 1140
0 1	**14,404 ± 1870**	3621 ± 1265	4083 ± 505
Medium	3215 ± 765	3215 ± 765	3215 ± 765

epitopes on the same molecule is not a necessary requirement for the induction of an antibody response to the 240-252 peptide.

The effect of co-immunization of 240-252 with the 258-277 peptide on the proliferative responses of lymphocytes to 240-252 peptide

Previously it has been shown that 240-252 peptide was able to induce antibody and proliferative responses in mice of H-2^s, H-2^k and H-2^b haplotypes but not in strains with the H-2^d or H-2^q haplotypes.[13] Since co-immunization of 240-252 with the 258-277 peptide resulted in the induction of anti-240-252 antibody response (Fig. 1) the possibility exists that the 258-277 peptide enhanced the induction of T-cell proliferative response to the 240-252 peptide in non-responding mice. Thus, BALB/c mice were co-immunized with 240-252 and 258-277 peptides to test this possibility. Table 2 shows that co-immunization did not result in a detectable 240-252 specific proliferative response.

Induction of antibody responses to the 240-252 peptide in non-responder BALB/c and SWR/J mice after co-immunization with the immunogenic 288-302 peptide

The observation that non-responsiveness to the 240-252 peptide could be overcome by co-immunization with an immunogenic peptide was further tested by using peptide 288-302 as a co-immunogen. This peptide behaves as a Th epitope in various mouse strains including BALB/c and SWR/J.[8,14] Following the same co-immunization protocol as above, mice of the H-2^d and H-2^q haplotype were shown to produce anti-240-252 antibody responses (Fig. 2). Furthermore, these responses were comparable to the response induced by the chimeric construct 288-P-240 in SWR/J mice but in BALB/c mice, responses were significantly lower. Both antigens must be injected in the same site since intraperitoneal injection of the immunogenic 288-302 peptide followed by a subcutaneous immunization of 240-252 peptide did not result in an anti-240-252 response in either of the strains of mice tested (Fig. 2).

Induction of antibody responses to the 188-199 peptide after co-immunization with the immunogenic 49-72 peptide

To test further the validity of the observation that B- and T-cell epitopes do not need to be linked in order to develop a T-cell-dependent anti-peptide response *in vivo*, BALB/c and SWR/J mice were immunized with a mixture containing the non-immunogenic B-cell epitope 188-199 and the immunogenic 49-72 peptides from the measles virus H glycoprotein. As shown in Fig. 3 both strains of mice developed a primary anti-188-199

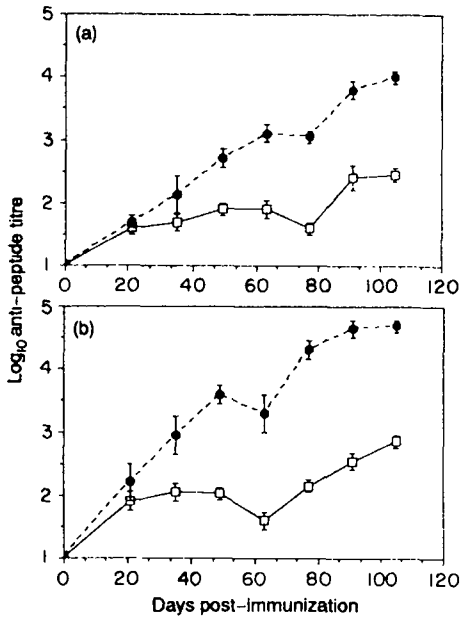

Figure 3. Kinetics of the antibody response to the 188-199 peptide in BALB/c (a) and SWR/J (b) mice after immunization with the 188-199 peptide alone (□) and after co-immunization with the 49-72 peptide (●). Results represent mean ± SD of ELISA titres of antibody in sequential serum samples from groups of four mice.

response which was greatly enhanced after a secondary immunization.

DISCUSSION

The results reported in this study demonstrate that covalent linkage of B and Th epitopes is not a necessary requirement for the generation of a T-cell-dependent antibody response *in vivo* to non-immunogenic synthetic peptides representing B-cell antigenic sites. That a Th-cell epitope such as 288-302 or 258-277 is needed to overcome non-responsiveness to the B-cell epitope 240-252, is shown by the failure of H-2^d and H-2^q mice to develop an antibody response unless a Th-cell epitope is provided, following either covalent linkage or co-immunization. The results presented here confirm early observations by Good *et al.*[12] that co-immunization of a non-immunogenic peptide from the circumsporozoite protein of malaria with KLH (a large molecule providing T-helper epitopes), overrides the genetically controlled unresponsiveness to the peptide. However, this system suffers from the following limitations: (1) the unsuitability of KLH for human use; (2) the protein carrier may induce epitopic suppression of the antibody response,[17] and (3) this strategy will not prime the host for a T-cell memory response to the pathogen. Thus, our findings extend these early observations by overcoming the requirement of a carrier by the use of a synthetic peptide representing a Th-cell epitope from the pathogen concerned. These results are consistent with those from a recent preliminary study in which the induction of anti-hapten antibodies did not require covalent linkage between the hapten and the peptide behaving as Th-cell determinant.[18]

A possible explanation for these findings is that the peptides cross-link via free sulphydryl (SH) groups on each peptide. However, since the T-cell epitope peptides 288-302 and 258-277 do not have free SH groups this explanation is not appropriate.

The inability of the 240-252 peptide to induce antibody responses in BALB/c and SWR/J mice may be due to defects in processing and presentation of the peptide by B cells or failure to make functional complexes with MHC molecules. The former possibility is unlikely since anti-240-252 antibody responses have been induced in both strains of mice after immunization with the chimeric construct 288-P-240. The 240-252 peptide is unable to induce proliferative responses in the above strains[13] which suggests that it cannot form functional complexes with MHC molecules. The observation that the Th-cell determinants (288-302 or 258-277) can provide help when they are co-immunized with the peptide 240-252 at the same site suggests that the local environment where the immunogens are administered is crucial for the 'communication' between B and Th cells. On the basis of our current understanding for the generation of help for a B-cell response it appears that during co-immunization, clones of B cells will recognize the immunogenic peptides (e.g. 258-277) and subsequently process and present them in conjunction with class II molecules to Th cells. Subsequent T-cell activation will provide the necessary help to the 258-277 B-cell clones for antibody production through the classical pathway of cognate help which requires the interaction of the T-cell receptor with the MHC-peptide complex on 258-277 specific B cells. In the subsequent effector phase of help, the interaction of activated Th cells and B cells is not MHC restricted and soluble factors such as interleukin-2 (IL-2), IL-4, IL-5, interferon-γ (IFN-γ) will provide the necessary signals not only to the 258-277 specific B cells but also to B-cell clones which have bound the 240-252 peptide via their surface immunoglobulin. This would thus result in anti-240-252 antibody production (bystander help). The concept of bystander help as a possible mechanism for overcoming non-responsiveness is further supported by the finding that co-immunization of the 258-277 peptide with the 240-252 peptide does not result in a detectable proliferative response to the 240-252 peptide. In addition, since aqueous mixtures of the peptides were co-emulsified in CFA, individual droplets may well have co-entrapped both peptides which may well have facilitated their uptake by the same B cells.

MHC restriction is a major concern for the prospects of the development of a synthetic peptide vaccine because peptides may only be recognized by a limited number of individuals in the genetically heterogeneous human population. Recently, a number of peptides with the ability to bind to multiple MHC alleles have been described both in mouse and human studies.[19-22] These findings have led to the construction of chimeric synthetic vaccines by pairing well-defined Th determinants with B-cell antigenic sites by polymerization,[23] covalent linkage[4] or co-linear synthesis.[5,6,8] However, it was subsequently found that chemical linkage has the disadvantage of the uncontrolled nature of the reaction and the risk of affecting the antigenicity of the peptides.[24] Moreover, orientation of the epitopes has been shown to have a profound effect on antibody specificity and antibody affinity,[25-28] suggesting that the stoichiometry and the amino acid composition of the epitopes determines the way chimeric peptides are processed and presented. The finding described in this report that genetically controlled unresponsiveness to synthetic peptides can be overcome by co-immunization

of peptides representing B- and T-cell epitopes rather than by covalent linkage of the epitopes, provides a new approach to the use of synthetic peptides as potential vaccines.

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