The effects of a flanking sequence on the immune response to a B and a T cell epitope from the fusion protein of measles virus

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A region of the fusion protein of measles virus (residues 240 to 252) was predicted to contain a B and a T epitope. A synthetic peptide representing this sequence was shown to induce both T and B cell reactivity in several inbred strains of mice, but the responses were clearly major histocompatibility complex-restricted. Elongation of this peptide by six residues at the C terminus on the basis of predictions for B cell epitopes resulted not only in increased peptide immunogenicity in some strains of mice but also produced strain-related positive and negative effects on the recognition of the peptide. BALB/c and SWR mice were non-responders to the short version of the peptide but responded well to the elongated form. On the other hand, the injection of the elongated peptide into C57BL/6 mice resulted in a loss of both B and T cell responsiveness seen with the short version. These results indicate the importance of flanking sequences on the immunogenicity and antigenicity of synthetic B and T cell epitopes and highlight the necessity to determine the most appropriate size of peptide to be used as an immunogen.

Introduction

Measles virus is a member of the morbillivirus subgroup of the paramyxovirus family (Kingsbury et al., 1978). The surface localization of the haemagglutinin (H) and fusion (F) glycoproteins is important in determining their role in mediating viral–cell membrane interactions during infection. The H protein is responsible for the first step in infection, the adsorption of the virus to receptors on the host cell, whereas the F glycoprotein is actively involved in the induction of membrane fusion (Choppin et al., 1981). Monoclonal antibodies against both H and F glycoproteins have been shown to neutralize the virus in vitro and to protect animals from infection when administered passively (Drillien et al., 1988; Malvoisin & Wild, 1990). Furthermore, the importance of antibodies to the F protein is illustrated by the observation that the immunopathological response of atypical measles is associated with the absence of antibodies to the F protein following immunization with formalin-inactivated virus (Mertz et al., 1980; Norrby et al., 1975).

Although the widespread use of attenuated measles vaccine has been successful in developed countries, the virus still remains one of the main causes of infant mortality in many parts of the world. Thus the development of a synthetic peptide vaccine based on the critical epitopes from the structural proteins of the virus appears to be a valid strategy for the control of measles. The recent availability of the complete amino acid sequences of these proteins has made it possible for this approach to be directly tested (Alkhatib & Briedis, 1986; Richardson et al., 1986).

Over the last few years much effort has been focused on the development of synthetic peptide vaccines against various pathogens. This work has led to a better understanding of the molecular events involved in antigen recognition by immunocompetent cells and has allowed the mapping of the critical antigenic sites from proteins of various pathogens which are involved in immunity (Milich, 1989). However, one problem with such vaccines is the genetic restriction to the antigen which results in the failure to generate T cell help for antibody production. Thus the identification of T cell determinants which are associated with protective immunity and can interact with a wide range of major histocompatibility complex (MHC) molecules is important in the design of synthetic peptide vaccines.

There appears to be a minimal length requirement for T helper (Th) cell determinants which can be defined in two ways: the shortest sequence which gives maximum responses (11 to 12 residues) or the sequence which generates any response (seven to eight residues). However, a recent analysis of peptides eluted from MHC class
II molecules has shown that naturally processed peptide fragments are 13 to 17 amino acids long (Rudensky et al., 1991). Furthermore, it appears that a central core within the sequence is necessary for binding to the MHC, whereas the flanking residues mainly make important contacts with the MHC protein or contribute to the ability of the peptide to fold into an appropriate conformation for optimal binding. Although substantial information has been accumulated characterizing the optimal peptide length necessary for Th cell activation, little is known about the effect of flanking sequences on antigen recognition. Vacchio et al. (1989) have shown the negative effects exerted by a flanking sequence on the recognition of an immunodominant Th cell determinant from staphylococcal nuclease by I-Ek-restricted T cell clones. Effects of peptide segments outside the minimal antigenic site of lysozyme and myoglobin have also been suggested (Gammon et al., 1987; Brett et al., 1988).

In this paper we describe studies on the immune responses to a synthetic peptide from the F protein of measles virus (residues 240 to 252) which is predicted to contain B and T cell antigenic sites, and the effect of elongation of the peptide at the C terminus upon these responses. Elongation of the peptide by six residues exerts both positive and negative effects on humoral and cellular responses. In addition, elongation is able to overcome non-responsiveness to the F1 : 240–252(C) peptide in BALB/c and SWR mice. However, elongation of the peptide prevented both humoral and cellular responses of C57BL/6 mice to the F1 : 240–252(C) peptide. Both peptides induced antibodies which reacted with the F protein and measles virus. These data thus demonstrate the successful prediction of B and T cell antigenic sites on the F protein of measles virus and illustrate the importance of flanking sequences on the development of immune responses. Such observations will be of importance in the design of synthetic vaccines.

**Methods**

**Selection of peptides.** The selection of peptide F1 : 240–252 (G-D-I-N-K-V-L-E-K-L-G-Y-S) as a potential Th sequence was based on the presence of a predictive motif of a charged residue followed by two hydrophobic residues and terminating with a polar amino acid (Rothbard & Taylor, 1988). Using the computer algorithm AMPHI (Margalit et al., 1987), this sequence was predicted to fold as an amphipathic α-helix.

**Extension of peptide F1 : 240–252 by six residues at the C terminus (G-G-D-L-L-G) was based on the presence of residues of moderate hydrophilicity (Kyte & Doolittle, 1982), flexibility (Karpplus & Schultz, 1985) and protrusion index (Thornton et al., 1986) along with the presence of a β-turn (Wilmot & Thornton, 1988). A cysteine residue at the carboxyl end of each peptide was introduced to increase immunogenicity (Francis et al., 1987).

**Peptide synthesis.** Peptides representing residues 240 to 252 and 240 to 258 were synthesized by manual solid-phase synthesis using Fmoc chemistry. Fmoc-protected amino acids were converted to the hydroxynbenzotriazole-activated esters by treatment with hydroxylbenzotriazole 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 diethyl ether, purified by preparative HPLC and their purity was assessed by analytical HPLC and amino acid analysis.

**Mice.** The following inbred strains of mice were purchased from the National Institute for Medical Research, Mill Hill, U.K.: A/J (H-2B), BALB/c (H-2d), CBA (H-2k), C57BL/6 (H-2b), SWR (H-2a) and SJL (H-2d). To mice (H-2d) were bred in the animal unit of the London School of Hygiene and Tropical Medicine, U.K.

**Immunization.** Antibody responses to F1 : 240–252(C) and F1 : 240-258(C) peptides were studied following intraperitoneal injection of female mice of various haplotypes (6 to 8 weeks old) with 100 μg of peptide emulsified in Freund’s complete adjuvant (FCA) (1:1). All animals were boosted by the same route and with the same dose of peptide in Freund’s incomplete adjuvant (FIA) 3 weeks later. Serum samples were collected 2 weeks after the boost. For lymphocyte stimulation assays groups of three to four mice were immunized via the hind footpads with 100 μg of each peptide emulsified in FCA.

**Antibody assays.** Anti-peptide antibody was assessed by solid-phase immunoassay employing microtitre plates coated with the peptide; bound antibody was detected by the addition of peroxidase-conjugated rabbit anti-mouse immunoglobulin antibody. Titres are expressed as log10 dilution of the serum sample giving an A492 corresponding to that given by a 1:10 dilution of serum from non-immunized mice of the appropriate strain. Anti-F antibody was assessed using microtitre plates coated with recombinant F protein (a generous gift from Dr U. Leibert, Würzburg, Germany).

**Anti-measles virus antibody was also assessed by a solid-phase assay.** Microtitre plates were coated with 3 × 10⁴ Vero cells/well and half of the wells were infected with measles virus (Schwartz strain) at an m.o.i. of 0.01 whereas the remaining wells contained medium alone (non-infected cells). Antisera were titrated in doubling dilutions and bound antibody was detected by the addition of peroxidase-conjugated rabbit anti-mouse immunoglobulin. Titres were expressed as log10 dilution of samples giving an A492 corresponding to that given by a 1:100 or 1:200 dilution of the same serum binding to non-infected cells.

**Lymphocyte stimulation assay.** Eight to 10 days after immunization, the draining lymph nodes (LNs) from groups of three to four mice were removed aseptically, pooled and the mononuclear cells collected by centrifugation. Viable, unfractionated LN cells (4 × 10⁶) in 0.2 ml RPMI 1640, 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 a humidified 5% CO₂ atmosphere at 37 °C for 5 days. The cells were pulse-labelled with 1 μCi [³H]thymidine in 10 μl volumes/culture to 18 h before harvesting. Thymidine incorporation was assessed by liquid scintillation spectrometry and the results were expressed as the mean c.p.m. from triplicate cultures corrected for background (A c.p.m.). For the comparison of antigenic potency of the synthetic analogues the ratio of the areas under the curves was calculated as the ratio sum of c.p.m. at all antigen concentrations where both analogues were tested.
Results

MHC-restricted proliferative responses to F₁:240–252(C) in inbred strains of mice

Mice of six inbred strains were primed with 100 μg of the F₁:240–252(C) peptide to determine whether their genetic background would influence the responsiveness of their lymphocytes upon in vitro stimulation with the homologous peptide. The LN cells of SJL, CBA, A/J and C57BL/6, but not those of BALB/c and SWR mice, showed a clear dose-dependent proliferative response upon in vitro stimulation with the peptide (Fig. 1). As a control for possible mitogenic effects of the peptide, it was shown that the peptide did not stimulate an in vitro proliferative response in LN cells from the responding strains of mice which had been injected with only PBS in FCA.

Positive and negative effects of a flanking sequence on the recognition of F₁:240–252(C) by lymphocytes

To determine the ability of F₁:240–258(C) to induce proliferative responses, mice of different haplotypes were primed with 100 μg of the peptide in FCA. As shown in Fig. 2, when CBA, A/J and TO mice were immunized with F₁:240–258(C), lymphocytes from the draining LNs gave a vigorous proliferative response when restimulated in vitro with the homologous peptide. In addition, elongation of the peptide resulted in a twofold increase of antigenic potency when compared to the responses induced by the F₁:240–252(C) peptide. Immunization of C57BL/6 mice with F₁:240–258(C) resulted in absence of response to the homologous peptide or F₁:240–252(C) (Fig. 3 a). Furthermore, when C57BL/6 mice were immunized with F₁:240–252(C), no response was observed following in vitro stimulation of lymphocytes with F₁:240–258(C) in contrast to the good proliferative responses induced by the F₁:240–252(C) peptide (Fig. 3 b).

Reactivity of peptide-primed lymphocytes with measles virus

Although peptides F₁:240–252(C) and F₁:240–258(C) were immunogenic in several strains of mice, only in H-2d mice could both peptides induce responses to measles virus (Table 1). Neither was antigenic in vitro for lymphocytes primed with recombinant F protein in vivo (data not shown).

Positive and negative effects of a flanking sequence on the antibody response to the F₁:240–252(C)

Since peptide F₁:240–252(C) has been shown to behave as a T cell determinant in certain strains of mice, and

Fig. 1. Proliferative responses of LN cells to F₁:240–252(C) peptide in inbred strains of mice. Mice of various haplotypes (a C57BL/6, b CBA, c SJL, d A/J, e SWR, f BALB/c) were injected in the footpad with either 100 μg of F₁:240–252(C) peptide (■) or PBS (*) emulsified in FCA. Draining LN cells were harvested 8 to 10 days later and cultured with different doses of the homologous peptide in vitro for 5 days. Proliferation was assessed by incorporation of [³H]Tdr into DNA and expressed as c.p.m. corrected for background (Ac.p.m.).
that the carboxyl flanking sequence 253 to 258 exerted both positive and negative effects on the induction of proliferative responses, the ability of the peptides to induce antibody responses in six strains of mice was studied. Mice were immunized with 100 μg of each peptide in FCA and boosted 3 weeks later with a similar dose in FIA. Serum samples were collected 2 weeks after the boost and analyzed for the presence of anti-peptide antibodies by indirect ELISA using the corresponding peptide coated on the plate.

As shown in Fig. 4, A/J, TO, CBA and C57BL/6 mice mounted an anti-F₁:240–252(C) response after two injections of the immunogen, with C57BL/6 mice responding significantly better (P < 0.001) than did the other strains. No response was observed in BALB/c and SWR mice. When peptide F₁:240–258(C) was used as an immunogen, the response in A/J, TO and CBA mice was significantly higher (P<0.001) compared to anti-F₁:240–252(C) peptide response, whereas C57BL/6 mice failed to respond (Fig. 4). In addition, both BALB/c and SWR mice responded well to F₁:240–258(C).

### Induction of proliferative cell responses to F₁:240–258(C) in non-responder mice by high dose immunization

Since peptide F₁:240–258(C) was shown to induce anti-peptide antibodies in BALB/c and SWR mice, the ability of this peptide to induce proliferative responses in the above strains was investigated. As shown in Fig. 5(a) and 6(a), lymphocytes from both BALB/c and SWR mice primed with 100 μg of F₁:240–258(C) peptide did not proliferate in the presence of the homologous peptide in vitro. Studies by Lehmann et al. (1989) have indicated that MHC molecules may bind peptides in quantities sufficient to cause competition, although these quantities may remain at the threshold of detectability of the
MV: flanking sequences and immunogenicity

1991

Fig. 4. Antibody responses to the F1:240–252(C) (■) and F1:240–258(C) (■■) peptides in strains of mice with different H-2 haplotypes. Mice were immunized intraperitoneally with 100 μg of peptide emulsified 1:1 in FCA and boosted in the same manner 3 weeks later with peptide in FIA. Two weeks later serum was taken and the presence of antibodies was determined by ELISA using the homologous peptide as the solid-phase test antigen. Mean ± s.d. of antibody titres are represented.

Fig. 5. Proliferative responses of LN cells to F1:240–258(C) peptide in BALB/c mice. Mice were injected in the footpad with either (a) 100 μg or (b) 500 μg of F1:240–258(C) peptide emulsified in FCA. Draining LN cells were harvested 8 to 10 days later and cultured with different doses of the F1:240–258(C) (■■) or F1:240–252(C) (■■) peptides in vitro for 5 days. Proliferation was assessed by incorporation of [3H]TdR into DNA and expressed as c.p.m. corrected for background (Ac.p.m.).

Fig. 6. Proliferative responses of LN cells to F1:240–258(C) peptide in SWR mice. Mice were injected in the footpad with either (a) 100 μg or (b) 500 μg of F1:240–258(C) peptide emulsified in FCA. Draining LN cells were harvested 8 to 10 days later and cultured with different doses of the F1:240–258(C) (■■) or F1:240–252(C) (■■) peptides in vitro for 5 days. Proliferation was assessed by incorporation of [3H]TdR into DNA and expressed as c.p.m. corrected for background (Ac.p.m.).

assays. Thus, the differences in responsiveness to F1:240–258(C) peptide between the responder strains (A/J, CBA and TO) and the non-responder strains (SWR and BALB/c) might be quantitative and so the non-responsiveness in BALB/c and SWR mice may well be overcome by increasing the immunizing dose of the peptide. Accordingly, mice were immunized with a higher dose of the peptide and the data given in Fig. 5(b) and 6(b) demonstrate that this is indeed the case. Lymphocytes from draining LN cells of BALB/c and SWR mice primed with 500 μg of peptide proliferated after restimulation in vitro with the homologous peptide in a dose-dependent fashion. Thus, for priming lymphocytes the dose required is fivefold higher than that used in A/J, TO and CBA mice. Furthermore, cross-reactivity with the F1:240–252(C) peptide was observed in both strains of mice. However, even when primed with the higher dose of the extended peptide, C57BL/6 mice remained non-responders to both F1:240–252(C) and F1:240–258(C) peptides (data not shown).

Anti-virus and anti-F protein antibody responses induced by the synthetic peptides

Immunization of various strains of mice with either the F1:240–252(C) or the F1:240–258(C) peptides resulted in
the induction of anti-peptide antibodies capable of recognizing the measles virus and F protein on ELISA plates. Antibodies raised against the elongated peptide \(F_1:240-258(C)\) reacted better with the F protein (Fig. 7) and measles virus (Fig. 8) than did \(F_1:240-252(C)\) antibodies in strains of mice where both peptides were immunogenic. Although peptide \(F_1:240-258(C)\) was found to be immunogenic in SWR mice, the antibodies failed to react with the virus or the F protein. In addition, none of the anti-peptide antibodies mentioned above neutralized or inhibited the fusion activity of measles virus in vitro.

**Discussion**

The results presented in this study demonstrate that in vitro T cell responsiveness to peptide \(F_1:240-252(C)\) which was predicted to form an amphipathic \(\alpha\)-helix and to contain a motif which correlates with T cell antigenicity was MHC-restricted. This genetic restriction imposed by molecules encoded by the I region of the MHC suggests that only those peptides capable of binding to class II molecules would subsequently activate T cells (Buus et al., 1987).

Elongation of the peptide sequence 240 to 252 by six residues at the C-terminus on the basis of predictions for B cell epitopes resulted in both positive and negative effects on the recognition of \(F_1:240-252(C)\) peptide depending on the haplotype of the mouse strain used. The positive effect of increasing length on peptide antigenicity observed in H-2\(^s\), H-2\(^k\) and H-2\(^a\) haplotypes is now well documented (Livingstone & Fathman, 1987) and the idea that the addition of residues outside the epitope increases antigenic potency by stabilizing appropriate secondary structure is clearly a plausible explanation. However, the possibility exists that these residues are not essential for stimulation but they may contribute directly to the affinity of the complex of T cell receptor–antigen–MHC by contacting the T cell receptor and/or the restricting class II molecule (Livingstone & Fathman, 1987). The surprising inhibitory effect exerted by the flanking sequence 253 to 258 on the recognition of the \(F_1:240-252(C)\) peptide in H-2\(^b\) mice suggests that it is unable to bind the I-A\(^b\) class II molecule and is subsequently proteolytically degraded. This possibility is supported by the finding that a 34 amino acid hen egg-white lysozyme peptide once bound to a class II molecule becomes inaccessible to proteolytic degradation (Donneymeyer & Allen, 1989). However, binding of the \(F_1:240-252(C)\) peptide to the I-A\(^b\) molecule may occur and the presence of the flanking sequence 253 to 258 beyond the binding site might sterically prevent the T cell receptor from interacting with the class II/antigen
The results presented here show for the first time both peptide with no stimulatory ability (Vacchio et al., 1989). The results presented here show for the first time both positive and negative effects of a single flanking sequence on the recognition of a peptide by Th cells which suggest that strain differences in antigen presentation may influence the immunogenicity of an epitope. The selective unresponsiveness observed in C57BL/6 mice might also arise from its ability to bind MHC molecules in different conformations (Bhayani & Paterson, 1989).

When the ability of these peptides to induce responses to measles virus was tested by lymphocyte proliferation, both peptides were found to induce responses cross-reactive with the virus only in mice possessing the H-2^d haplotype. This suggests that either the processing of the virus yields peptides larger than the minimal epitope and the presence of the flanking sequence might affect the conformation of the epitope required for T cell recognition (Brett et al., 1988) or the binding of the peptides by the I-A^k class II molecules is significantly better than that in other strains. However, other factors intrinsic or extrinsic to the antigen affecting immunodominance might account for this observation (Berzofsky et al., 1987).

The positive and negative effects that the flanking sequence 253 to 258 exerted on the proliferative responses to the F_1:240-252(C) peptide correlated with the antibody responses in mice of different MHC haplotypes. Peptide F_1:240-252(C) was immunogenic in certain strains of mice, and this ability was dependent on the restriction element capable of recognizing the Th cell determinant(s) present on this peptide. However, the immunogenicity of this peptide was increased by elongating the sequence by six residues at the C terminus. Two strains of mice which were non-responders to the F_1:240-252(C) peptide responded to the elongated peptide. Thus the addition of flanking sequence (253 to 258) not only resulted in an increase in the immunogenicity of the F_1:240-252(C) peptide but also overcame non-responsiveness in two strains of mice. Elongation of peptide length was also shown to have negative effects on the induction of antibody responses in C57BL/6 mice.

Hyperimmunization of BALB/c and SWR mice with the peptide F_1:240-258(C) resulted in a proliferative response of lymphocytes which was low and sensitive to changes in antigen concentration. However, following hyperimmunization H-2^b mice remained non-responders to F_1:240-258(C). Similar observations made by Lehmann et al. (1989) have shown that peptide HEL 46-61 which does not bind to the I-A^d molecule and is not immunogenic in H-2^d mice can in fact induce a proliferative response in H-2^b mice after hyperimmunization. Collectively these data suggest that the low efficiency for antigen presentation of F_1:240-258(C) observed in SWR and BALB/c mice might be influenced by the affinity of MHC molecule for the antigenic and competitor (self) peptide. Therefore, for BALB/c and SWR mice hyperimmunization results in a sufficient concentration of the antigenic peptide to compete effectively for presentation.

Both peptides were able to induce antibodies that reacted with the fusion protein and the virus but there were strain differences. Thus in SWR mice, although peptide F_1:240-258(C) was immunogenic, the antibodies failed to react with the virus or the F protein, perhaps owing to the induction of low affinity antibodies by this strain of mice (Petty et al., 1982). These findings suggest that this region represents a B cell epitope exposed on the native measles virus F protein.

The results presented here suggest that although prediction methods are useful tools in identifying potential antigenic epitopes, considerable attention should be given to determine the most appropriate size of peptide to be used as an immunogen. Recent reports of the successful elution and characterization of peptides from MHC molecules (Rudensky et al., 1991; Falk et al., 1991; Jardetzky et al., 1991) suggest that such an approach might be the most appropriate for defining the optimum size of the epitopes.

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References


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