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Protection against Measles Virus-Induced Encephalitis by Anti-mimotope Antibodies: The Role of Antibody Affinity

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Synthetic peptides mimicking a conformational B-cell epitope (M2) of the measles virus fusion protein (MVF) were used for the immunization of BALB/c mice and the anti-peptide and anti-virus antibody titers induced were compared. Of the panel of tested peptides, a chimeric peptide consisting of two copies of a T-helper epitope (residues 288–302 of MVF) and one copy of the mimotope M2 (TTM2) and a multiple antigen peptide with eight copies of M2 (MAP-M2) induced the highest titers of anti-M2 and anti-MV antibodies. Furthermore, peptides TTM2 and MAP-M2 induced antibodies with highest affinity for the mimotope and highest avidity for measles virus. Immunization with the MAP-M2 construct induced high titers of high-affinity anti-M2 antibody despite the absence of a T-helper epitope, and lymphocyte proliferation data suggest that the addition of M2 to the MAP resulted in the generation of a structure capable of stimulating T-cell help. Sera with anti-M2 reactivity were pooled according to affinity values for binding to M2, and high- and low-affinity pools were tested for their ability to prevent MV-induced encephalitis in a mouse model. The high-affinity serum pool conferred protection in 100% of mice, whereas the lower affinity pool conferred protection to only 50% of animals. These results indicate the potential of mimotopes for use as synthetic peptide immunogens and highlight the importance of designing vaccines to induce antibodies of high affinity.
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INTRODUCTION

The successful identification of T- and B-cell epitopes from viral proteins has contributed to the concept of the synthesis of chimeric peptide immunogens composed of different combinations of both types of epitopes to induce T-helper cell responses and antibody production. Recent experiments have shown that the amino acid composition, the number and the orientation of the epitopes, as well as the use of an adjuvant and the route of vaccine delivery may influence the level and affinity of the antibodies elicited (Partidos *et al.,* 1992a, b; Obeid *et al.,* 1996). This suggests that different ways of antigen processing and presentation may result in the production of immunochemically distinct antibodies, and the appropriate selection of epitopes to stimulate antibody and T-cell responses is fundamental to the design of effective peptide vaccines. The inclusion of promiscuous T-cell epitopes in such a vaccine will ensure the stimulation of responses in individuals of different MHC haplotypes (Lairmore *et al.,* 1995). A number of such promiscuous T-cell epitopes have been identified; these include those from tetanus toxoid (residues 830–843 and 947–967) (Ho *et al.,* 1990) and the measles virus F protein (residues 288–302) (Partidos and Steward, 1990). Immunization with chimeric peptides made up of the latter peptide coupled to a linear B-cell epitope (residues 404 to 414 of measles virus fusion (MVF) protein) (peptides TB and TTB) have been shown to induce anti-peptide- and anti-virus-specific antibodies. Additionally, the TTB peptide induced protective immune responses against *in vivo* challenge with MV and canine distemper virus (Obeid *et al.,* 1995).

It is very likely that a significant proportion of antibody responses to viral proteins and glycoproteins are directed toward conformational rather than linear B-cell epitopes and combinatorial peptide libraries have been used to generate peptide mimics (mimotopes) of these epitopes, several of which have been identified and characterized. These include those mimicking two different epitopes from the human hepatitis B virus envelope protein (HBsAg) (Folgoriet *et al.,* 1994), an epitope from the repeated region of *Plasmodium falciparum* circumsporozoite surface protein (CSP) (Stoute *et al.,* 1995), an epitope from the F protein of respiratory syncytial virus (Chargelegue *et al.,* 1998), and an epitope from the MVF protein (Steward *et al.,* 1995). The latter (mimotope M2) was selected from a solid-phase 8-mer random combinatorial peptide library by screening with a monoclonal antibody to the F protein of MV. Immunization with M2

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induced antibodies that cross-reacted with MV as well as conferring protection against fatal encephalitis induced by challenge with neuroadapted MV. Immunization with mimotopes synthesized as multiple antigenic peptides (MAP) (Tam and Lu, 1989; Dedoort *et al.,* 1992; Chai *et al.,* 1992) has been shown to induce neutralizing anti-RSV antibodies able to reduce virus load and mononuclear cell infiltration in the lungs of RSV-challenged mice (Chargelegue *et al.,* 1998).

It is generally accepted that the level of antibodies, their subclasses, and avidity to infectious agents are important factors in preventing disease (Steward *et al.,* 1991; Chargelegue *et al.,* 1995); however, the precise immunochemical characteristics of biologically effective antibodies are not known. High-affinity antibodies are biologically more effective than low-affinity antibodies but conversely, their production may be associated with progressive tissue damage in the course of autoimmune disease (Steward and Chargelegue, 1997). The inability to produce a high-affinity antibody response may also be associated with an excessive production of low-affinity antibodies and this would represent a form of immunodefficiency leading to susceptibility to chronic immune complex disease (Steward, 1979). High-affinity binding of membrane-bound immunoglobulin to antigen can generate or inhibit the expression of certain epitopes during antigen processing (Watts and Lanzavecchia, 1993) since the processing would affect the complex rather than the antigen itself. In this manner the affinity of antibodies may influence which T-cell epitope will dominate and may change the focus of the response, possibly by revealing cryptic epitopes (Watts *et al.,* 1989).

In the work described in this paper, the immunogenicity of different synthetic peptide constructs containing the sequence of mimotope M2 was compared and it was shown that the affinity of the antibodies induced plays an important role in protection against MV-induced encephalitis.

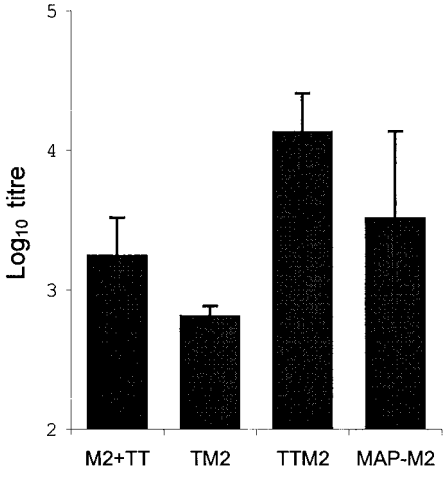


FIG. 1. Anti-peptide ELISA antibody titers in BALB/c mice immunized with different peptides containing mimotope M2. Results are expressed as the reciprocal of the $\log_{10}$ dilution of serum giving an absorbance of 0.2 at 492 nm. Values represent means and standard deviations for groups of four mice bled 2 weeks after booster immunization.

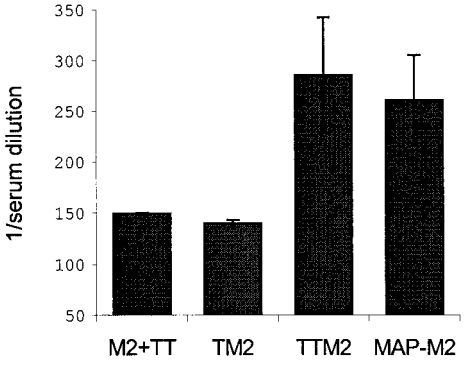


FIG. 2. Anti-MV ELISA antibody titers in BALB/c mice immunized with different peptides containing mimotope M2. Results are expressed as the reciprocal of the serum dilution giving an absorbance of 0.2 at 492 nm. Values represent means and standard deviations for groups of four mice bled 2 weeks after booster immunization.

RESULTS

Comparison of immunogenicity of synthetic peptides containing mimotope M2

Serum samples were obtained weekly and were analyzed to assess the comparative immunogenicity of the peptides. Data presented in Fig. 1 were obtained from enzyme-linked immunosorbent assay (ELISA) analysis of serum samples taken 2 weeks after the booster immunization from mice immunized ip with TM2, TTM2, or MAP-M2 or coimmunized with M2 plus TT. The results show the presence of antibodies to M2 in all groups of mice. However, higher titers were obtained in sera from animals immunized with the chimeric peptide consisting of two copies of the T-helper epitope (TTM2) or with MAP-M2, when compared with groups immunized with peptide TM2 or coimmunized with M2 plus TT. Similarly, peptides TTM2 and MAP-M2 generated higher levels of antibodies reactive with MV than peptides M2 + TT or TM2 ($P < 0.01$) (Fig. 2).

The affinity of the anti-peptide antibodies in these sera for M2 was determined and the highest affinity values were detected in sera from mice injected with MAP-M2 ($P < 0.001$) (Fig. 3). An ELISA-based dissociation assay was performed with MV on the solid phase to estimate avidity indices of the antibodies for MV. Avidity indices of the antibodies for MV following immunization with the chimera TTM2 and with MAP-M2 (Fig. 4) were significantly higher than those of antibodies induced following immunization with M2 plus TT or with TM2 ($P < 0.001$).

In vitro proliferative responses of splenocytes primed with MAP-M2

Lymphocyte proliferation assays were performed with splenocytes obtained from mice immunized intranasally

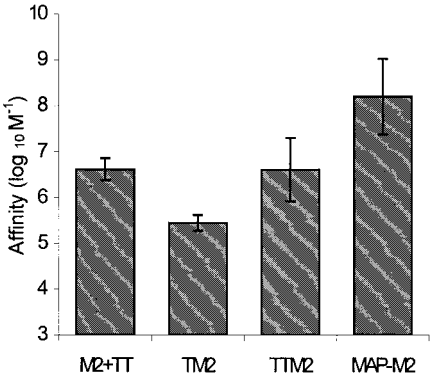


FIG. 3. The affinity of antibodies for the mimotope M2 induced by immunization with the indicated peptide. Results are expressed as means and standard deviations ($\log_{10} M^{-1}$) for groups of four mice bled 2 weeks after booster immunization.

with either MAP-M2 with mucosal adjuvants or MAP-M2 in saline. The results obtained show that upon *in vitro* restimulation with MAP-M2, splenocytes from mice immunized with MAP-M2 with adjuvants proliferated in a dose-dependent fashion (Fig. 5a). Proliferative responses of the T-cell-enriched population of cells to MAP-M2 were similar to those of unfractionated splenocytes. However, no responses were observed when cells were restimulated with free M2 peptide or with the unrelated control peptide MAP-B. No responses were detected in the group of mice immunized intranasally with MAP-M2 in saline (Fig. 5b).

The effect of passive transfer of high-affinity and low-affinity antibodies on encephalitis induced by intracranial challenge with neuroadapted MV

For these experiments, the affinity for the mimotope M2 was assessed for every serum sample obtained at different time points postimmunization with the different immunogens containing M2. Sera containing either high-

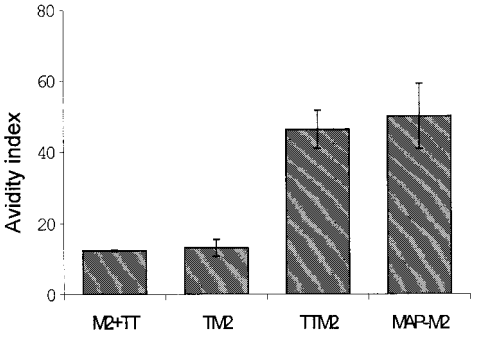


FIG. 4. Avidity for MV measured by the DEA dissociation method. To assess the correlation of the results obtained using this method with results from another technique, 13 samples of serum were tested by both inhibition ELISA and DEA dissociation. Statistical analysis revealed that both methods rank sera similarly with a correlation coefficient of 0.71. Results are presented as avidity indices.

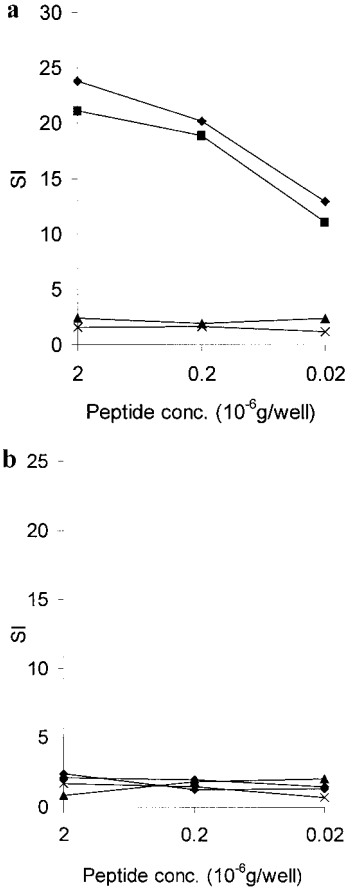


FIG. 5. Lymphoproliferative responses of splenocytes from mice immunized intranasally with MAP-M2 + adjuvant (a) or MAP-M2 + saline (b). Splenocytes were restimulated *in vitro* with MAP-M2 (◆), M2 (▲), or MAP-B (×) or T-cell-enriched splenocytes were restimulated with MAP-M2 (■). Data are presented as means of three SIs. Maximal SI with Con A was 5.96 for the population of splenocytes and 5.05 for T-cell-enriched splenocytes.

or low-affinity anti-M2 antibodies were pooled to give two serum pools. The affinities of antibodies in these pools for M2 were determined as 3.36×10^7 M^{-1} (high-affinity pool) and 1.2×10^3 M^{-1} (low-affinity pool). Since the anti-M2 antibody titers differed in these pools, they were appropriately diluted to give the same OD values in ELISA prior to passive transfer to mice (Table 1). Neither pool had demonstrable *in vitro* measles neutralizing activity nor were there significant differences in immunoglobulin classes and subclasses between the pools (data not shown). Following intracranial challenge with neuroadapted MV, animals were monitored daily for signs of encephalitis. At the end of the experiment (day 31 postchallenge), all mice in the group that had been passively immunized with the high-affinity antibody were still alive (100% survival, $P = 0.0001$, compared to mice receiving normal serum). Four of eight animals receiving the low-affinity antibody survived (50% survival, $P = 0.08$, compared to animals receiving normal mouse serum) (Fig. 6). The difference in survival between mice

TABLE 1

Characterization of High- (H-A) and Low- (L-A) Affinity Serum Pools Used for the Passive Transfer Experiment

Serum	Anti-M2 antibody titer ($\log_{10}$)	Anti-MV antibody titer ($\log_{10}$)	Affinity for M2 (M^{-1})	Avidity for MV	*In vitro* neutralization
H-A diluted	2.3	1.04	3.36×10^7	47.93	−ve
L-A	2.3	1.1	1.2×10^3	31.77	−ve

receiving high-affinity and low-affinity antibodies was significant ($P = 0.02$).

DISCUSSION

The work described in this paper is part of a program of research to investigate the potential of synthetic peptides as vaccines. In this context, the appropriate presentation of synthetic B- and T-cell epitopes to immune cells is critical for the induction of antibodies of high affinity, titer, and immunological memory. Earlier studies have shown that effective anti-peptide and anti-virus antibodies could be induced in mice following immunization with chimeric peptides consisting of either one or two copies of a promiscuous T-helper epitope (T) from the F protein of MV covalently coupled to a single copy of a linear B-cell epitope representing residues 404–414 (B) from the F protein (TB or TTB, respectively). The TTB chimera was more immunogenic and induced antibodies of higher affinity than did the TB peptide. However, the TTB peptide did not induce antibodies with demonstrable MV-neutralizing activity. It is important to note that immunization with peptides corresponding to T or TT alone did not induce antibodies that reacted with the virus and had no influence on the response to virus challenge (Obeid *et al.,* 1995).

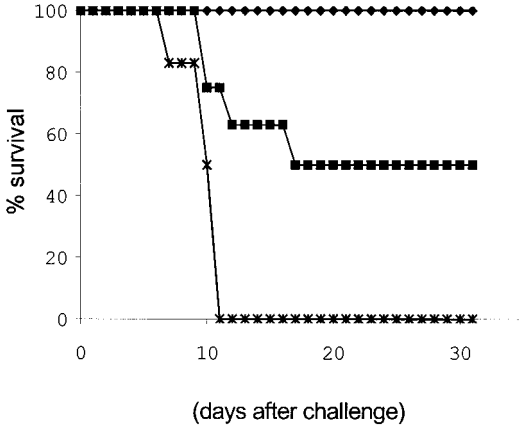


FIG. 6. Effect of passive immunization of BALB/c mice with high-affinity and low-affinity antibodies on the response to challenge with the neuroadapted strain of MV. Groups of eight 12-day-old mice received 200 μl of high-affinity (◆) ($\log_{10}$ antibody titer, 2.3), low-affinity (■) ($\log_{10}$ antibody titer, 2.3) or normal mouse serum (*) intraperitoneally and were challenged 24 h later with MV. Values represent the percentage survival in the three groups of mice over 31 days after challenge.

In the present studies, we have used peptides containing a mimotope (M2) of a conformational B-cell epitope, which does not induce antibodies in the absence of T-cell help (Steward *et al.,* 1995). A peptide containing two copies of the T epitope coupled to the mimotope M2 (TTM2) was more effective than a peptide consisting of only one copy of the T epitope plus M2 (TM2) in terms of immunogenicity and the affinity of the anti-M2 antibodies induced. It therefore appears that the mimotope is best presented in the form of TTM2 and it is possible that the number of copies of a helper T-cell epitope in a peptide construct influences B-cell activation, perhaps by increasing the production of cytokines. This increased "help" may result in increased levels and affinity of antibody. It is also possible that the length of the construct containing two T-helper epitopes creates a conformation that is more efficiently processed and presented than that formed by the shorter peptide (M2 coimmunized with TT or TM2). However, MAP-M2 does not include a T-helper epitope in its structure and despite this, T-help appears to have been generated. The construct is shown here to be immunogenic and able to induce both anti-peptide and anti-MV reactivity. When the affinities of the anti-peptide antibodies for M2 generated by the different constructs were compared, MAP-M2 was shown to induce significantly higher affinity antibodies than did the chimeric peptides (TM2 and TTM2) (Fig. 3, $P < 0.001$). Thus, it is possible that the addition of lysine residues from the backbone to the sequence of M2 could create a conformation that may stimulate helper T-cells or that more than one M2 sequence is involved in forming a T-helper epitopic structure. In experiments to investigate this phenomenon, we have shown that the MAP-M2 peptide induced *in vitro* proliferation of splenocytes from mice primed with MAP-M2 and adjuvant whereas M2 and the unrelated peptide MAP-B did not induce proliferation (Fig. 5a) and proliferative responses were not observed with splenocytes from mice immunized with MAP-M2 in saline (Fig. 5b). These results suggest that it is the combination of the M2 amino acid sequence with the MAP backbone that creates the epitopic structure capable of providing T-cell help. This phenomenon seems to be unique to the present system, since in related work, we have demonstrated that mimo-

topes derived from the F protein of respiratory syncytial virus presented as MAP constructs in fact required T-helper coimmunization to induce anti-mimotope antibodies with anti-virus reactivity (Chargelegue *et al.,* 1998). While we have not formally compared the antibody responses to the linear 404–414 epitope and the mimotope in this study, antisera to the two epitopes have similar anti-MV antibody titers and avidity and are predominantly of the IgG2a subclass, and some anti-M2 antisera have MV neutralizing activity (data not shown). Anti-404–414 sera are not MV neutralizing (Steward *et al.,* 1995).

There is a considerable body of literature concerning the significance of antibody affinity and avidity in determining the efficacy of antibodies in *in vitro* reactions and in disease associations *in vivo.* However, there is little information on the importance of these parameters of antibodies specific for infectious agents in protection *in vivo* (Steward and Chargelegue, 1997). In the experiments described here, the role of antibody affinity and avidity in the protective efficiency of antibodies specific for MV was formally tested. BALB/c mice were passively immunized with similar amounts of either high- or low-affinity MV-specific anti-mimotope antibody prior to intracranial challenge with neuroadapted MV. The anti-mimotope antibodies specifically reacted with, but did not neutralize, MV *in vitro.* All mice receiving the high-affinity antibodies were protected against MV challenge, whereas 50% of mice receiving the low-affinity antibodies were protected. None of the control mice that received normal serum survived the challenge. Although the difference between groups receiving high- and low-affinity anti-M2 antisera was statistically significant ($P = 0.02$, Fisher's exact), half of the animals survived, even though they received low-affinity serum. However, since differences in avidity indices of the anti-peptide antibodies for MV were not as marked as the differences in affinity for M2 (Table 1), it is possible that the avidity for MV in the lower affinity serum pool was close to, or just above, the protection threshold (Bachmann *et al.,* 1997), although the affinity values for the mimotope were very significantly different. These results clearly indicate the importance of affinity and avidity in antibody-mediated protection against MV encephalitis.

It is interesting to note that neither of the serum pools used neutralized MV *in vitro* although they both reacted well with the virus in an ELISA-type assay.

Even though virus neutralization is clearly an important property of anti-virus antibodies, it is apparent from a number of studies that *in vitro* virus neutralization does not always correlate with protective efficacy *in vivo* (Lefrancois, 1984; Schmalijohn *et al.,* 1982). The present results are consistent with the observation that antibody-mediated protection against vesicular stomatitis virus was independent of neutralization, immunoglobulin subclass, and antibody avidity and, above a minimum avidity threshold, was dependent upon a minimum antibody concentration (Bachman *et al.,* 1997).

In conclusion, the data presented here suggest that mimotopes may have potential as components of synthetic peptide vaccines. They are not naturally occurring sequences and, providing their sequences are checked against the known sequences of human proteins, the risk of inducing autoimmunity would be minimal. The data presented here highlight the importance of antibody concentration and avidity in antibody-mediated protection and indicate the necessity for new vaccines to induce antibodies of appropriate avidity. In the case of synthetic peptide vaccines, indications are emerging as to how to design the vaccine to induce a high-affinity and -avidity antibody response. It is likely, however, that vaccines for individual infectious agents will require their own particular synthetic approach.

MATERIALS AND METHODS

Selection and synthesis of peptides

Conventional solid-phase peptide synthesis using Fmoc chemistry was used to synthesize the mimotope M2 and the chimeric peptides TT, TM2, and TTM2 where T represents residues 288–302 of the fusion protein (F) of MV (Partidos and Steward, 1990), a promiscuous T-helper epitope able to provide help for B-cells. Chimeric peptides with one or two copies of the T-cell epitope were colinearly synthesized at the amino-terminus of one copy of the M2 epitope with two glycine residues as a spacer. MAP-M2 was synthesized using commercially available resin with a polylysine backbone (Nova Biochem) to which eight M2 sequences were added using Fmoc chemistry. MAP-B, where B represents a linear B-cell epitope (residues 404–414) of the F protein of MV (Partidos *et al.,* 1992a), was synthesized similarly. The purity of the peptides was assessed by high-pressure liquid chromatography and mass spectrometry. The amino acid sequences of the peptides used are given in Table 2.

Mice

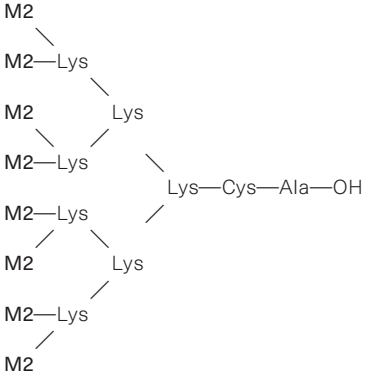
Inbred female BALB/c (H-2^d) mice were purchased from the National Institute of Medical Research (Mill Hill, UK) and maintained at the London School of Hygiene and Tropical Medicine (London, UK).

Measles virus

The Edmondston strain of MV was used for the determination of anti-measles antibody titers and antibody avidity to the virus in ELISA assays. Rodent-neuroadapted MV was originally provided by Dr U. G. Liebert (Würzburg, Germany). The virus was injected intracranially into suckling mice and brain homogenates were used as the source of virus for challenge studies.

TABLE 2

Notation and Sequences of Peptides Used in the Study

Peptides	Sequences
T	LSEIKGVIVHRLEGV
M2	NIIRTKKQ
TMS	T - Gly - Gly -M2
TTM2	TT - Gly - Gly -M2
MAP-M2	

Immunization of mice

Five- to eight-week-old mice were immunized intraperitoneally with 100 μg of peptide TM2, TTM2, or MAP-M2 or coimmunized with M2 plus TT in Freund's complete adjuvant. After 2 weeks the mice were boosted with the same dose of peptide in Freund's incomplete adjuvant. Mice were bled weekly and sera were stored at −20°C.

For intranasal immunization, mice (four per group) were given 50 μg of MAP-M2 together with 10 μg of each of the mucosal adjuvants LTR72 (mutant of heat-labile toxin of *Escherichia coli,* provided by R. Rappuoli, Siena, Italy) and CpG repeats with nucleotide sequence TCCATGACGTTCCTGACGTT (synthesized by Pharmacia Biotech). Animals were immunized on days 0, 7, 14, and 28 with a total volume of 30 μl per mouse. A further group of mice was similarly immunized with 50-μg doses of MAP-M2 in saline.

Antibody titers: ELISA

Anti-peptide and anti-MV antibody titers were assessed by a solid-phase ELISA on microtiter plates (Nunc, Roskilde, Denmark). Plates were coated overnight at 4°C with 50 μl of a 5 μg/ml solution of M2 per well or with 50 μl of a 5 μg/ml of purified MV in 0.1 M carbonate–bicarbonate buffer (pH 9.6) per well. The plates were blocked with 1% BSA in PBS (pH 7.3). Serial twofold dilutions of sera in phosphate-buffered saline–0.05% Tween 20–1% BSA (final volume 50 μl) were added to the plates, which were incubated at 37°C for 1 h and then washed. Fifty microliters of a 1:2000 dilution of peroxidase-conjugated rabbit anti-mouse IgG (heavy and light chains) (Nordic, The Netherlands) was added to each well and the plates were incubated for 1 h at 37°C. Unbound conjugate was removed by washing and 50 μl of 0.04% *o*-phenylenediamine–hydrogen peroxidase in citrate/phosphate buffer was added to detect bound enzyme. The reaction was stopped after 10 min by the addition of 25 μl of 2 M sulfuric acid per well and absorbance (A_{492}) was determined in an automatic plate reader (Dynex MRX). Titers of anti-peptide and anti-virus antibodies are expressed as $\log_{10}$ of the reciprocal of the serum dilution, giving an absorbance of 0.2.

Antibody affinity: Inhibition ELISA

The affinity of anti-peptide antibodies for M2 was assessed by a solid-phase enzyme inhibition assay (Rath *et al.,* 1988). ELISA plates were coated with M2 at 5 μg/ml and doubling dilutions of sera from mice immunized with chimeric peptides were added. The assay was continued, as described above, to assess the dilution of antibody giving an A_{492} of 0.6, which was then used in the second stage of the assay. Serial dilutions of a 3 mM solution of M2 were used to inhibit the binding of homologous antibody to the solid-phase antigen. The relative affinity of antibody was calculated as the reciprocal of the concentration of peptide giving 50% inhibition ($I_{0.5}$) of the binding in the absence of the peptide. These values represent an estimation of "average" antibody affinity.

Antibody avidity: ELISA-based dissociation assay

The method is based on the fact that chaotropes, such as diethylamine (DEA), break noncovalent bonds between antigen and antibody (Steward and Chargelegue, 1997). Low-avidity antibodies can be easily dissociated from the antigen in the presence of DEA, while higher avidity antibodies are more difficult to dissociate. Several dilutions of DEA were tested to determine the optimal concentration giving significant antibody dissociation but not affecting the virus on a plate and 20 mM was chosen. Four replicates of each working dilution of the sera were incubated on microtiter plates coated with 5 μg/ml of MV as described above. After six washes, each duplicate received either 100 μl of PBS or 100 μl of 20 mM DEA in PBS, respectively. The samples were incubated for 15 min at room temperature and washed and the assay was performed as described above. The avidity index (AI) was calculated as follows:

$$\text{AI} = \frac{\text{mean of } A_{492} \text{ treated with DEA}}{\text{mean of } A_{492} \text{ treated with PBS}} \times 100.$$

To maximize the sensitivity of the assay, sera working dilutions were chosen to give 50% of the maximum OD of the sample treated with PBS.

Protection study

The affinity, avidity, and titers of antibodies for M2 from different bleeds from mice immunized with the different immunogens were assessed and samples of low affinity (L-A; affinity values less than 10^4 M^{-1}) and high affinity (H-A; affinity values over 10^6 M^{-1}) were pooled separately. The titer and affinity of anti-M2 antibodies in the two pools were determined and, because the titers in the two pools differed, the H-A serum pool was diluted to give the same OD values as the L-A serum pool in ELISA. Two hundred microliters of either the L-A or the H-A serum was injected ip into 12-day-old suckling mice. Normal mouse serum was used in a control group. Twenty-four hours later, mice were intracranially challenged with 10^4 PFU of neuroadapted MV and animals were monitored daily for 31 days for the development of clinical signs.

Neutralization assay

The assay was performed as described (Giraudon and Wild, 1985). Briefly, serial serum dilutions were incubated at 37°C in a CO_2 incubator with 50 PFU of MV per well in 199 Earl's medium supplemented with 5% FCS, 0.2% penicillin/streptomycin, and 5% HEPES. A total of 5×10^4 Vero cells per well were added after 90 min and further incubated for 3 h at 37°C in 5% CO_2. Appropriate cell and virus controls were included. The medium was discarded and cells were overlaid with 199 Earl's medium reconstituted in carboxymethyl-cellulose solution supplemented with FCS and penicillin/streptomycin. Incubation was carried for 4–6 days for the development of cytopathic effect. After the cells were fixed with methanol and stained with crystal violet, the dilution of serum giving a 50% reduction in plaques was determined. Sera failing to reduce plaque numbers by 50% were classified as nonneutralizing.

Lymphocyte proliferation assay

Spleens from MAP-2 immunized mice were removed aseptically 2 weeks after the last immunization, transferred to transport medium, teased, and passed through a sieve. Cells were spun at 1200 rpm for 5 min, the supernatant was discarded, and red blood cells were lysed using lysing buffer (0.14 M ammonium chloride, 20 mM Tris, pH 7.5). B lymphocytes were removed by nylon-wool chromatography (Julius *et al.,* 1973; Croll *et al.,* 1986). Eluted cells are referred to as the T-cell-enriched population. After three washes in RPMI 1640, cells were resuspended in complete medium (2% FCS, 0.2% penicillin/streptomycin, 1% glutamine, 1% HEPES buffer in RPMI 1640). Three 10-fold dilutions of peptides MAP-M2, M2, and MAP-B and concanavalin A were dispensed into wells of 96 round-bottomed tissue culture plates. Negative controls were included. All tests were performed in triplicate. A total of 2×10^5 cells were added to each well and plates were incubated for 3 days at 37°C in 5% CO_2. Cells were pulsed on the fourth day with tritiated thymidine (1 μCi/well) and harvested 18 h later on glass microfiber papers. Thymidine incorporation was assessed by liquid scintillation spectrometry. Results are expressed as stimulation indices of the mean counts per minute (cpm) from triplicate cultures in the presence of antigen divided by mean cpm of triplicate cultures with medium only. Values equal to or higher than 2 were considered positive.

Statistical analysis

Antibody titers and values of affinity and avidity were analyzed by a Tukey–Kramer Multiple Comparisons Test. Fisher's exact two-tailed test was used for the comparison of survival curves in the protection study.

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