JOURNAL OF VIROLOGY, Aug. 1988, p. 3032–3036
0022-538X/88/083032-05$02.00/0

Vol. 62, No. 8

Vaccination against Lethal Coronavirus-Induced Encephalitis with a Synthetic Decapeptide Homologous to a Domain in the Predicted Peplomer Stalk

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Received 28 December 1987/Accepted 11 April 1988

A surface probability method was used to select a decapeptide homologous to residues 993 to 1002 of the peplomer protein E2 of murine hepatitis virus strain JHM, a neurotropic coronavirus. This sequence of amino acids corresponded to a minor peak on a hydrophilicity plot. Immunization of mice with the chemically synthesized peptide coupled to keyhole limpet hemocyanin elicited high levels of neutralizing antibody and protected against lethal virus challenge. Protection correlated with a critical level of antipeptide antibody, which could be reached after a single inoculation. These results suggest that an appropriate antibody response to a highly restricted, surface-exposed domain of this viral protein is critical in determining the outcome of infection of the central nervous system. This sequence is located in the C-terminal fifth of the E2 peplomers, between two predicted coiled-coil structures.

Chemically synthesized oligopeptides have become increasingly popular tools for molecular characterization of the interactions between various proteins and the immune system. They allow the fine mapping, on proteins of infectious agents, of areas that are critical for induction of a protective immune response. This technique has led to the concept of synthetic immunogens for vaccination against infectious diseases (1, 38, 40). The feasibility of synthetic viral vaccines is suggested by the successful vaccination of guinea pigs (3) and more recently cattle (14) against foot-and-mouth disease, of mice against herpes simplex virus (16, 46) or influenza virus (33, 37), and of chimpanzees against hepatitis B virus (19). Finally, synthetic peptides could provide safe and accessible tools for the serodiagnosis of infections (7, 21; M. Lacroix, M. Stern, F. Bellini, and G. Dionne, 1st Can. AIDS Res. Conf. 1988) or the production of custom-made antibody reagents for the detection of pathogens (6).

Potential immunogenic peptides are often selected from the amino acid sequence of the viral protein, which can be deduced from the DNA sequence of the cloned gene. With the assumption that immunogenic domains are exposed on the virion's surface, the protein sequence is usually analyzed for regions of hydrophilicity (23, 27) and bends in the protein structure (8). However, these predictive algorithms are not always successful. Recently, Emini et al. (17) and Boger et al. (J. Boger, E. A. Emini, and J. A. Schmidt, 6th Int. Cong. Immunol. 1986, abstr. no. 2.75.3) described the use of a potentially more reliable technique based on the probability that an amino acid will be exposed on the outer surface. The algorithm uses indices of fractional surface exposure probability which were determined from a group of proteins of known three-dimensional structure, with a surface residue defined as one with >2.0 nm^2 of water-accessible surface (24).

The JHM strain of murine hepatitis virus (MHV-JHM) is a neurotropic member of the *Coronaviridae*, a family of enveloped viruses that contain single-stranded RNA of positive polarity. The agent is involved in encephalitis and demyelination in mice and rats (39, 47). This experimental model for virus-induced disease of the central nervous system may have direct relevance in the search for potential viral agents of neurological disorders such as multiple sclerosis (MS) (25). Human coronavirus infections are ubiquitous (29), and direct involvement in MS has been suggested by reports of intrathecal synthesis of anticoronavirus antibodies (35), isolation of coronaviruses (5), or the presence of coronalike virus particles (45) in MS patients. Three major structural polypeptides compose the MHV-JHM virion (39, 47). Much attention has recently been focused on the external spike glycoprotein E2, which appears to be a critical determinant of virus biology and neurovirulence (4, 9, 10, 18, 20, 42, 43, 48). Monoclonal antibodies have allowed the delineation on E2 of at least three antigenic sites involved in virus neutralization (42, 48), although precise physical mapping has not been reported. A first step in that direction has been rendered possible by the recent cloning and sequencing of the E2 gene (36). In the present study, we have used a synthetic peptide selected from the predicted amino acid sequence of the E2 polypeptide to locate a critical determinant of viral pathogenesis.

MHV-JHM was obtained from the American Type Culture Collection (Rockville, Md.), plaque-purified twice, and passaged four times at a multiplicity of infection of 0.01 on DBT cells (11) prior to use as inoculum. Female, 6- to 7-week-old, MHV-seronegative BALB/c mice (Charles River, St-Constant, Québec, Canada) were used in all experiments, including the in vivo titration of the viral stock by intracerebral inoculation. One 50% lethal dose (LD_{50}) corresponded to 0.4 PFU (34).

Selection and synthesis of the peptide. From the predicted amino acid sequence of the E2 polypeptide of MHV-JHM (36), the surface probability predictive algorithm (17, 24; Boger et al., abstr.) was used, and aside from the area around the predicted cleavage site (36), the highest peak on the surface probability profile corresponded to residue 997, a relatively minor peak on a hydrophilicity profile (Fig. 1). A decapeptide homologous to residues 993 to 1002 was

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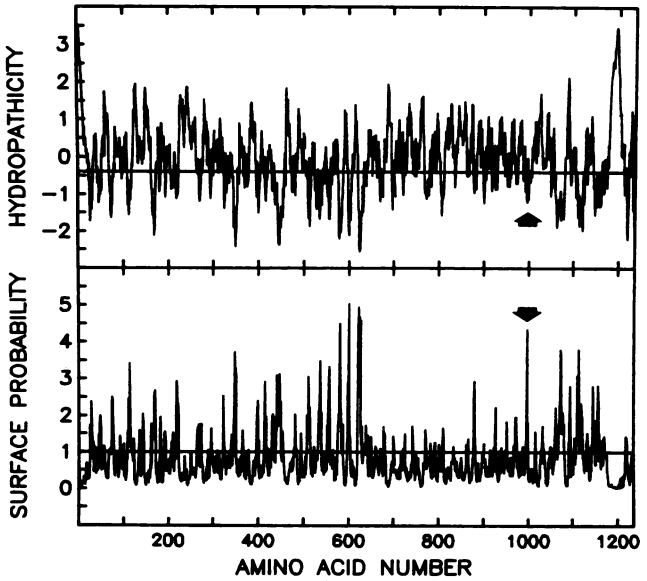

FIG. 1. Hydropathicity (top) and surface probability (bottom) profiles calculated from the predicted amino acid sequence of the E2 protein of MHV-JHM. A window of nine residues was used for hydropathicity, with hydrophilic peaks extending downwards from the average hydropathicity of a random sequence (27). Surface probabilities were calculated as described in the text, with the value obtained from a random sequence plotted as a solid horizontal line. Arrows point at the center of the synthetic peptide used in the present study.

chemically synthesized, which had the following sequence: H_2N–Cys-993–Val–Lys–Ser–Gln–Thr–Thr–Arg–Ile–Asn-1002–COOH. The peptide was synthesized on a *p*-methylbenz-hydrylamine resin (IAF BioChem International Inc., Laval, Québec, Canada). Each amino acid was coupled by the dicyclohexylcarbodiimide–1-hydroxybenzotriazole method. Completeness of coupling was monitored by the ninhydrin test (26). The α-amino group of each amino acid was protected with 9-fluorenylmethoxycarbonyl, and the side-chain-protecting groups were as follows: *tert*-butyl for serine and threonine, *tert*-butyloxycarbonyl for lysine, tosyl for arginine, and *p*-methoxybenzyl for cysteine. The 9-fluorenylmethoxycarbonyl group was cleaved between couplings with 20% piperidine in dimethylformamide. A terminal ^{14}C-labeled glycine residue was added on the N-terminus to assess the efficiency of coupling to carriers. Cleavage of the peptide from the resin and removal of the *tert*-butyl- and *tert*-butyloxycarbonyl-protecting groups were achieved by treatment with 55% trifluoroacetic acid in methylene chloride in the presence of 5% anisole. The remaining protecting groups were cleaved by HF treatment for 60 min at 0°C in the presence of 5% anisole and 5% ethanedithiol as scavengers. The peptide was purified to 95% homogeneity by reverse-phase high-pressure liquid chromatography on a Vydac C_{18} column (2.2 by 25 cm). Amino acid analysis, after total hydrolysis in 6 N HCl (containing 0.05% phenol) at 110°C for 48 h, yielded the expected amino acid composition. The peptide was coupled to keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) through the cysteine residue, with sulfosuccinimidyl 4-(*p*-maleimidophenyl) butyrate (Sulfo-SMPB; Pierce Chemical Co., Rockford, Ill.) as the coupling reagent (28). The conjugated peptide was dialyzed against Dulbecco phosphate-buffered saline (PBS), pH 7.0, and coupling efficiency was determined by measuring the remaining radioactivity. The average peptide/carrier ratio of the conjugates was 36 for KLH and 21 for BSA. All conjugates were stored in portions at −20°C.

TABLE 1. Vaccination of mice against coronavirus encephalitis[a]

Virus challenge (LD_{50})	No. of survivors/no. immunized (% surviving)[b]		
	Peptide-KLH	KLH control	PBS control
10	19/25 (76)**	6/17 (35)*	0/7 (0)
100	7/16 (44)**	1/16 (6)*	0/8 (0)

[a] Groups of mice were primed intraperitoneally with the synthetic peptide (50 μg) coupled to KLH and emulsified in complete Freund adjuvant. Control groups received either an equivalent amount of KLH or PBS, both in the same adjuvant. A booster inoculation was given 21 days later in incomplete Freund adjuvant. All animals received an intracerebral challenge 7 days later with either 10 or 100 LD_{50} of MHV-JHM.

[b] Statistical significance (Fisher exact test): *, not significant versus PBS; **, $P < 0.05$ versus KLH or PBS.

Vaccination experiments. Mice were inoculated twice with 36 nmol (50 μg) each time of the chemically synthesized peptide, coupled to KLH and emulsified in complete (day 0) or incomplete (day 21) Freund adjuvant. Animals in control groups received either an identical amount of unconjugated KLH or PBS in adjuvant. A large proportion of animals that were vaccinated with the peptide were protected against lethal challenge with as much as 100 LD_{50} of MHV-JHM (Table 1). Nonprotected mice died between 5 and 10 days after virus challenge, with histopathological evidence of meningoencephalomyelitis in animals sacrificed from parallel groups. Protection required Freund adjuvant and was not observed with lower peptide doses of 1 or 10 μg. A partial but statistically nonsignificant protective effect was observed in the group of animals inoculated with KLH and challenged with 10 LD_{50} of virus. Unlike the apparent nonspecific partial protection against influenza virus (37), complete Freund adjuvant did not contribute to the protection observed in our study, since all animals that had received PBS in this adjuvant died after virus challenge (Table 1). Protection levels conferred by peptide immunization were reduced following challenge with a 10-fold-higher virus dose, and the nonspecific KLH-induced partial protection was no longer apparent in these conditions (Table 1). The level of peptide-induced immunity could not protect mice inoculated with higher virus doses of 10^3 or 10^4 LD_{50}. On the other hand, a single peptide inoculation conferred protection from a 10-LD_{50} viral challenge given 21 days later to a similar extent as two peptide immunizations (data not shown). Finally, free peptide (50 μg) did not alter virulence when mixed with the 10-LD_{50} virus inoculum, which suggests that competition for viral receptors on brain cells cannot account for the protective effect of this peptide. Protection apparently correlated with greatly reduced viral replication in the brain, as indicated by a 46-fold decrease (70 versus 3,200 PFU/g) in infectious viral titer recovered 7 days after challenge from the brains of survivors. (Brain tissue was homogenized on ice to 10% [wet weight] per volume in PBS, using a Polytron homogenizer [Brinkman Instruments, Rexdale, Ontario, Canada].) Protected animals have now survived for at least 7 months, although histopathological examination of some mice 7 days after viral challenge did show subclinical signs of encephalitis, as observed after passive antibody protection (4) or inoculation with virus mutants (10, 18) or recombinants (30).

Antibody response. Recent studies on various proteins have indicated that antigenic sites involved in B-cell immune

response are distinct from those implicated in T-cell immunity (see, for example, reference 31). Indeed, antigenic sites recognized by T cells appear to have different structural requirements (13) that do not correspond to the features of the peptide selected for the present study. Moreover, previous work on the neurotropic coronavirus MHV-JHM has indicated that an appropriate antibody response to restricted domains of the surface-exposed viral protein E2 is critical in determining the outcome of infection (4, 44). Thus, we wanted to determine whether the observed protection conferred by the synthetic peptide correlated with the presence of neutralizing antibody. Indeed, high levels of such antibodies, especially when measured by plaque assay, were observed before virus challenge in peptide-vaccinated animals, unlike the background levels observed in KLH-immunized mice. In this experiment for induction of neutralizing antibody after immunization with synthetic peptide, groups of 12 mice were inoculated with peptide-KLH or KLH in adjuvant at days 0 and 21 and bled from the retroorbital plexus, with heparinized capillary tubes, on day 27. Pooled plasma samples were analyzed for their ability to neutralize either 50 PFU or 100 50% tissue culture-infective doses of MHV-JHM in either a plaque assay (11) or by cytopathic effect (34), respectively. For immunizations with peptide-KLH, the neutralizing titers were 2,048 in the plaque assay and 316 in the cytopathic effect assay; for KLH immunizations, the values were 32 and 24, respectively. (Values are reciprocals of the plasma dilution required to neutralize 50% of the virus on monolayers of DBT cells.) Finally, the synthetic peptide, coupled to a different carrier to prevent detection of antibodies to KLH, was used as the antigen in a solid-phase enzyme immunoassay for quantitation of peptide-specific antibodies in mice subjected to various immunization protocols. The assay was performed as described previously (42), except that antigen consisted of 4 μg per well of peptide coupled to BSA, or an equivalent amount of BSA. The peptide-specific endpoint titer was determined as the reciprocal of the dilution of plasma, expressed on a log 2 scale, which gave an optical density of 0.32, a value determined as 3 standard deviations above the mean optical density obtained with BSA as the antigen (n = 320). As shown in Fig. 2, protection correlated with the presence of a critical level of antibody prior to virus challenge. This level of antibody was estimated to correspond to an endpoint titer of ≥ 16 $\log_2$ (1:65,000), as evaluated from the data presented in Fig. 2 and two additional experiments in which titers were evaluated in individual mice (data not shown).

Since the E2 polypeptide of MHV-JHM has been predicted to contain 1,235 amino acid residues (36), the decapeptide used in the present study represents only 0.8% of this sequence. Thus, our results directly confirm the importance of a highly restricted domain on the E2 protein for the induction of a protective immune response against this neurotropic agent. This conclusion had previously been reached indirectly from passive monoclonal antibody protection studies (4, 20, 48; P.J.T., unpublished data) and work with virus mutants resistant to neutralization by monoclonal antibody (10, 18). A recent study has made use of MHV recombinants with cross-overs within the E2 gene to localize neuropathogenic determinants at the C-terminal one-third of the protein (30), which is consistent with the localization of the critical decapeptide described in the present study. It would be interesting to determine whether this decapeptide represents at least part of an epitope recognized by the previously described monoclonal antibodies and whether it is conserved among various MHV strains. In this respect,

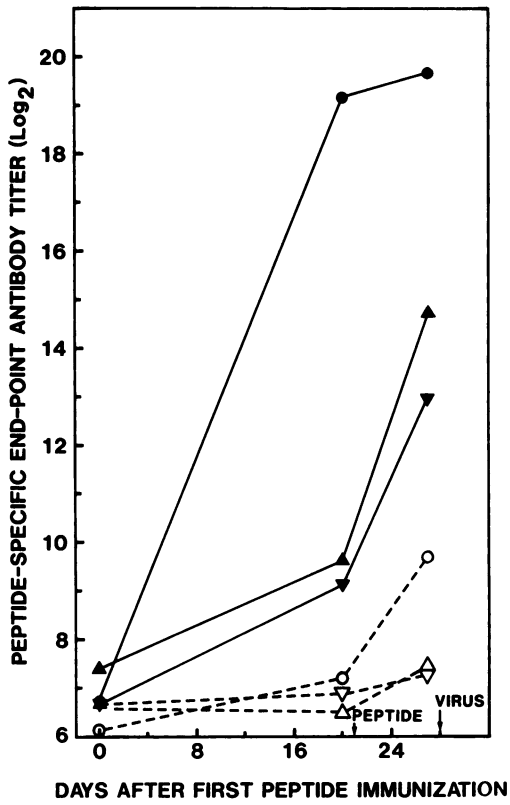

FIG. 2. Antibody response of mice to the synthetic peptide. Groups of 6 to 12 mice were inoculated intraperitoneally with the synthetic peptide coupled to KLH (solid symbols) or an equivalent amount of unmodified KLH (open symbols) at days 0 and 21. Intracerebral challenge with 10 LD_{50} of MHV-JHM was done on day 28. Symbols: ●,▼, 50 or 10 μg (amount of peptide only), respectively, of peptide-KLH in complete (day 0) or incomplete (day 21) Freund adjuvant; ▲, 50 μg of peptide-KLH without adjuvant at days 0 and 21; ○,▽, equivalent doses of unmodified KLH with adjuvant or (△) without adjuvant at days 0 and 21. Mice were bled on days 0, 20, and 27. Peptide-specific antibody titers were determined by an enzyme immunoassay with pooled plasma samples from each group. Only mice that received two injections of 50 μg of peptide, coupled to KLH and in adjuvant (●), were protected from a viral challenge given on day 28.

part of the predicted amino acid sequence of the E2 from the A59 strain of MHV has been published and compared with E2 sequences from feline and avian coronaviruses (12). From this comparison, two conserved heptad repeat patterns indicative of coiled-coil structures were identified in the C-terminal portion of E2, which was predicted to form an elongated stalk between a globular N-terminal portion and the viral envelope. Alignment of this partial MHV-A59 E2 sequence (12) with that of MHV-JHM (36) identified the minor repeat near the membrane as residues 1120 to 1179 and the major repeat as residues 859 to 967. Thus, the decapeptide comprising residues 993 to 1002 is homologous to a region between these two predicted coiled-coil structures in the E2 peplomer stalk. The presence of neutralization epitopes in the stalk region of coronavirus peplomers is in contrast with the localization of the four antigenic sites of the influenza virus hemagglutinin in the globular region (49). Presumably, the C-terminal portion of the peplomer becomes exposed after proteolytic cleavage into two subunits of similar size and activation of the fusion activity (41), as

suggested previously (30). Moreover, a conformational change in acidic endosomes, analogous to that seen with influenza virus (2), could also expose the C-terminal portion, which appears to contain fusion determinants (30). Presumably, appropriate antibody could prevent this conformational change, as suggested for West Nile virus (22).

We have not yet determined the importance of the carrier-hapten effect (32) in protection, since the preservation of peptide immunogenicity in the absence of carrier has been reported to require a structural modification of the peptide, such as cyclization (15), addition of a hydrophobic tail for insertion into liposomes (46), or the linkage of two epitopes with a diproline spacer (14).

Finally, the fact that this peptide, selected by a surface probability strategy, corresponded to a minor peak on hydrophilicity plots emphasizes the usefulness of this recently introduced predictive algorithm. The study of coronavirus immune determinants and the use of synthetic peptides may ultimately be of relevance in the prevention and diagnosis of some human neurological disorders.

We thank Suzanne Lemieux, Christopher Richardson, Michael Stern, and Michel Trudel for critically reviewing the manuscript; Francine Lambert and Francine Allard for excellent technical assistance; Jean-Paul Descôteaux for histopathological examinations; Jean Pellerin for computer expertise; Mary Désy for statistical analysis; and Lucie Summerside for typing the manuscript.

This work was supported by a grant from the Medical Research Council of Canada (MA-9203). P. J. Talbot is the recipient of a university research scholarship from the National Sciences and Engineering Research Council of Canada.

ADDENDUM

The complete sequence of the E2 polypeptide of MHV-A59 shows conservation of the decapeptide used in the present study (W. Luytjes, L. S. Sturman, P. J. Bredenbeek, J. Charite, B. A. M. van der Zeijst, M. C. Horzinek, and W. J. M. Spaan, Virology **161**:479–487, 1987).

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