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Protective immunity against murine hepatitis virus (MHV) induced by intranasal or subcutaneous administration of hybrids of tobacco mosaic virus that carries an MHV epitope

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ABSTRACT **Hybrids of tobacco mosaic virus (TMV) were constructed with the use of fusion to the coat protein peptides of 10 or 15 amino acids, containing the 5B19 epitope from the spike protein of murine hepatitis virus (MHV) and giving rise to TMV-5B19 and TMV-5B19L, respectively. The TMV hybrids were propagated in tobacco plants, and the virus particles were purified. Immunogold labeling, with the use of the monoclonal MAb5B19 antibody, showed specific decoration of hybrid TMV particles, confirming the expression and display of the MHV epitope on the surface of the TMV. Mice were immunized with purified hybrid viruses after several regimens of immunization. Mice that received TMV-5B19L intranasally developed serum IgG and IgA specific for the 5B19 epitope and for the TMV coat protein. Hybrid TMV-5B19, administered by subcutaneous injections, elicited high titers of serum IgG that was specific for the 5B19 epitope and for coat protein, but IgA that was specific against 5B19 was not observed. Mice that were immunized with hybrid virus by subcutaneous or intranasal routes of administration survived challenge with a lethal dose ($10 \times LD_{50}$) of MHV strain JHM, whereas mice administered wild-type TMV died 10 d post challenge. Furthermore, there was a positive correlation between the dose of administered immunogen and protection against MHV infection. These studies show that TMV can be an effective vaccine delivery vehicle for parenteral and mucosal immunization and for protection from challenge with viral infection.**

Several plant viruses have been developed for use as vectors for the expression and delivery of foreign peptides (1–7). These examples include immunogenic epitopes that can be used in vaccines to confer protective immunity against human and animal diseases. We have demonstrated that a hybrid tobacco mosaic virus (TMV), containing the 13 amino acid sequence of the murine zona pellucida ZP3 epitope fused to a region near the C terminus of the coat protein (CP) successfully elicited the production of anti-ZP3 antibody in parenterally immunized animals. Anti-ZP3 antibody accumulated around the ova of immunized animals; however, it did not prevent fertilization (5).

Murine hepatitis virus (MHV), a member of the *Coronaviridae* family, is responsible for a variety of acute and chronic diseases in its natural host. The strain JHM of MHV induces demyelinating encephalomyelitis with high mortality, and surviving mice exhibit chronic demyelination. MHV contains at least three dominant structural proteins: the membrane glycoprotein (M protein), the nucleocapsid protein (N protein), and the spike glycoprotein (S protein) (8). The S protein is posttranslationally processed to yield the S1 and the S2 proteins (9). The S protein has been the focus of many studies because of its important role in viral attachment to target cellular receptors (10), its membrane penetration, and its ability to induce cell fusion (11). The S protein is a critical determinant of viral pathogenicity and has been shown to contain major immunodominant neutralization domains. Protective neutralizing antibodies were induced in mice by immunization with purified S protein (12) and synthetic peptides derived from the S2 protein (13, 14).

The monoclonal antibody 5B19.2 was shown to protect against lethal challenge by passive immunization (15, 16). Epitope-mapping and peptide-scanning studies were used to map the 5B19.2 antibody recognition sequence to a 10-amino acid segment, L^{900}LGCIGSTCA^{909} of the S2 glycoprotein of MHV strain A59 (17, 18).

In this communication, we show that intranasal and subcutaneous immunization of mice with TMV hybrid viruses carrying the MHV 5B19 epitope elicited protective immunity against challenge infection with MHV strain JHM. Mice immunized with wild-type (w.t.) TMV produced antibody only against TMV coat protein; they were not protected against MHV infection. These studies demonstrate the successful use of TMV as a vaccine carrier and delivery vehicle for parenteral and mucosal routes of immunization.

MATERIALS AND METHODS

Construction of Hybrid TMV CP Molecules. Physical maps of hybrid TMV molecules used in this study are shown in Fig. 1. Peptides 5B19 (L^{900}LGCIGSTCA^{909}) and 5B19L (P^{899}LLGCIGSTCAEDGN^{913}) containing the 5B19 epitope (underlined sequence) from MHV strain JHM S2 glycoprotein (15, 18) were inserted between amino acid residues Ser-154 and Gly-155 of the TMV CP by the following method. A double-stranded oligonucleotide encoding 10 amino acids of the 5B19 peptide (L^{900}LGCIGSTCA^{909}) with *Ahd*I cohesive ends was formed by annealing two oligonucleotides 5′-t TTA TTA GGA TGT ATA GGA TCT ACT TGT GCT-3′ and 5′-GC ACA AGT AGA TCC TAT ACA TCC TAA TAA ag-3′ (sequences in upper case represent the 5B19 peptide coding sequence; the coding frame is indicated). The annealed oli-

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Abbreviations: CP, coat protein; MHV, murine hepatitis virus; TMV, tobacco mosaic virus; w.t., wild type; S protein, spike protein.

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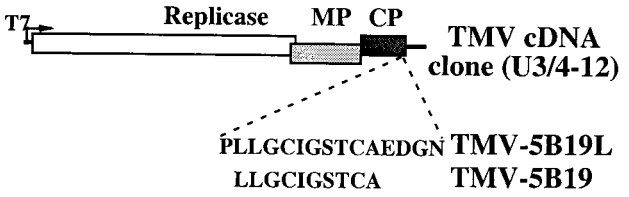

FIG. 1. Map of hybrid TMV cDNA clones. The position of the 5B19 and 5B19L peptides between Ser-154 and Gly-155 of the TMV coat protein (CP) is indicated. The T7 promoter was used to produce viral RNA in *in vitro* transcription reactions. MP, Movement protein.

gonucleotides were inserted into the *Ahd*I site of the TMV cDNA clone U3/4–12 (19). The resulting hybrid is an infectious full-length cDNA clone referred to as TMV-5B19; it carries the mutant CP gene CP-5B19.

To construct the TMV-5B19L hybrid virus, the 5B19L peptide was inserted in the plasmid pKN2 (which contains the TMV CP gene) (20) by using PCR-based, site-directed mutagenesis and the following primers: 5′-GT ATA GGA TCT ACT TGT GCA GAG GAC GGA AAT ggt cct gca act tga gg-3′ and 5′-CC GTC CTC TGC ACA AGT AGA TCC TAT ACA TCC TAA TAA TGG aga ggt cca acc caa ac-3′ (sequences in upper case represent the 5B19L peptide coding sequence). These primers placed the 5B19L coding sequence between Ser-154 and Gly-155 of the TMV CP, and were used in the PCR reactions in combination with the AATTAACCCTCACTAAAGGG (T3) and TAATACGACTCACTATAGGG (T7) primers, respectively. The amplified fragments contain partial sequences of the CP and the overlapping coding sequence of the 5B19L peptide; the fragments were used in PCR in combination with the T3 and T7 primers to produce a full-length CP sequence that contained the 45 nucleotides encoding the 5B19L peptide. The amplified fragment was digested with *Xba*I and *Kpn*I and cloned into the vector pKN2 to produce the plasmid pCP. The fragment *Nco*I–*Kpn*I from the plasmid pCP:5B19L was used to replace its homologue in the TMV cDNA clone U3/12–4 (19) linearized with *Nco*I and *Kpn*I. These reactions resulted in the generation of an infectious full-length cDNA clone referred to as TMV-5B19L; it contains the hybrid CP gene CP-5B19L.

Plant Inoculation and Virus Purification. Infectious RNA transcripts were generated *in vitro* from the w.t. TMV, TMV-5B19, and TMV-5B19L cDNA clones by using T7 RNA polymerase as described previously (19). Transcripts were inoculated to 4-week-old *Nicotiana tabacum* Xanthi-nn and Xanthi-NN plants. Wild-type TMV and TMV-5B19L were purified from infected leaves 2 weeks postinoculation as previously described (21). TMV-5B19 was purified as follows: infected leaves were ground to a fine powder and suspended in 50 mM sodium phosphate buffer (pH 7.0) at a ratio of 1.5 ml/g of leaf material. The slurry was centrifuged at 5,000 × *g* for 20 min, and the supernatant was mixed with 5% (wt/vol) diatomaceous earth and cleared at 12,000 × *g* for 30 min at 4°C. Virus-like particles in the supernatant were pelleted by ultracentrifugation at 90,000 × *g* for 90 min. After the supernatant was removed, the pellet was resuspended in 1× PBS with short ultrasonic disruption.

SDS/PAGE and Western Immunoblot Analysis. Tobacco leaves were ground to fine powder and homogenized in Laemmli sample loading buffer (22). Total protein extracts were boiled and fractionated by electrophoresis on a 12.5% polyacrylamide gel containing SDS (SDS/PAGE). Separated proteins were either stained with Coomassie blue or transferred onto nitrocellulose membranes and analyzed by using Western immunoblots (23). To detect TMV CP, membranes were first reacted with rabbit anti-TMV polyclonal antibody followed by a mouse anti-rabbit Ig conjugated with horseradish peroxidase (Amersham). Specific binding reactions were detected by using the chemiluminescence ECL-kit (Amersham) according to the manufacturer's instructions. To detect the MHV-5B19 epitope, the anti-TMV antibody was stripped off the membrane, and the blots were reprobed with the anti-5B19 monoclonal antibody MAb5B19 (15). A goat anti-mouse Ig conjugated with horseradish peroxidase was used as the secondary antibody and detected as described above.

Mass Spectrometry. CP purified from w.t. TMV and TMV-5B19L particles, either nondigested or treated with trypsin, was subjected to matrix-assisted laser absorption/desorption ionization MS (24) analysis as described (25).

Electron Microscopy and Immunogold Labeling. Purified w.t. TMV and TMV-5B19L particles were applied to carbon-coated copper electron-microscopy grids and used in immunogold labeling experiments as described (26). The antibody MAb5B19 (15) was used as first reactant and a goat anti-mouse IgG antibody conjugated with 50 nm gold particles (Ted Pella, Redding, CA) was used as second antibody. The grids were negatively stained with 1% phosphotungstic acid solution (pH 5.8) for 2 min and examined on an electron microscope (model CM100, Philips) at 39,000× magnification.

Immunization of Mice and MHV Protection Assays. Female BALB/cBj mice that were TMV- and MHV-seronegative were obtained from The Scripps Research Institute rodent-breeding colony. Six different protocols were used to administer immunogens to mice. Mice were lightly anesthetized by inhalation of Metofane (methoxyfluorane) before administration of the antigen. The immunization schedules are shown in Fig. 4. Six groups of six mice each were administered w.t. TMV, TMV-5B19L, or TMV-5B19. The mice in groups 1, 2, and 3 were intranasally administered TMV-5B19L (in 10 μl of PBS), 3 d per week (50 μg of virus per administration) for 10, 6, and 4 weeks prior to challenge, respectively. Mice in groups 4 and 5 were intranasally immunized with a single dose per week (50 μg of virus per administration) for 6 and 4 weeks, respectively. Mice in group 6 were immunized by subcutaneous injection with 50 μg of TMV-5B19 emulsified with MPL-TDM adjuvant (RIBI Immunochemicals Research) at d 1, 14, and 28. During each regime of immunization, each mouse in a group of six was administered w.t. TMV. Serum samples were prepared from blood obtained by tail bleeding at 10-d intervals and stored at −70°C until they were analyzed.

One week after completion of the immunization schedules, mice in all of the groups were challenged on the same day by intranasal injection with a single batch of inoculum consisting of 10 × LD_{50} [112 plaque-forming units (pfu)/ml] of MHV strain JHM. Mice were observed daily and disease symptoms and death were scored.

Antibody Titration of Mouse Serum. Titers of anti-TMV or anti-5B19 IgG and IgA were determined by limiting dilution of sera in ELISAs for mice in each group. Plates (Maxisorp,

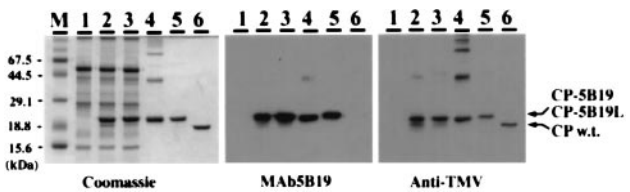

FIG. 2. Accumulation of hybrid TMV coat protein in tobacco leaves. Total leaf proteins extracted from infected tobacco tissues or partially purified proteins were fractionated on duplicate 12.5% SDS/12.5% PAGE. One gel was stained with Coomassie blue (*Left*), the other gel was used for immunoblot analysis with the monoclonal antibody to 5B19, MAb5B19 (*Center*), or with the polyclonal antibody specific for TMV (*Right*). M, Protein molecular mass. Lane 1, Extract from non-infected plant; lane 2, extract from TMV-5B19 infected plant; lane 3, extract from TMV-5B19L infected plant; lane 4, partially purified TMV-5B19; lane 5, purified TMV-5B19L; lane 6, purified w.t. TMV.

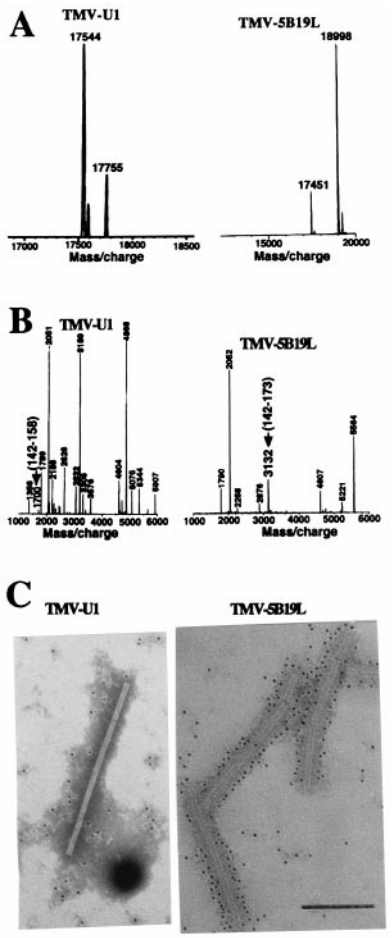

FIG. 3. (*A* and *B*) MS analysis of w.t. CP (*Left*) and CP-5B19L (*Right*) purified from virus particles. Molecular mass of the CPs untreated (*A*) or treated with trypsin (*B*) was determined by matrix-assisted laser absorption/desorption ionization MS. (*B*) The mass and the amino acid coordinates (between parentheses) of the fragment containing the 5B19L peptide and its homologue in w.t. CP are indicated by arrows. (*C*) Electron microscopy and immunogold labeling of TMV-5B19L and TMV-U1. Purified virus was subjected to treatment with a monoclonal antibody against the 5B19 peptide followed by goat anti-mouse antibody conjugated to 5-nm gold particles. Samples were negatively stained and analyzed and viewed at 39,000×. (Bar = 200 nm.)

Nunc) were precoated with TMV (250 ng per well) or with a synthetic peptide SPLLGCIGSTCAEDGNK (250 ng per well) containing the 5B19 epitope (underlined; MHV S-protein amino acid residues 898–914) and additional flanking sequences, after conjugation to BSA (27). Plates were blocked with 2% BSA in 1× Tris-buffered saline and 0.05% Tween 20 (TBST) for 1 h at 37°C. Sera from immunized mice were applied in duplicate wells by using a 2-fold dilution series in PBS containing 2% BSA. Preimmune serum was serially diluted and used as the control. After 2 h of incubation at 37°C, the plates were washed with TBST and the secondary anti-mouse IgG or anti-mouse IgA, conjugated with horseradish peroxidase (Bio-Rad) and diluted 1:5000 in 2% BSA in TBST was applied to each well and incubated for 2 h at 37°C. An ELISA plate reader (Bio-Rad) was used to quantitate reactions. Antibody titers were defined as the highest dilution that produced an A_{405} 150% greater than the mean value of the preimmune serum.

RESULTS

Characterization of Hybrid Viruses TMV-5B19 and TMV-5B19L. The TMV coat protein gene was modified by site-directed mutagenesis to encode a hybrid CP that contained either the peptide 5B19L (residues 899–913 of MHV strain JHM S2 protein) or 5B19 (residues 900–909) placed between residues Ser-154 and Gly-155 of the TMV coat protein (Fig. 1). Plasmids containing the modified full-length cDNAs TMV-5B19L and TMV-5B19 were used to produce infectious viral RNA *in vitro*. TMV-5B19L and TMV-5B19 systemically infected *N. tabacum* Xanthi-nn plants and induced typical mosaic symptoms (data not shown). Infectious virus was recovered from systemically infected leaves and passaged to other plants.

SDS/PAGE and immunoblot assays performed with protein extracts from leaves infected with TMV-5B19L and TMV-5B19 showed high levels of accumulation of the hybrid coat proteins (Fig. 2). The hybrid proteins CP-5B19L and CP-5B19 migrated more slowly than the w.t. CP on SDS/PAGE and were specifically recognized by MAb5B19 (Fig. 2).

TMV-5B19 was quite different from TMV-5B19L. More than 90% of the TMV-5B19 was recovered in an insoluble fraction and ultrasonication was required to solubilize the TMV-5B19 particles. In contrast, TMV-5B19L was purified by using the same method as for purification of the w.t. TMV. These data suggest that the differences in solubility between the hybrid viruses result from the presence or absence of specific residues of the S2 epitope sequence.

The molecular mass of the hybrid CP in the purified TMV-5B19L particles was analyzed by MS (Fig. 3 *A* and *B*). A signal corresponding to the predicted mass of CP-5B19L was observed, demonstrating the presence of the full-length chimeric CP. Furthermore, digestion of the CP-5B19L with trypsin (Fig. 3*B*) revealed the presence of a fragment, the mass of which corresponds to the mass of the peptide containing amino acids 142 to 173, which represents the full amino acid sequence of the 5B19L peptide and the amino acids 142 to 154 and 155 to 158 of the w.t. CP.

Immunogold-labeling experiments performed with purified TMV-5B19L and TMV-U1 by using the MAb5B19 (Fig. 3*C*) revealed that the antibody specifically recognized TMV-5B19L, whereas there was no reaction with the w.t. TMV (Fig. 3*C*).

Immunogenicity of Hybrid TMV in Mice. The serum antibody titers in the mice immunized with TMV-5B19, TMV-5B19L, or w.t. TMV were determined by limiting dilution of ELISA. Mice from group 1, which were immunized intranasally with TMV-5B19L 3 d per week for 10 weeks (Fig. 4), had high titers of IgG and moderate titers of IgA specific to the S2 peptide (Fig. 5*A* and *B*, respectively). Measurable amounts of anti-5B19 IgG were detected approximately 10 d after the first administration of the immunogen, whereas the anti-5B19 IgA response occurred 10 to 15 d later. High IgG and moderate IgA

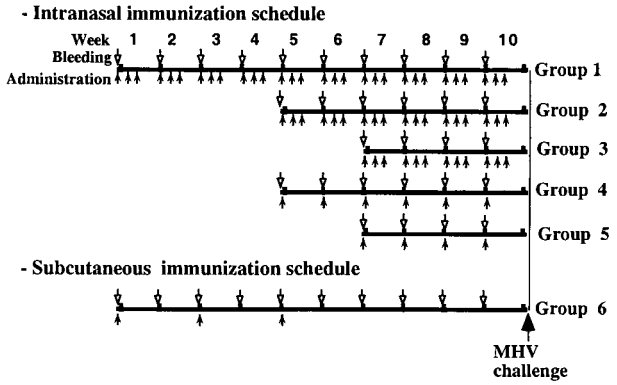

FIG. 4. Schedule of immunization of groups of mice. Solid arrows represent times of administration; open arrows represent times of sample collection.

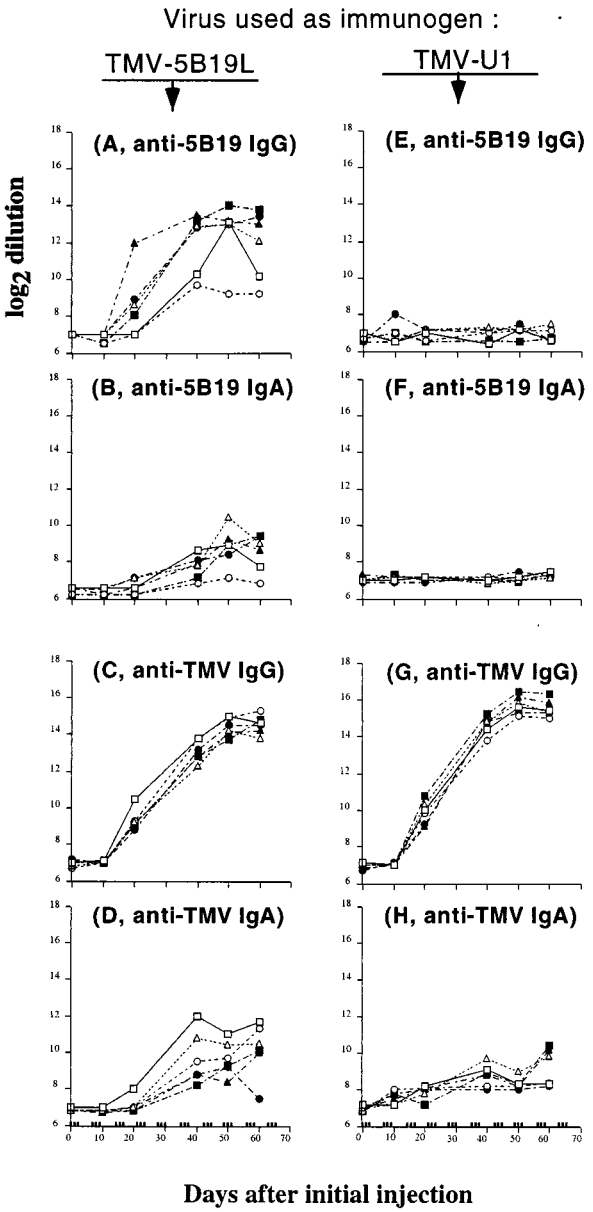

FIG. 5. Immune response of group 1 mice (treated as indicated in Fig. 4). IgG and IgA antibodies specific to the 5B19 peptide (*A, B, E,* and *F*) or TMV (*C, D, G,* and *H*) were determined in ELISA. Serum IgG and IgA antibodies were detected with goat anti-mouse IgG or anti-mouse IgA antibodies conjugated with horseradish peroxidase. (*A–D*) Serum titers of mice immunized with TMV-5B19L; (*E–H*) Serum titers of the mice immunized with w.t. TMV. Short vertical bars on the *x* axis indicate the days of immunization. Each line represents the response of a single mouse.

titers specific to TMV were also induced (Fig. 5 *C* and *D*, respectively).

In animals immunized with the w.t. TMV, high anti-TMV IgG and moderate IgA titers were obtained (Fig. 5 *G* and *H*), but neither IgG nor IgA specific to the 5B19 epitope was detected (Fig. 5 *E* and *F*). Anti-TMV or anti-5B19 IgGs were detected 20 d after the first administration, and after approximately 50 d, serum IgG and IgA reached maximal levels (Fig. 5).

To determine the minimal doses of TMV-5B19L that are required to elicit antibody responses, mice groups 2, 3, 4, and 5 (Fig. 4) were intranasally administrated one or three doses per week for 4 and 6 weeks. In mice immunized with three doses (group 2) or one dose (group 4) per week for 6 weeks, similar levels of anti-TMV and anti-5B19 serum IgG and IgA antibody were observed (Fig. 6: compare *A* to *E* and *C* to *G* for IgG titers, and *B* to *F* and *D* to *H* for IgA titers). Measurable amounts of anti-TMV IgA were observed approximately 20 d after the first injection (Fig. 6). In mice that received TMV-5B19L for 4 weeks (group 3), lower but detectable amounts of serum antibodies were observed by 20 d after the first injection (data not shown). These data suggest that a minimum 6-week regimen of immunization with this immunogen is necessary to reach maximal antibody levels.

Because TMV-5B19 was highly insoluble it was difficult to purify the virus in the quantities necessary for intranasal administration. Therefore a protocol to use subcutaneous administration was adopted. After subcutaneous injections, serum IgG and IgA titers specific for TMV and for the 5B19 peptide were measured by ELISA. High titers of anti-TMV CP IgG and anti-5B19 IgG were induced approximately 10 d after the first injection (Fig. 7). However, no measurable anti-TMV CP IgA or anti-5B19 IgA were induced in the immunized mice (Fig. 7). These data show that the 10 amino acid 5B19 epitope sequence elicited an immune response in mice by subcutaneous administration similar to that in mice immunized by intranasal administration with the 5B19L peptide.

Protection of Mice from Viral Challenge. To determine whether immunization with the TMV that contained the 5B19 epitope (TMV-5B19L or TMV-5B19) gave protection against MHV infection, mice were challenged intranasally with 10 × LD_{50} (112 pfu/ml) of MHV strain JHM. Groups of mice were challenged on the same day, 1 week after completion of the immunization schedule with the same batch of MHV strain

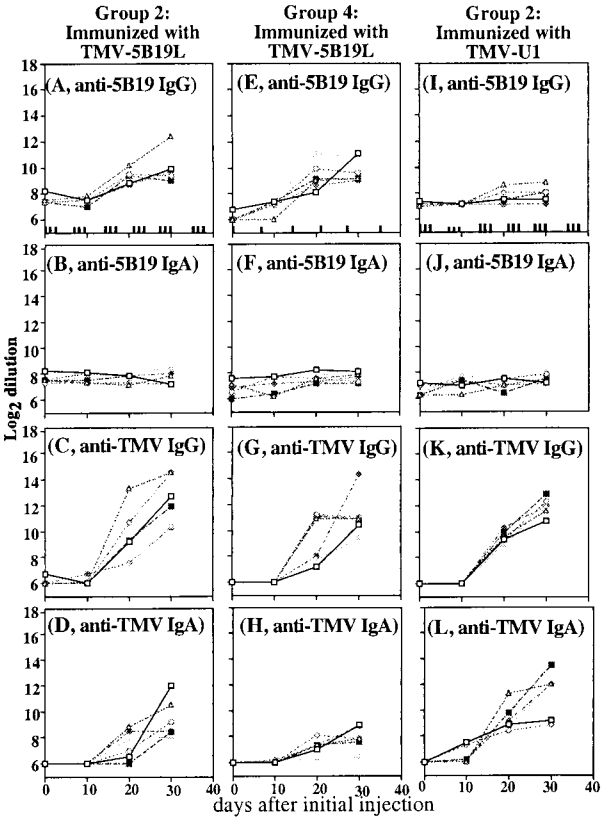

FIG. 6. Immune responses of mice that received low regimen of immunization with TMV-5B19L by intranasal injection. IgG and IgA antibodies specific to peptide (*A, B, E, F, I,* and *J*) or TMV (*C, D, G, H, K,* and *L*) were determined in mouse sera by using as antigen 5B19 peptide-BSA conjugate or with TMV, respectively, in ELISAs as in Fig. 5. The regime of immunization of each group is shown in Fig. 4. The small vertical bars on the *x* axis indicate the days of immunization. Each line represent the response of an individual mouse.

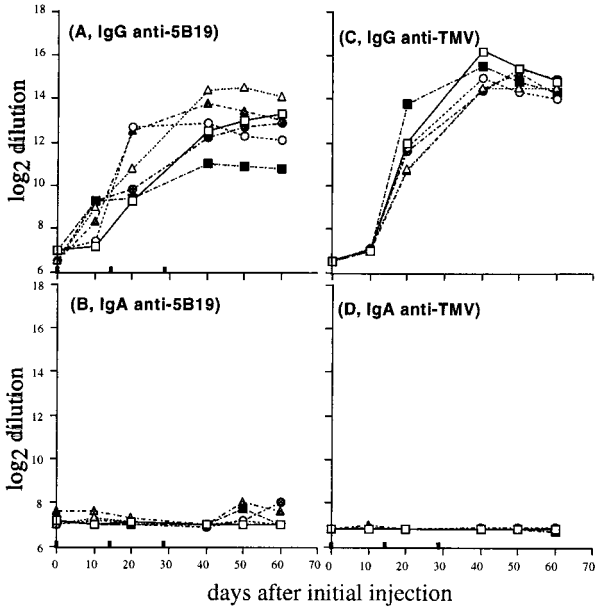

FIG. 7. Immune responses of mice immunized with TMV-5B19 by subcutaneous injection (group 6 in Fig. 4). Antibodies specific to the peptide (*A* and *B*) or TMV (*C* and *D*) were determined with ELISA as in Fig. 5. The immunization regimen of each group is shown in Fig. 4. Short vertical bars on the *x* axis indicate the days of immunization. Each line represents the response of an indivdual mouse.

JHM (Fig. 4). Mortality was scored daily for 21 d after challenge (Fig. 8). Five of the six mice immunized intranasally for 10 weeks (group 1) or by subcutaneous injection (group 6) survived through the 21-d period, whereas two of six mice intranasally immunized for 6 weeks (group 2) and only one of six mice intranasally immunized for 4 weeks (group 3) survived (Fig. 8*A*). Although some surviving mice showed mild signs of infection within 4 or 5 d after challenge with MHV, the disease did not progress. All mice immunized intranasally with a single weekly dose of hybrid TMV containing the 5B19 epitope for 6 and 4 weeks (groups 4 and 5) and all of the mice immunized with the w.t. TMV died 11 d after MHV challenge (Fig. 8).

DISCUSSION

We report here the successful use of TMV as a carrier and delivery system for a vaccine against MHV. Synthetic peptides that contain the 5B19 epitope of the MHV glycoprotein elicit production of protective neutralizing antibodies when administered intraperitoneally (13) or subcutaneously (14) to mice. In the present work, we determined whether the 5B19 epitope fused to the CP of TMV could elicit protective immunity against MHV strain JHM when administered intranasally or subcutaneously. The advantages of using TMV as a carrier and delivery vector include the high yield of the peptide fused to the CP, the simplicity of growth, and the ease of purification of large amounts of the vaccine. Both peptides selected for the present study span the immunodominant domain containing the 5B19 epitope, here referred to as 5B19 and 5B19L, which contain 10 or 15 amino acids, respectively.

TMV-5B19L produced typical mosaic symptoms in infected tobacco plants and virus particles were readily purified by using a standard TMV-purification method in quantities similar to those obtained with the w.t. TMV. The purified TMV-5B19L virus particles were similar in shape and length to the w.t. TMV particles, and they retained infectivity; the epitope was not lost after two or three repeated passages in tobacco plants. Analyses by MS showed the presence of the 5B19L peptide fused to the CP, and immunogold-labeling

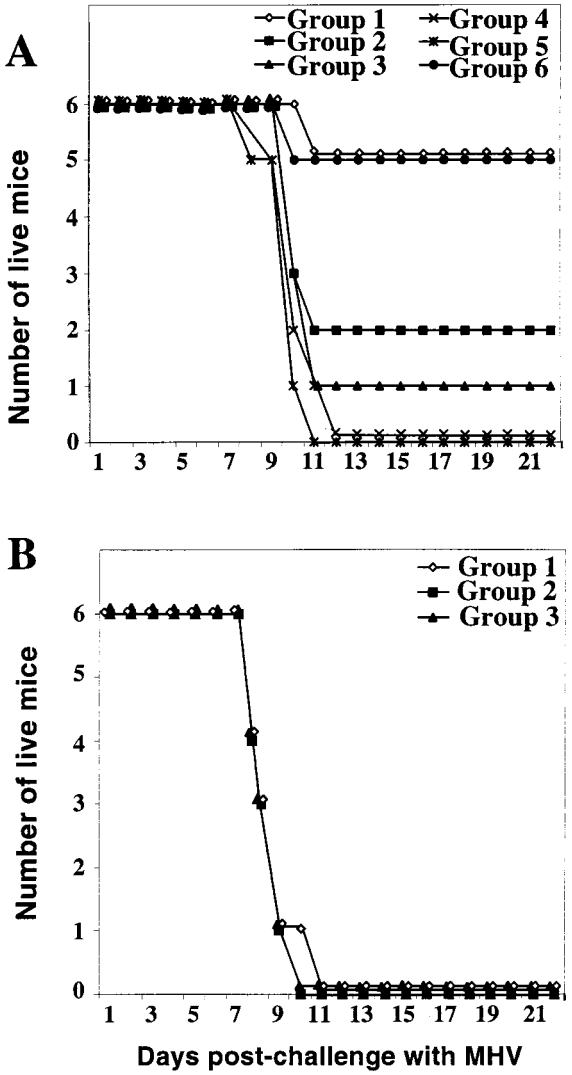

FIG. 8. Protection of immunized mice against MHV strain JHM. Groups of six mice were infected with $10 \times LD_{50}$ (112 pfu/ml) of MHV strain JHM, and survival was scored over a 21-d interval. The immunization regimen of each group is shown in Fig. 4. (*A*) Survival of mice immunized with TMV-5B19L or TMV-5B19. (*B*) Survival of mice immunized with w.t. type TMV.

experiments showed the specific reaction of TMV-5B19L virus particles. These results demonstrate that the full-length sequence of the peptide is maintained on virus particles and that the antigenic sequence on the 5B19 epitope is exposed on or near the surface of the assembled virus particles.

Although TMV-5B19 assembled to form virus-like particles and systemically infected *N. tabacum* Xanthi-nn plants, purification was difficult because of the high insolubility of the virus. Most of the virions remained in the insoluble fraction and precipitated with the cell debris, resulting in a very low yield of purified TMV-5B19. We reported similar insolubility problems when the murine zona pellucida ZP3 epitope was similarly fused to the C terminus of the TMV CP (5). The reasons for insolubility remain unclear; we suggest that insertion of the 5B19 peptide near the C terminus of the CP may create a domain that interacts with cellular membranes and anchors virus particles to cellular debris during extraction (26).

The 5B19 epitope elicited antibody response in mice administered the high dose regimen of hybrid TMV-5B19L; high titers of antigen-specific IgG antibody were detected between 10 and 20 d after initial inoculation. At that time mice had received six to nine doses, and the equivalent of 300 to 450 μg

of virus (groups 1, 2, and 3). Studies of the minimal dose of hybrid virus needed to induce IgG production indicated that a single dose per week for 2 weeks (group 4; Fig. 6) was sufficient. However, the antibody titers in these animals were slightly lower than they were in the mice that were immunized three times per week. Anti-5B19 IgA was also induced in mice that were administered high doses of TMV-5B19L. However, the titers of IgA were very low with lower amounts of immunogen or with shorter times of administration. These results demonstrate that TMV is an immunogenic delivery vehicle for this epitope and that it acts in a dose-dependent manner.

Despite the solubility problems of TMV-5B19, we succeeded in recovering sufficient amounts of the chimeric virus for subcutaneous administration. These experiments showed that the 5B19 epitope displayed on TMV can induce a high level of anti-5B19 IgG. However, in these mice little or no anti-5B19 IgA was produced in comparison with mice that were immunized intranasally (e.g., group 1). The difference in antibody induction by TMV-5B19 and TMV-5B19L was probably because of the different routes of administration, i.e., intranasal for TMV-5B19L versus subcutaneous for TMV-5B19. Mice that were intranasally administered TMV-5B19L, TMV-5B19, or w.t. TMV had high titers of anti-TMV IgG and IgA. The immunogenicity of TMV did not interfere with the immunogenicity of the 5B19 epitope in mice that were administered TMV-5B19L or TMV-5B19.

Mice that produced high titers of anti-5B19 antibody were protected against MHV strain JHM infection. Even though all of the mice challenged with MHV showed minor clinical signs of MHV strain JHM infection, those that received the high dose regimens of immunization (three injections per week for 10 weeks) or that were immunized by subcutaneous injection had a higher survival rate than those that received lower doses. Therefore, the regimen of immunization positively correlated with the amount of antibody and the degree of protection against MHV-JMH infection. However, the amount of subcutaneously administered immunogen that was required to achieve effective protection was at least 10 times less than that required for intranasal administration.

The question of whether IgG, IgA, or both are responsible for the protection against MHV infection is not completely answered here. However, it is likely that at least a part of the inoculum was neutralized by anti-5B19 IgA, resulting in the reduction of acute disease. Thus the mice that received low levels of immunogen and had high anti-5B19 IgG titers, but low IgA titers were not protected as well as the mice treated with the high regime of immunization that resulted in higher anti-5B19 IgA titers.

These results demonstrate that TMV can be used as a carrier and delivery vehicle for a protective epitope from MHV. This work correlates with previous reports of studies in which mice that were subcutaneously immunized with the 5B19 peptide alone or that received the monoclonal antibody 5B19.2 by passive transfer were protected against MHV infection (14, 15). The advantage of the vector described here is the simplicity of the expression system, involving a nonpathogenic virus (to humans and animals) that yields high quantities of the immunogenic peptide at low cost.

The present system of vaccine delivery may be suitable for oral vaccine development. Arakawa *et al*. (28) showed that transgenic potatoes that express the cholera toxin B-subunit pentamer elicited a mucosal protective immune response in mice that were fed such potatoes. Similarly, an immune response was induced in humans who consumed transgenic potatoes containing the *Escherichia coli* heat-labile toxin B (29). Combining a similar system of mucosal targeting with the TMV system may lead to the development of an alternative low-cost oral vaccine.

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