

Mouse hepatitis virus spike and nucleocapsid proteins expressed by adenovirus vectors protect mice against a lethal infection

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Infection with the mouse hepatitis coronavirus (MHV) provides an excellent model for the study of viral diseases of the central nervous system and the gastrointestinal tract. With the ultimate aim of studying mucosal immunity to MHV we have cloned the genes encoding the structural proteins of MHV strain A59 (MHV-A59) into the E3 region of a human adenovirus type 5 vector. Infection of HeLa cells with the resulting recombinant adenoviruses AdMHVS, AdMHVN and AdMHVM revealed the correct expression of the spike (S), nucleocapsid (N) and membrane (M) proteins, respectively. Intraperitoneal inoculation of BALB/c mice with the recombinant viruses elicited serum antibodies which specifically recognized the respective MHV proteins in an immunoprecipitation assay. Only antibodies to the S protein neutralized MHV-A59 *in vitro* but titres were low. When analysed by ELISA or by

immunofluorescence only the antibody response to the N protein was significant; weak responses or no detectable response at all were found for S and M, respectively. Upon intracerebral challenge with a lethal dose of MHV-A59 we found that a significant fraction of animals vaccinated with adenovirus vectors expressing either the S protein or N protein were protected. This protective effect was significantly stronger when the animals were given a booster immunization with the same vector prior to challenge. No protection was induced by AdMHVM. Interestingly, enhanced protection resulted when AdMHVS and AdMHVN were applied in combination as compared to survival after single immunizations. The results indicate that both the N and S proteins generate a protective immune response and suggest that this response is enhanced by combined expression of the two proteins.

Introduction

Murine hepatitis viruses (MHVs) are enveloped positive-stranded RNA viruses which belong to the Coronaviridae family (Spaan *et al.*, 1988). In contrast to most other coronaviruses, they have a broad tissue tropism causing respiratory, neurological and gastrointestinal disorders in addition to hepatitis. The MHV strain A59 virion is composed of three proteins, the spike glycoprotein (S), the membrane glycoprotein (M) and the nucleocapsid protein (N). The S protein forms conspicuous peplomer structures on the surface of the virus particle and is responsible for many important biological properties: it mediates attachment to the target cell, causes fusion of infected cells and triggers humoral and cell-mediated immune responses in the infected host.

Antibodies directed to this protein not only neutralize the virus *in vitro* (Spaan *et al.*, 1990), but also provide protection of mice against a lethal MHV challenge (Hasony & MacNaughton, 1981; Daniel & Talbot, 1990). Protection can also be induced by immunogenic peptides derived from the S protein (Talbot *et al.*, 1988; Koolen *et al.*, 1990).

Although the S protein is the immunodominant structural component, MHV infection also induces a humoral response to the M and N proteins. Antibodies directed against the N protein do not neutralize virus *in vitro* but in some cases passive transfer of monoclonal antibodies (MAbs) specific to the N protein of MHV-2 (Nakanaga *et al.*, 1986) and MHV-3 (Lecomte *et al.*, 1987) protects mice from a lethal MHV infection. Furthermore, some MAbs directed against the M protein, in one case with virus-neutralizing activity *in vitro*, were shown to protect mice against a lethal MHV-4-induced hepatitis (Fleming *et al.*, 1989).

Since the early 1980s, adenoviruses, herpesviruses and poxviruses have been engineered as live carrier vaccine

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candidates. These replicating vectors possess the advantage over inactivated vaccines that their expressed foreign antigens are presented to the immune system in a manner which may mimic a natural infection. Human adenoviruses have a number of advantages over other viral vectors (Graham, 1990; Graham & Prevec, 1991). They are stable DNA viruses that can easily be manipulated and propagated in culture and possess low pathogenicity in man and animals. Adenoviruses replicate in the upper respiratory and gastrointestinal tracts thereby inducing mucosal immune responses including the triggering of cellular immunity. These features make them a useful carrier for vaccination against influenza (Ebata *et al.*, 1992), herpes simplex virus (Johnson *et al.*, 1988; McDermott *et al.*, 1989) and rotavirus (Andrew *et al.*, 1993). Human adenovirus vectors are also able to induce immune responses in dogs (Natuk *et al.*, 1992) and in mice (McDermott *et al.*, 1989; Prevec *et al.*, 1989, 1990).

In this study we used the human adenovirus (Ad5) system to express the MHV proteins in mice. We analysed the immune responses and determined their protective effects upon challenge with a lethal dose of MHV-A59.

Methods

Mice, cells, viruses and plasmids. Female MHV seronegative BALB/c mice were obtained from Credo/IFA (Brussels, Belgium). The A59 strain of MHV (MHV-A59) was originally obtained from the American Type Culture Collection. The virus was propagated in Sac⁻ cells and titrated on L cells (Spaan *et al.*, 1981); these were also used for the virus neutralization assays.

Ad5-transformed human embryo kidney cells (293 cells) were used for rescue of recombinant Ad5 (Graham *et al.*, 1977). Titration and growth of Ad5 were performed in 293 cells and HeLa cells. Monolayer cultures of cells were maintained in MEM-F11 medium (Gibco-BRL) supplemented with 5% horse serum (Gibco-BRL) for 293 cells and in DMEM supplemented with 5% fetal calf serum (FCS) for HeLa cells.

The enzymes used for recombinant DNA work were purchased from Pharmacia, Gibco-BRL and Boehringer-Mannheim. Plasmid DNA was prepared using the alkaline lysis method, with minor modifications (Birnboim & Doly, 1979). Cloning techniques were performed essentially according to Sambrook *et al.* (1989).

Preparation of recombinant Ad5 viruses. The S, M and N gene were excised with *Bam*HI from vectors in which they had been cloned previously: the S gene from pBlueScript (Luytjes *et al.*, 1987), the M gene from pTZ19R (Krijnse Locker *et al.*, 1992) and the N gene from pBS(+) (Bredenbeek, 1990). These genes were cloned into the *Bam*HI site of plasmid pSV2X3 (Prevec *et al.*, 1990), a derivative of pSV2neo (Southern & Berg, 1982). pSV2X3 has the simian virus 40 (SV40) promoter sequence and poly(A) signal sequence separated by a polylinker that is used to insert genes of interest. The cassette is bounded by *Xba*I sites.

The *Xba*I cassettes containing the MHV S, M and N genes (designated pGG1, pGG2 and pGG3, respectively) were each subcloned into the *Xba*I site present in the partially deleted E3 region of the Ad5 transfer vector pFG144K3 (Hanke *et al.*, 1991) such that the

orientation of the SV40 promoter was parallel to that of the E3 promoter (designated pJW5, pJW6 and pJW7, respectively). The MHV S gene was also cloned in the reverse orientation with respect to the E3 promoter (pJW5L). pFG144K3 consists of the rightward 40% of the Ad5 genome fused to a plasmid containing an origin of replication and a kanamycin resistance gene. The Ad5 part of pFG144K3 has a deletion in the essential E1 region [1.3 to 10.6 map units (m.u.)] and a deletion in the non-essential E3 region (78.5 to 84.7 m.u.; Fig. 1). The plasmids pJW5, pJW6, pJW7 and pJW5L were each cotransfected with a second transfer vector, pFG173 (Hanke *et al.*, 1991), into 293 cells (Graham *et al.*, 1977) using the calcium phosphate precipitation technique (Graham & Van der Eb, 1973). pFG173 can complement sequences from the left end of Ad5 necessary for virus replication and carries a lethal deletion comprising the region between 75.9 and 85.0 m.u. of the Ad5 genome (Fig. 1).

After 6 to 10 days individual plaques were isolated, plaque-purified three times, and viral DNA was analysed by restriction enzyme digestion and agarose gel electrophoresis. The recombinant adenoviruses obtained were stable upon *in vitro* passaging. Analysis of viral DNA after several passages in 293 cells or HeLa cells revealed the maintenance of the correct MHV inserts.

Analysis of expressed MHV-A59 proteins. HeLa cells infected with 10 p.f.u./cell of recombinant adenovirus were labelled with [³⁵S]methionine (50 µCi/ml) for 1 h starting at 16 h post-infection (p.i.). Cells were lysed in TES (20 mM-Tris HCl pH 7.4, 1 mM-EDTA, 100 mM-NaCl) containing 1% Triton X-100 and the lysates cleared by low-speed centrifugation. To 0.1 ml cell lysate, 1 ml detergent solution (52.5 mM-EDTA, 50 mM-Tris-HCl pH 8.0, 1% v/v NP40, 0.4% w/v

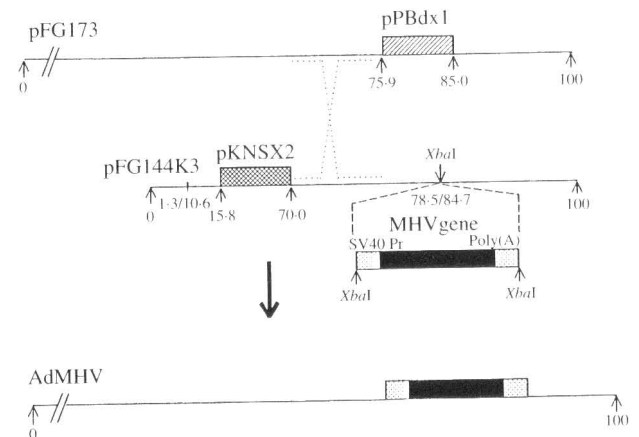


Fig. 1. Strategy for rescue of MHV sequences into Ad5. The genes encoding the MHV structural proteins (black bar) were cloned into the *Bam*HI site of plasmid pSV2X3 (Prevec *et al.*, 1990) which contains the SV40 promoter (Pr) sequence and poly(A) addition sequence (stippled regions) separated by a multiple cloning site. The *Xba*I cassettes containing the MHV genes were each subcloned into the *Xba*I site of adenovirus transfer vector pFG144K3 which is located in the partially deleted E3 region (Hanke *et al.*, 1991). pFG144K3 consists of the rightward sequences of Ad5 (m.u. 70 to 100) fused to plasmid pKNSX2 (cross-hatched bar) which contains an origin of replication and a kanamycin resistance gene. The Ad5 part has a deletion in the essential E1 region and a deletion in the non-essential E3 region (m.u. 78.5 to 84.7). The resulting plasmids were cotransfected with pFG173 into 293 cells (Graham *et al.*, 1977) to generate the desired recombinant viruses. pFG173 (Hanke *et al.*, 1991) contains plasmid pPBdx1 sequences (hatched bar) inserted at a position of the Ad5 genome where the E3 region has a deletion extending into the L4 region.

sodium deoxycholate) containing 0.25% SDS was added followed by 2 µl of a rabbit anti-MHV serum (K134; Rottier *et al.*, 1981). After overnight incubation at 4 °C 20 µl staphylococcus A (Gibco-BRL) was added to each sample and the incubation was continued for 2 h. The bacteria were pelleted by low-speed centrifugation and washed three times with radioimmunoprecipitation assay buffer (25 mM-Tris-HCl pH 8.0, 150 mM-NaCl, 0.5 mM-MgCl₂) containing 0.1% (v/v) Triton X-100. The adsorbed proteins were eluted into Laemmli sample buffer and analysed without heating by SDS PAGE and fluorography as described by Laemmli (1970).

Animal experiments. Four-week-old female BALB/c mice were immunized intraperitoneally (i.p.) using 0.5 ml inoculum volumes and subsequently challenged with MHV-A59 by the intracerebral (i.c.) route (0.05 ml of inoculum). In single immunization experiments mice were given 5×10^7 to 1×10^8 p.f.u. of Ad5 recombinant virus per animal followed 3 weeks later by a challenge with 10^1 or 10^5 p.f.u. of MHV-A59, as specified in the figures. When booster immunizations were applied the primary inoculum contained 1×10^7 to 5×10^7 p.f.u. of Ad5 recombinant virus per mouse which was followed 2 weeks later by 5×10^7 to 1×10^8 p.f.u. per mouse. Two weeks after the booster immunization the mice were challenged with 10^1 or 10^5 p.f.u. of MHV-A59. Control immunizations were performed with wild-type Ad5 virus, with a recombinant carrying the S gene in the reverse orientation or with PBS.

Sera were obtained by bleeding from the retro-orbital plexus using heparinized capillary tubes 1 day before immunization, 1 day before challenge, and 4 to 6 days after challenge. Blood was centrifuged at 12000 g for 5 min and the supernatant was decanted by heating at 56 °C for 20 min. All mice were kept in special pathogen-free cages which were located in a level C containment laboratory. The experimental protocols were approved by the institutional animal welfare committee.

Assay of antibodies in mouse sera

(i) *Virus neutralization assay.* Mouse sera were analysed for their ability to neutralize MHV-A59 virus. Serum dilutions were made in DMEM containing 5% FCS and were incubated for 1 h at 37 °C with approximately 100 p.f.u. MHV-A59. Hyperimmune mouse serum (post-challenge serum) and serum from non-infected mice were taken as positive and negative controls, respectively. Residual infectivity was evaluated in a c.p.e. inhibition assay by applying the virus/plasma samples to confluent monolayers of L cells in 96-well plates. After incubation for 1 h at 37 °C, culture medium was added to each well and plates were incubated for 1 to 2 days at 37 °C. Neutralization titres were determined from the reduction of plaque formation and the titres were calculated as the reciprocal serum dilution giving 50% virus neutralization.

(ii) *Radioimmunoprecipitation.* Sac⁻ cells infected with 10 p.f.u./cell of MHV-A59 were labelled with [³⁵S]methionine (50 µCi/ml) for 3 h starting at 6 h p.i. They were then washed once with PBS and lysed in TES 1% Triton X-100 to which 1% aprotinin and 1 mM-PMSF were added. Nuclei were pelleted at 12000 g for 5 min and the supernatant was used as a source of antigen for immunoprecipitation.

Of the cell lysate 7.5 µl was mixed with 0.3 ml TES-0.1% Triton X-100, 2 µl mouse serum was added and the mixture was incubated overnight at 4 °C. KCl was added to 0.6 M and the immune complexes were adsorbed onto staphylococcus A (20 µl of a 10% w/v suspension) for 2 h at 4 °C. The bacteria were pelleted and washed three times with TES-0.1% Triton X-100. The final pellet was resuspended in 25 µl Laemmli sample buffer. The immunoprecipitated proteins were heated for 5 min at 95 °C (except for the immunoprecipitates of the M protein which aggregates upon heating) and analysed by SDS-PAGE and fluorography as described by Laemmli (1970).

(iii) *ELISA and immunofluorescence (IF).* Titration of individual sera in an ELISA, using frozen and thawed MHV-infected cell cultures as a solid-phase antigen source (Peters *et al.*, 1979), and by indirect IF on ethanol-fixed MHV-A59-infected Sac⁻ cells, was performed according to standard techniques.

Results

Construction of Ad5 recombinant viruses

Adenoviruses expressing the S, M or N gene of MHV-A59 were constructed by cloning the genes in a cassette containing the SV40 promoter and SV40 poly(A) signal sequences (see Methods). Subsequently, the cassettes containing the MHV structural genes flanked by the SV40 sequences were subcloned into the Ad5 transfer vector pFG144K3 (Fig. 1) such that the MHV genes were in the same orientation as the Ad5 E3 promoter. In one construct the MHV S cassette was also cloned in the reverse orientation with respect to the E3 promoter. These transfer vectors were each cotransfected into 293 cells (Graham *et al.*, 1977) with a second plasmid (pFG173) which is able to complement sequences from the left part of the Ad5 genome necessary for virus replication. This plasmid carries a deletion of the E3 region of the Ad5 genome (Fig. 1). In this way each of the plasmids is non-infectious by itself and any viral plaque obtained from the cotransfection must be generated by recombination.

Recombinant adenoviruses thus obtained were designated AdMHVS, AdMHVN, AdMHVM and AdMHVS2, the latter containing the S gene in the reverse orientation with respect to the E3 promoter. After three rounds of plaque purification viral DNA was isolated and analysed by restriction enzyme digestion and agarose gel electrophoresis to analyse the viral DNA structure and verify the particular MHV gene insert. The purified Ad5 viruses were grown on 293 or HeLa cells to prepare virus stocks which were used in further experiments.

Expression of MHV genes by Ad5 recombinant viruses

Since vectors AdMHVS, AdMHVS2, AdMHVM and AdMHVN are unrestricted for replicative growth in human cell lines, HeLa cells rather than 293 cells were used for subsequent experiments. To determine whether the MHV structural proteins were correctly expressed by the respective Ad5 recombinant viruses, HeLa cells were infected and labelled from 16 to 17 h p.i. with [³⁵S]methionine. MHV-specific proteins were immunoprecipitated from lysates prepared from these cells using a rabbit anti-MHV serum and analysed by SDS-PAGE and fluorography.

As seen in Fig. 2 each Ad5 recombinant virus expressed

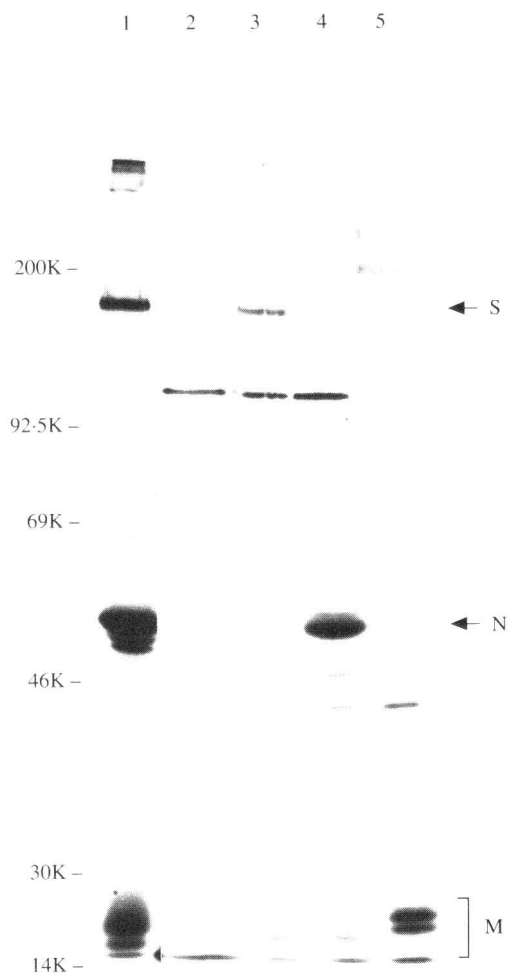


Fig. 2. Expression of MHV proteins by Ad5. Proteins were immunoprecipitated with a rabbit anti-MHV serum from lysates of radio-labelled HeLa cells infected with wild-type Ad5 (lane 2) or with the recombinant adenoviruses AdMHVS (lane 3), AdMHVN (lane 4) and AdMHVM (lane 5). For reference, a similarly prepared immunoprecipitate from MHV-A59-infected *Sac*⁻ cells is shown in lane 1. The positions of the S and N proteins are indicated by arrows; the position of the different forms of the M protein is indicated by a bracket. Numbers on the left refer to M_r markers.

the expected MHV structural protein. AdMHVS specified a protein of approximately 150K (lane 3) which comigrated with the MHV spike precursor glycoprotein synthesized in MHV-A59-infected cells (lane 1). AdMHVN induced a protein of about 54K comigrating with the coronavirus nucleocapsid protein (lane 4). A characteristic set of proteins in the M_r range of 23K to 25K was expressed by the AdMHVM recombinant representing the unglycosylated and different *O*-glycosylated forms of the MHV M protein (lane 5). The expression of the MHV proteins appeared to be driven by the Ad5 E3 promoter or the major late promoter of

Ad5 and not by the SV40 promoter. This was concluded from the fact that the vector containing the MHV S gene in the reverse orientation with respect to the E3 promoter (AdMHVS2) did not detectably express the S protein (results not shown) and is consistent with results from several other similar Ad5 vectors (Graham & Prevec, 1991).

It is interesting to note that the Ad5 vector apparently can package over 4000 bp which is generally considered to be the limit the virus can incorporate. We have, however, indications that the uptake of approximately 4500 bp, as is the case with the MHV S expression cassette, was accompanied by the introduction of one or more deletions elsewhere in the genome.

Antibody induction by Ad5 recombinant viruses

To evaluate the immunogenicity of the MHV proteins expressed by the recombinant Ad5 viruses, infection experiments were performed in mice. BALB/c mice were immunized i.p. with the recombinant viruses AdMHVS, AdMHVN, AdMHVM and with wild-type Ad5, followed 2 weeks later by a booster immunization. After another 2 weeks, sera were prepared and analysed for the presence of specific antibodies in a RIPA using an MHV-A59-infected cell lysate as the antigen source (Fig. 3). Antibodies reactive with MHV structural proteins were detected with the AdMHVS virus (lane 4), the AdMHVN virus (lane 5) and also with the AdMHVM virus, although the response to the M protein was rather weak (lanes 6 and 7). Similar responses were observed as early as 2 weeks after single immunizations. Animals inoculated with wild-type Ad5 (lane 3) or injected with PBS (lane 2) failed to produce MHV-specific antibodies.

The sera were also analysed in a plaque reduction assay to detect virus-neutralizing antibodies. No neutralizing activity was detected in response to AdMHVM and AdMHVN, whereas a low neutralizing titre (10) was observed in response to AdMHVS and only in some mice. Such an incidental low response was also found when mice were immunized with a combination of AdMHVS and AdMHVN. Interestingly, only the N protein induced a significant titre when measured by ELISA (12 of 18 mice and five of six mice were positive in two independent experiments) or the IF test (eight of 15 and all of five mice in the same experiments). The S protein evoked no ELISA titres and only a weak IF signal (titre of 16 in one of three, and all of five mice, respectively). Immunization with AdMHVN plus AdMHVS yielded positive ELISA and IF titres in all mice tested but the results of these assays did not exceed background levels in the case of the M protein. Together the data indicate that although Ad5 replicates only poorly in mouse cells (Prevec *et al.*, 1989, 1990), the Ad5

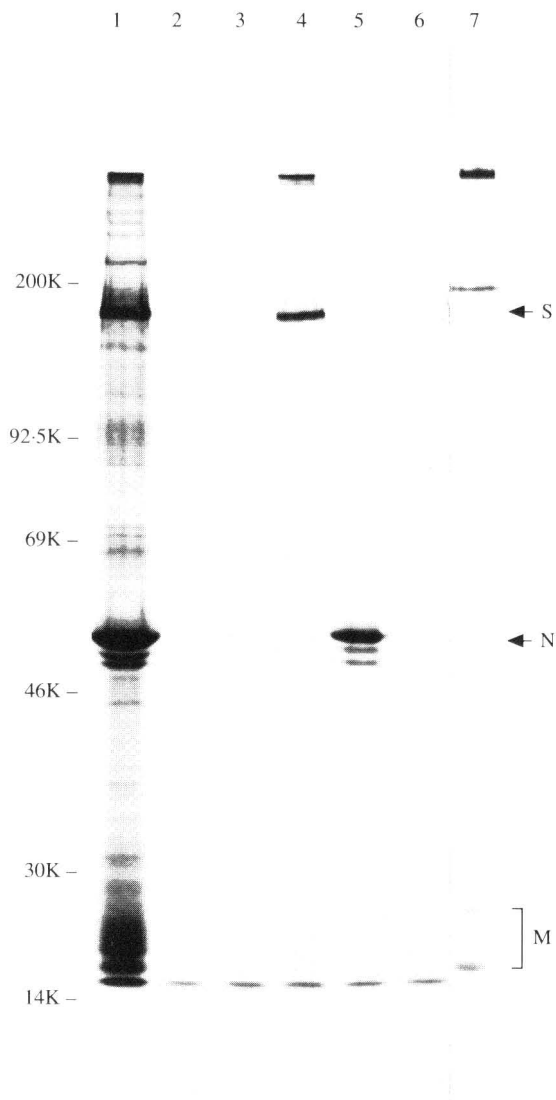


Fig. 3. Immunogenicity of expressed MHV-A59 proteins. Groups of five 4-week-old BALB/c mice were immunized with wild-type or recombinant adenovirus by injection of 1×10^7 p.f.u. followed 2 weeks later by a booster of 1×10^8 p.f.u. After another 2 weeks sera were collected, pooled for each group and tested for the presence of MHV-specific antibodies in an immunoprecipitation assay using a radio-labelled lysate of MHV-A59-infected Sac⁺ cells. The fluorograph shows the analyses of the sera from mice immunized with wild-type Ad5 (lane 3), AdMHVS (lane 4), AdMHVN (lane 5) and AdMHVM (lanes 6 and 7) and from mice injected with PBS only (lane 2). Lane 7 is a longer exposure of an immunoprecipitation similar to the one shown in lane 6. For reference, a precipitate prepared using a polyclonal anti-MHV serum is shown in lane 1. The positions of the MHV structural proteins S and N are indicated by arrows; the positions of the different forms of MHV M are indicated by a bracket.

recombinant viruses were capable of expressing MHV proteins to an extent sufficient to induce antibodies which in the case of the S protein can neutralize MHV *in vitro*.

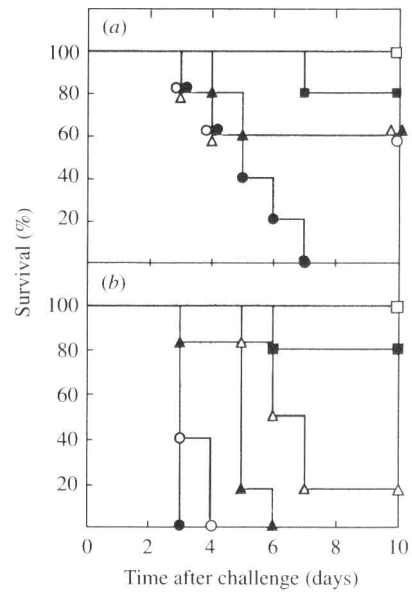


Fig. 4. Comparison of different MHV infection routes. Six groups of 8-week-old BALB/c mice ($n = 7$) were infected i.p. (a) or i.c. (b) with different doses of MHV-A59 ranging from 10^1 to 10^6 p.f.u. (\square , 10^1 p.f.u.; \blacksquare , 10^2 p.f.u.; \triangle , 10^3 p.f.u.; \blacktriangle , 10^4 p.f.u.; \circ , 10^5 p.f.u.; \bullet , 10^6 p.f.u.). Survival of the animals was monitored daily.

Challenge route of MHV-A59

To set up a challenge model for the evaluation of *in vivo* protection against MHV-A59 infection elicited by the recombinant Ad5 viruses, three different infection routes were tested. Intranasal inoculation resulted in variable survival rates and was generally ineffective: up to 10^6 p.f.u. of MHV-A59 was required before any mortality was detected (data not shown). Subsequently, we compared i.p. and i.c. inoculation. As Fig. 4 demonstrates, both routes had a dose-dependent effect which was, however, not linear in the case of the i.p. application: doses varying from 10^3 to 10^5 p.f.u. per animal yielded similar levels of survival (Fig. 4a). Moreover, this route was relatively ineffective: about 10^6 p.f.u. of MHV-A59 per animal was required to achieve 100% death (Fig. 4a), whereas 10^4 p.f.u. was sufficient for the same result when using i.c. inoculation (Fig. 4b). From Fig. 4(b) an LD_{50} value of about 500 p.f.u. was deduced for the i.c. MHV-A59 infection in BALB/c mice. Based on these results this route was chosen for subsequent challenge experiments.

Recombinant Ad5 virus-induced protection against MHV-A59

Several experiments were performed to determine the protective effect of the recombinant Ad5 viruses against lethal MHV challenge. In the experiment of Fig. 5, the

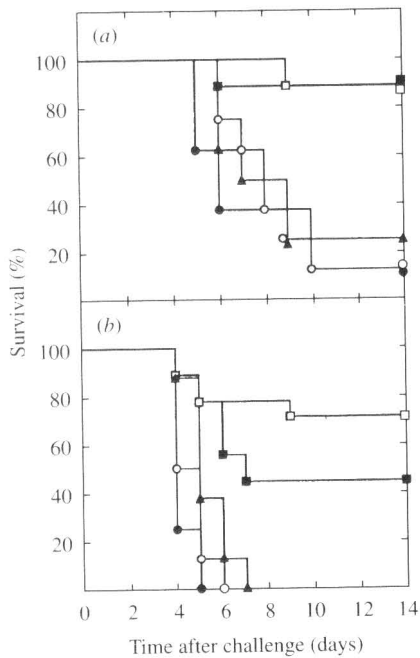


Fig. 5. Protective effects of recombinant adenoviruses expressing the MHV structural proteins against lethal infection with MHV-A59. Groups of 4-week-old BALB/c mice ($n = 20$) were inoculated i.p. with PBS (●) or with wild-type (○) or recombinant adenoviruses (▲, AdMHVM; □, AdMHVN; ■, AdMHVS) (5×10^6 p.f.u. per animal). After 2 weeks the animals were given a booster dose of 5×10^7 p.f.u. of virus. Two weeks thereafter each group was divided into two parts ($n = 7$ to 8), one of which was challenged i.c. with 10^4 p.f.u. MHV-A59 per animal (a) while the other received 10^5 p.f.u. (b). The mice were monitored daily for survival.

recombinant viruses were applied twice, in doses of 5×10^6 p.f.u. per animal (first immunization), and 5×10^7 p.f.u. given 2 weeks later. The mice were challenged i.c. 2 weeks after the booster immunization either with 10^4 p.f.u. MHV-A59 per animal (Fig. 5a) or with 10^5 p.f.u. MHV-A59 (Fig. 5b). The results show clearly that the recombinant viruses expressing the S and the N proteins did induce a protective immunity against MHV-A59. Most animals were protected when challenged with the lower MHV dose (10^4 p.f.u.), whereas 50% (AdMHVS) or more (AdMHVN) of the mice survived the higher dose. Those animals which survived the challenge showed symptoms of general malaise and neurological disease similar to those of the non-protected mice. These disorders gradually disappeared within about 2 weeks after infection. In contrast, no significant protective effect was seen for the recombinant Ad5 virus expressing the M protein.

To confirm the unexpected protection induced by the expressed N protein we extended the experiment to include immunizations with a combination of AdMHVN and AdMHVS. In addition, we tested the effect of booster immunizations. Finally, knowing that mice can

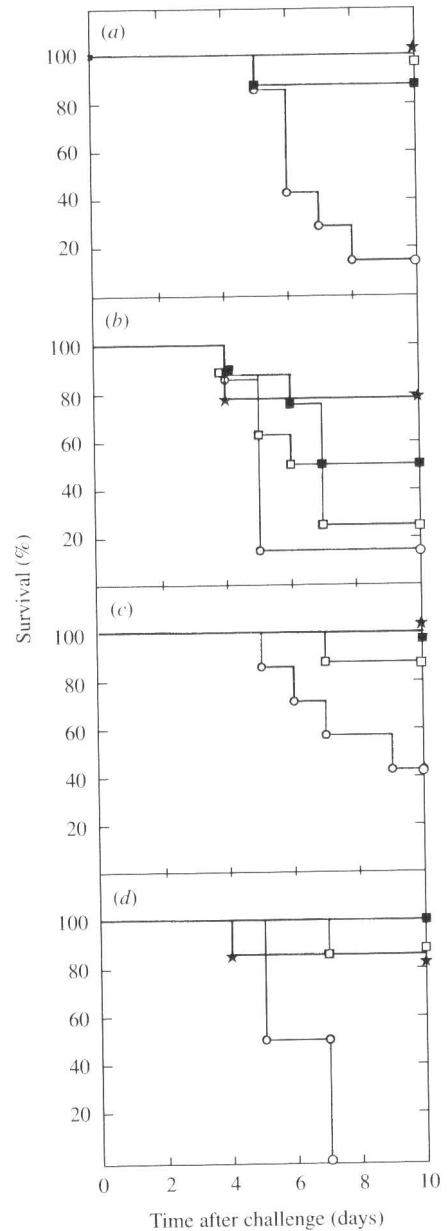


Fig. 6. Protective effects of recombinant adenoviruses expressing the N and S proteins of MHV. Four groups of 4-week-old BALB/c mice ($n = 14$ to 18) were immunized i.p. using 1×10^8 p.f.u. per animal with wild-type Ad5 (○), or AdMHVN (□), or AdMHVS (■) or with a 1:1 mixture of these two recombinant viruses (★). Three weeks later each group of animals was divided into two subgroups ($n = 7$ to 8), one of which was challenged i.c. with 10^4 p.f.u. MHV-A59 (a), the other with 10^5 p.f.u. MHV-A59 virus (b). To study the effect of boosting, four other groups of animals ($n = 14$ to 17) were immunized twice, first with 1×10^7 p.f.u. per animal followed 2 weeks later by 1×10^8 p.f.u. Two weeks after the booster each group of mice was again split ($n = 7$ to 8) and challenged i.c. either with 10^4 p.f.u. MHV-A59 (c) or with 10^5 p.f.u. MHV-A59 virus (d). The timing of the experiment was such that all animals were challenged on the same day.

cope with higher doses of adenoviruses without noticeable ill effects (McDermott *et al.*, 1989; Prevec *et al.*, 1990) we increased the vaccine dose to 1×10^8 p.f.u. per mouse (single immunizations) and 1×10^7 p.f.u. plus 1×10^8 p.f.u. per mouse (dual immunizations).

The combined results of the experiment are shown in Fig. 6. Both the expressed S protein and the N protein provided a strong protection against MHV-A59 infection when the animals received a low challenge dose [10^4 p.f.u.; (a, c)]. Protection levels of 100% were observed when mice were given a combined administration of AdMHVS and AdMHVN, even when the booster immunization was omitted (a, c). The level of protection against the high challenge dose (10^5 p.f.u.), on the other hand, was clearly improved when using a booster immunization regimen (b, d). Combined administration of the two recombinant viruses similarly protected the animals against MHV infection. This combination applied in a single immunization even enhanced the protective effect against the high challenge dose as compared to that elicited by the separately expressed N and S proteins (b). The results suggest that the exposure of epitopes of the N protein in combination with those of the S protein triggers the immune system in such a way that a better protection is achieved than with either of the MHV proteins individually.

Discussion

In this paper we describe the expression of the genes encoding the MHV structural proteins using Ad5 as a vector. The proteins produced in recombinant virus-infected HeLa cells were indistinguishable by their electrophoretic behaviour and recognition by specific antisera from those produced by the neurotropic A59 strain of MHV from which the inserted genes had been derived.

Inoculation of mice with the recombinant viruses elicited antibodies which specifically precipitated the S, N and M proteins of MHV, confirming the proper *in vivo* expression from each recombinant. Thus, although mice are considered semi-permissive for Ad5 replication (Prevec *et al.*, 1989), the levels of the MHV proteins induced were apparently high enough to trigger the immune system. From these and other results (Prevec *et al.*, 1989, 1990; McDermott *et al.*, 1989; Ginsberg *et al.*, 1991) it is obvious that human adenovirus vectors can effectively express antigens in non-human hosts.

The vaccination experiments demonstrate the efficacy of the recombinant viruses in inducing protection against lethal MHV infection. Remarkably, expression not only of the S protein but also of the N protein was effective. The protective effect of AdMHVS is in line with earlier

findings which showed that antibodies to the S protein can neutralize MHV *in vitro* (Collins *et al.*, 1982; Wege *et al.*, 1984; Talbot *et al.*, 1984; Gilmore *et al.*, 1987) and can protect against MHV infection after passive transfer (Buchmeier *et al.*, 1984; Wege *et al.*, 1984). Accordingly, protection was also observed after immunization either with the S protein (Hasony & MacNaughton, 1981; Daniel & Talbot, 1990) or with synthetic peptides derived from the protein (Talbot *et al.*, 1988; Koolen *et al.*, 1990).

The protective effect elicited by the recombinant adenovirus specifying the N protein was quite surprising. Data on the role of this protein in immunity against MHV are somewhat confusing. Whereas antibodies to N reportedly have no neutralizing activity *in vitro* (Buchmeier *et al.*, 1984; Talbot *et al.*, 1984; Nakanaga *et al.*, 1986; Gilmore *et al.*, 1987) a MAb to the protein prevented the c.p.e. of MHV in L cells (Lecomte *et al.*, 1987). This antibody as well as another, non-neutralizing MAb (Nakanaga *et al.*, 1986) protected mice from acute disease, in contrast to the lack of passive *in vivo* protection reported with several other anti-N antibodies (Hasony & MacNaughton, 1981; Buchmeier *et al.*, 1984; Talbot *et al.*, 1984). The mechanism by which the N protein in our studies provided such a strong protection is unclear. It did induce antibodies but these could not be demonstrated to neutralize MHV *in vitro*. As we are presenting the N protein in the animals through a replicating expression vector we speculate that the protection may involve a cell-mediated defence mechanism, e.g. cytotoxic T lymphocyte (CTL) activity or T cell-derived antiviral cytokines.

Cell-mediated immunity is probably required for control of MHV infection as adoptive transfer of MHV-specific CD4⁺ and CD8⁺ cells was shown to mediate virus clearance and to prevent virus-induced acute encephalitis (Sussman *et al.*, 1989; Yamaguchi *et al.*, 1991). Accordingly, *in vivo* depletion experiments using T cell-specific antibodies also showed that both CD4⁺ and CD8⁺ T cells are required for clearance of MHV (strain JHM) from the central nervous system of mice (Williamson & Stohlman, 1990). CTL recognition of the N protein after MHV infection has been described (Stohlman *et al.*, 1992). In rats a strong CD4⁺ T cell response specific for N was detected during acute infection with the JHM strain, and these T cells conferred protection to challenge after transfer (Körner *et al.*, 1991). CD4⁺ cells provide help for B cells. Stimulation of N-reactive helper T cells supporting virus-specific B cell responses might account for the enhancement of protection that we observed after combined immunization with the N and S proteins (Fig. 6b).

In our studies the expressed M protein, although inducing specific antibodies, failed to protect against

MHV challenge. This observation is at variance with the data of Fleming *et al.* (1989) who showed that two M-specific MAbs, one of which was neutralizing *in vitro*, were able to protect mice passively against a lethal challenge. The reason for this discrepancy is unclear but it might merely reflect the observed weak immune response to the expressed protein.

The data presented demonstrate the usefulness of the recombinant adenovirus system in the analysis of the immune responses to MHV infection. It will now be interesting to define further the nature of the protective immune responses, in particular the response to the N protein. In addition, due to the features of the carrier these recombinant adenoviruses are very attractive tools for studying the role of mucosal immunity in protection against natural respiratory and enteric MHV infections.

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