Induction of protective immunity against coronavirus-induced encephalomyelitis: evidence for an important role of CD8+ T cells in vivo*

Coronavirus MHV-JHM infections of rats provide useful models to study the pathogenesis of virus-induced central nervous system disease. To analyze the role of the immune response against defined MHV-JHM antigens, we tested the protective efficacy of vaccinia virus (VV) recombinants expressing either the nucleocapsid (N) or the spike (S) protein. A strong protection was mediated in animals by immunization with recombinant VV encoding a wild-type S protein (VV-Swildtype), whereas VV recombinant expressing a mutant S354C protein (VV-S354C) had no protective effect. Recombinant VV encoding N protein (VV-N) induces a humoral and a CD4+ T cell response, but did not prevent acute disease regardless of the immunization protocol. In these experiments, challenge with an otherwise lethal dose of MHV-JHM was performed prior to the induction of virus-neutralizing antibodies and studies with the anti-CD8+ monoclonal antibody, MRC OX8 showed that elimination of the CD8+ subset of T cells abrogates the protective effect. This result indicates that CD8+ T cells primed by recombinant VV expressing wild-type S protein are a primary mechanism of immunological defense against MHV-JHM infection in rats.

1 Introduction

Coronavirus infections of the central nervous system (CNS) are interesting models to analyze mechanisms of inflammatory demyelination and virus persistence. In particular the murine coronavirus strain MHV-JHM induces different courses of encephalomyelitis in mice or rats [1, 2].

In Lewis rats, different forms of the disease have been observed [3-7]. The acute encephalomyelitis (AE) is a rapidly progressing disease which leads to death of the animal. The subacute demyelinating encephalomyelitis (SDE) is a paralytic disease characterized by selective loss of myelin and inflammations in the white matter of the CNS.

The host’s immune response to MHV-JHM plays a critical role in protection from acute disease and also in modulating the development of chronic disease associated with demyelination. Both cellular (T cell-mediated) and humoral (B cell-mediated) responses are involved, although the role of the different components of the immune system with regard to defined virus proteins and the course of the infection is not clear. There is evidence that the passive transfer of antibodies specific for the spike (S) protein of the virus can provide protection from lethal infection. Moreover, anti-viral antibodies can alter the phenotype of CD8+ T cells in vivo* disease in mice and rats [8-11]. Also, protective neutralizing antibodies can be induced by immunization of mice with purified S protein or an S protein decapeptide [12, 13]. These results implied that the humoral immune response may be sufficient for the observed protection against MHV-JHM infection.

A T cell-mediated immunity is also believed to be crucial for the control of coronavirus infections and in adoptive transfer experiments with CD4+ T cells, protection against MHV-JHM infections has been demonstrated [14-18].

In the experiments reported here we have used recombinant vaccinia viruses (VV) expressing specific MHV proteins to determine the ability of different viral antigens to induce antiviral immunity early after immunization. Furthermore, by the depletion of CD8+ T cell subsets in vivo, we have analyzed the role of these cells in the protective immune response in infected rats.

2 Materials and methods

2.1 Construction and expression of MHV-JHM proteins using VV recombinants

cDNA copies of the MHV-JHM nucleocapsid (N) protein gene [19] and the S protein gene [20] were cloned into the SmaI site of the transfer vector pSC11 (kindly provided by B. Moss, National Institute of Health, Bethesda, MD). Two different S gene cDNAs were used. First, a “wild-type” S gene cDNA which differs from the S gene sequence published by Schmidt et al. [20] at positions 793: G to C; 794: C to G; 1855: A to G and 3281: C to T. The S protein expressed from this gene is fusogenic [21]. Second, a variant S gene cDNA which differs from the published sequence at positions 793: G to C; 794: C to G; 1090: T to C and 1855: G to T. The S protein expressed from this gene is not fusogenic [unpublished observation]. These plasmids were used to introduce each of the MHV-JHM genes into
the thymidine kinase (TK) gene of wild-type VV (VV-wt) by homologous recombination [22]. The recombinants are referred to as VV-N, VV-S\textsubscript{wildtype} and VV-S\textsubscript{354CR}, expressing the N, S or non-fusogenic variant S protein, respectively.

The expression of MHV-JHM proteins by VV-N, VV-S\textsubscript{wildtype} and VV-S\textsubscript{354CR} was tested by immunofluorescence. For indirect immunofluorescence cultures of DBT cells were infected with VV-wt, VV-S\textsubscript{wildtype} and VV-S\textsubscript{354CR} at a multiplicity of infection (m.o.i.) of 1 [10, 21, 23, 24]. The cells were fixed with 3.5% buffered formaldehyde and incubated with monoclonal antibodies against MHV-JHM-N or S proteins, followed by incubation with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Dako, Copenhagen, Denmark).

2.2 Animals, virus and immunization protocol

Specified pathogen-free Lewis rats (MHC-RT1) were purchased from the Zentralinstitut für Versuchstierzucht Hannover, FRG. Lewis rats (3 to 4 weeks old) were inoculated either once (single shot immunization) or three to four times (multiple shot immunization) at 5- to 6-day intervals by intraperitoneal (i.p.) injections of 1 \times 10^7 to 7.5 \times 10^7 PFU of VV-recombinant. Seven days after single shot immunization or 4 days after the last VV inoculation, the animals were challenged by intracerebral (i.c.) inoculation with 5x or 32x LD\textsubscript{50} of MHV-JHM in a volume of 40 \textmu l into the left brain hemisphere. For this purpose, we used a MHV-JHM wild-type virus that was passaged three times in mouse brain and twice in rat brain [3]. This virus was designated as SM3SR2. Animals were observed for the appearance of clinical signs of encephalomyelitis. Lewis rats were dissected for histologic examination in the brain at various intervals post-infection (p.i.) [3, 7].

2.3 Serologic assays and virus isolation

Antiviral IgG in the serum of infected animals was measured by ELISA on microplates coated with purified virus AG as described previously [6, 10]. The ratios were calculated relative to a graph obtained with a standard antiserum from MHV-JHM immunized rats. The protein specificity of antiviral antibodies was tested by immunostaining of Western blots [23]. As Ag we used purified MHV-JHM virus which was separated by 15% PAGE-SDS and electroblotted onto nitrocellulose membranes. Virus-neutralizing antibodies were measured by plaque number reduction assays on DBT cells. The antibody titer resulting in 50% reduction of plaque number (PRD\textsubscript{50}) was calculated as described previously [10, 23]. For the detection of infectious virus in homogenates of CNS tissue, DBT cell cultures were inoculated with the virus-suspension in 24-well cluster plates. The number of plaques were counted to determine the amount of virus present in the brain [23].

2.4 Depletion of CD8\textsuperscript{+} T cells in vivo

Lewis rats vaccinated with VV-recombinants were depleted by i.p. inoculation with anti-CD8 mAb, MRC OX8 at a concentration of 3-5 mg IgG/ml [17, 25]. One dose (200 \textmu l) was given 3 days before infection, followed by a second dose (200 \textmu l) 1 day prior to MHV-JHM infection. The efficiency of the depletion was monitored by FACS\textsuperscan analysis (Hewlett-Packard) in control rats 13 days post-MHV-JHM challenge. Superficial cervical lymph nodes were prepared as single-cell suspensions and stained with antibody (tissue culture supernatant), OX8 (CD8 specific) and isotype control antibody OX21 (human C3b inactivator) against rat T cell surface molecules [25].

3 Results

3.1 Expression of MHV-JHM proteins using VV recombinants

The expression of the MHV-JHM N, S\textsubscript{wildtype} and S\textsubscript{354CR} proteins was confirmed by indirect immunofluorescence using mAb against MHV-JHM N or S protein, respectively (Fig. 1). Diffuse membrane staining and extensive syncytia formation was observed in DBT cells infected with VV recombinants VV-N (b), VV-S\textsubscript{354CR} (c) and VV-S\textsubscript{wildtype} (d). The cells were incubated with the corresponding anti-MHV-JHM monoclonal antibodies followed by fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin; (a) no staining of VV-wt infected cells with a mixture of anti-N and anti-S monoclonal antibodies (control); cytoplasmatic staining with anti-N monoclonal antibodies (b) and anti-S monoclonal antibodies (c); diffuse membrane staining with anti-S monoclonal antibodies with syncytia formation (d).
formation caused by the fusogenic activity of recombinant S protein was seen in VV-Swildtype-infected DBT cells. By contrast, N or S protein was localized in the cytoplasm of VV-N- or VV-S354CR-infected DBT cells.

### 3.2 Induction of MHV-JHM protein-specific humoral immune responses

The potential of the recombinant VV to induce antibodies specific for MHV-JHM structural proteins was determined by vaccination of Lewis rats. Pooled serum samples were tested by ELISA for antiviral IgG and virus neutralizing activity was monitored. The protein specificity was demonstrated by immunostaining of Western blots with purified MHV-JHM virus as Ag. The fusogenic VV-Swildtype recombinant induces a humoral immune response after a single shot immunization (Table 1). A high level of S-specific antibodies with virus-neutralizing activity was present 21 days after immunization. By contrast, the VV recombinant expressing the non-fusogenic S protein does not induce any specific humoral immune response, whether in a single shot or in multiple shot vaccination. The VV-N recombinant elicits a low level of nucleocapsid-specific antibodies only after four immunizations. In the neutralization assays, these antibodies have no virus-neutralizing activity.

### 3.3 Influence of VV recombinants on MHV-JHM-induced encephalomyelitis

To evaluate the protective effect of individual coronavirus proteins on MHV-JHM-induced encephalomyelitis, adult Lewis rats were vaccinated intraperitoneally with recombinant VV or VV-wt 7 days prior to intracerebral (i.c.) infection with a lethal dose (5x LD50) of MHV-JHM (SM3SR2). Uninfected or VV-wt vaccinated animals and Lewis rats immunized with VV-N or VV-S354CR developed an acute encephalomyelitis within 7–9 days after MHV-JHM challenge (5x LD50) and died. In contrast, most animals vaccinated once with VV-Swildtype overcame this acute phase even when a higher MHV-JHM challenge dose (32x LD50) was applied (Table 2).

In the attempt to increase the protective effect of VV-N or VV-S354CR immunization, rats were injected four times with the respective VV recombinants and challenged i.c. 4 days after the last VV inoculation with a lethal dose of MHV-JHM. No protective effect was observed. The rats immunized with either VV-wt or VV-S354CR and VV-N invariably developed an acute lethal encephalomyelitis.

### Table 2. Protection of VV-Swildtype immunized rats from MHV-JHM induced acute encephalomyelitis

| VV-R immun.a) | No. of immun. | No. of rats | MHV-JHM challenge | Dead/Total |
|---------------|---------------|-------------|-------------------|-----------|
| –             | 1 × 6         | 5 × LD50    | 6/6               |
| VV-wt         | 1 × 14        | 5 × LD50    | 14/14             |
|               | 4 × 4         | 32 × LD50   | 4/4               |
| VV-N          | 1 × 18        | 5 × LD50    | 18/18             |
|               | 4 × 6         | 32 × LD50   | 6/6               |
| VV-S354CR     | 1 × 10        | 5 × LD50    | 10/10             |
|               | 4 × 6         | 32 × LD50   | 6/6               |
| VV-Swildtype  | 1 × 26        | 5 × LD50    | 5/26              |
|               | 14            | 32 × LD50   | 3/14              |

a) Lewis rats were immunized with VV recombinants and challenged with 5 or 32 × LD50 of MHV-JHM 7 days later. For multiple shot immunization the rats were immunized four times at 5- to 6-day intervals with VV recombinants. Four days after the last VV immunization the animals were infected with MHV-JHM.

In the neutralization test (NT) virus infectivity was measured by cultivation of homogenized brain tissue on DBT cells. At day 6 p.i. the brain tissue of rats contained 2.1 × 10⁴ PFU of MHV-JHM after prior vaccination with VV-wt. By contrast, in animals immunized with VV-Swildtype no infectious virus was detected after 6, 10 and 18 days after the i.c. challenge. At 3 days p.i. brain tissue from only one out of five Lewis rats yielded a single MHV-JHM virus plaque. These observations indicate that the clearance of infectious MHV-JHM from the CNS occurs very fast if rats were primed by VV-Swildtype immunization.

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### 3.4 Virus elimination and humoral immune response after MHV-JHM challenge in VV-Swildtype-protected rats

Virus reisolation was performed at various time points p.i. by cultivation of homogenized brain tissue on DBT cells. At day 6 p.i. the brain tissue of rats contained 2.1 × 10⁴ PFU of MHV-JHM after prior vaccination with VV-wt. By contrast, in animals immunized with VV-Swildtype no infectious virus was detected after 6, 10 and 18 days after the i.c. challenge. At 3 days p.i. brain tissue from only one out of five Lewis rats yielded a single MHV-JHM virus plaque. These observations indicate that the clearance of infectious MHV-JHM from the CNS occurs very fast if rats were primed by VV-Swildtype immunization.

The antiviral and neutralizing antibody response of serum samples of rats immunized with VV-Swildtype were analyzed in a plaque number reduction assay and ELISA test. Control rats which were vaccinated with VV-wt and then challenged with MHV-JHM developed no measurable response.
humoral immune response against MHV-JHM and died 7 to 9 days after infection. The antiviral antibody response of VV-Swildtype-immunized rats was significantly enhanced following MHV-JHM challenge compared to sera from unchallenged rats at the same time points. In agreement with these findings the MHV-JHM challenge resulted also in an earlier raise and increased amount of virus-neutralizing antibodies. However, infectious virus was already eliminated from brain tissue before detectable amounts of neutralizing antibodies were measured.

3.5 Effect of CD8+ T cell depletion in rats immunized by VV-Swildtype and challenged with MHV-JHM

If activated CD8+ T cells are important for restricting the spread of MHV-JHM virus, their depletion should abrogate the protective immune response. We depleted the CD8+ T cell repertoire by treatment of VV-Swildtype immunized rats with the CD8-specific mAb OX8. The efficiency of the CD8+ depletion was monitored by FACS analysis of cervical lymph node cells obtained from control rats 13 days after challenge with MHV-JHM. This CD8 depletion protocol resulted in a physical loss of CD8+ T cells.

In the absence of CD8+ T cells, VV-Swildtype immunized rats did not survive an intracerebral challenge with a lethal dose of MHV-JHM (Table 3). Despite immunization with VV-Swildtype the CD8-depleted rats developed an acute encephalomyelitis and died within the same time as control rats when challenged with 5 × LD50 of MHV-JHM.

4 Discussion

To analyze the contribution of the different virus proteins for cellular and humoral immunity during the course of coronavirus infections, we immunized Lewis rats selectively with VV recombinants to establish antiviral immunity. We challenged the vaccinated rats with MHV-JHM at a time point before virus-neutralizing IgG antibodies are detectable. Our results indicate that the presence of CD8+ T cells is an indispensable requirement to overcome an infection with MHV-JHM and that the cellular immune response is primarily responsible for protection and virus elimination during the acute phase of the disease process. Furthermore, we detected no pathological changes in histological analysis of brain tissue from those rats dissected at various time points after MHV-JHM challenge (data not shown). We assume therefore that neutralizing antibodies are not the essential factor in mediating protection during the acute stage of disease. This conclusion is also supported by the observation that antibodies produced within the CNS of MHV-JHM-infected rats do not prevent the onset of acute viral encephalomyelitis [26, 27].

In mice, CD8+ CTL are the predominant effector cells for elimination of infected cells [15, 16]. To find out whether activated, S protein-specific CD8+ T cells are capable of restricting virus spread, CD8+ T cells were eliminated from VV Swildtype-immunized rats by treatment with monoclonal antibodies. Similar to VV-wt-immunized control rats, animals which were CD8 depleted died within 7 to 9 days after MHV-JHM challenge. In experiments employing a less efficient depletion of CD8+ T cells we observed that VV-Swildtype-immunized rats remained protected if a low percentage of CD8+ T cells were detectable (data not shown). These results indicate that in the rat system CD8+ MHV-JHM S protein-specific lymphocytes contribute to protection and virus clearance.

A pronounced protective immunity was induced by vaccination with the fuscogenic VV-S recombinant. Surprisingly, by vaccination with VV-S354CR we could not induce a protective immunity regardless of the vaccination protocol. Preliminary experiments indicate that within the cell compartment this variant S protein is not processed beyond the gp150 precursor stage (data not shown). We assume therefore that the antigen processing for this protein does not result in an immune response which is relevant for protection.

In a number of virus-host systems the immune response against the nucleocapsid protein alone can mediate protection [28-32]. The results vary to some extent, if different systems and immunization schedules are considered. For example, immunization with purified influenza nucleoprotein (NP) can induce protection, whereas vaccination with a VV-recombinant expressing NP induced no protective immunity [33, 34]. In our experiments, a nucleocapsid-specific humoral and CD4+ T cell response was induced by VV-N [18]. A low delay in death was noticed in VV-N vaccinated rats compared to control animals (data not shown). But nevertheless vaccination with the VV-N recombinant did not prevent acute disease regardless of the immunization protocol. However, we assume that the N protein is a dominant T cell antigen in rats and mice [17, 18, 35].

The protection mediated by VV-Swildtype is dependent on the presence of S-specific CD8+ T cells and without essential participation of antibodies. As an indirect measure for a specific CD4+ T cell response, we established T cell lines from VV recombinant immunized rats and measured their specificity by proliferation assays. Although S-specific CD4+ T cells are demonstrable in lymphoid organs from rats diseased with SDE [17], no such response was observed following immunization with VV-Swildtype and VV-S354CR. By contrast, VV-N elicits a specific CD4+ T cell response [18]. Furthermore, the biological potential of activated, MHV-JHM protein-specific CD4+ T cells to mediate protection was revealed by transfer experiments [17, 18].
Therefore, for unprimed rats efficient virus elimination and survival of the acute disease stage require both CD4+ and CD8+ T cell interactions, whereby the relative impact of each subset may strongly depend on the epitope specificity for each MHV protein.

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