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Measles Virus Nucleocapsid Protein Protects Rats from Encephalitis

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Lewis rats immunized with recombinant vaccinia virus expressing the nucleocapsid (N) protein of measles virus were protected from encephalitis when subsequently challenged by intracerebral infection with neurotropic measles virus. Immunized rats revealed polyvalent antibodies to the N protein of measles virus in the absence of any neutralizing antibodies as well as an N protein-specific proliferative lymphocyte response. Depletion of $CD8^+$ T lymphocytes did not abrogate the protective potential of the N protein-specific cell-mediated immune response in rats, while protection could be adoptively transfered with N protein-specific $CD4^+$ T lymphocytes. These results indicate that a $CD4^+$ cell-mediated immune response specific for the N protein of measles virus is sufficient to control measles virus infections of the central nervous system.

Measles is a highly contagious disease which can be prevented by a live attenuated measles virus (MV) vaccine. Nevertheless, the infection is not controlled in most parts of the world, and up to two million patients still die annually from measles (20). Most measles-associated deaths result from bacterial superinfections of the respiratory and gastrointestinal tracts. However, central nervous system (CNS) complications occur in about 1 in 1,000 children and remain a significant cause of permanent neurological morbidity and mortality. In addition, MV is etiologically involved in subacute sclerosing panencephalitis (SSPE), which develops after persistent MV infection in brain tissue (1, 26). Moreover, a rise in measles cases was recently observed in industrialized countries. More than 50% of these measles cases were seen among previously vaccinated patients (5).

While the role of the humoral measles-specific immune response, particularly against the viral hemagglutinin (H) and fusion (F) glycoproteins, for the prevention of measles is well established, little is known about cell-mediated immune (CMI) reactions. Clinical studies have shown that overcoming acute measles requires a competent CMI system to clear the MV infection. Although it was shown that lymphocytes of human high responders can be stimulated to proliferate when cultured in the presence of purified MV H, F, phosphoprotein (P), nucleocapsid (N), and matrix (M) proteins (22), data are not available about the role of individual MV structural proteins for the induction of CMI reactions and its involvement in acute measles as well as in MV complications of the CNS. The aim of the present study was to investigate the effect of an immune response to the MV N protein expressed by recombinant vaccinia virus (VV) with regard to MV-induced encephalitis in Lewis rats.

MATERIALS AND METHODS

Construction of VV recombinants. VV strain Copenhagen and the temperature-sensitive mutant *ts7* (obtained from A. von Brunn, Deutsches Krebsforschungzentrum, Heidelberg, Germany) was used to obtain VV recombinants expressing the MV N protein. The 1,720-bp *Not*I-*Xho*I fragment of the MV *N* gene of strain CAM/R40 was excised from a Bluescript vector (4) and inserted downstream of the p7.5 early/late promoter into the transfer vector pSC11 (a kind gift from B. Moss, National Institutes of Health, Bethesda, Md.). The resultant pSC11-CN recombinant plasmid was used to insert the MV *N* gene into the thymidine kinase gene of wild type VV (VV-wt) by homologous recombination (18), creating VV-MVN.

The VV-MVN-directed expression of the MV N protein was tested by Western immunoblotting, radioimmunoprecipitation (RIPA), and indirect immunofluorescence. Lysates prepared from infected and noninfected Vero cells were separated by 12.5% polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulfate (SDS), electroblotted onto Immobilon transfer membranes (Millipore), and immunostained with absorbed human serum from a patient with SSPE that contained high amounts of MV-specific antibodies or with monoclonal antibodies directed against the N protein, respectively. For RIPA, Vero cells infected with VV-wt and the recombinant VV-MVN were pulse labeled with [^{35}S]methionine as described before (21). Cell lysates were immunoprecipitated with polyclonal or monoclonal antibodies directed against MV or the MV N protein. Parallel VV-wt and VV-MVN-infected Vero cells were processed for immunofluorescence, actone fixed, and incubated with antibodies against MV or the MV N protein, respectively, followed by fluoroisothiocyanate conjugates.

Animals and experimental protocol. Lewis rats were purchased from the Zentralinstitut für Versuchstiere, Hannover, Germany, and kept under specific-pathogen-free conditions in the animal facilities of the Institut für Virologie und Immunbiologie, Würzburg, Germany. Weanling rats were immunized either once or twice (at 5- to 7-day intervals) by intraperitoneal (i.p.) injections of 10^7 PFU of VV-wt or VV-MVN. Five days after immunization or 3 days after the booster injection, the animals were challenged by intracerebral (i.c.) infection with MV (volume, 25 μl). For this purpose we used our neurotropic rat brain-adapted MV strain CAM/R40 as a 10% homogenate of newborn rat brain (CAM/RBH) (15). The titer of our stock preparation was 3.2×10^5 50% tissue-culture-infective doses ($TCID_{50}$) per ml. A dose of 0.5×10^4 $TCID_{50}$ induced fatal encephalitis in 3- to 4-week-old Lewis rats (17).

Animals were weighed daily beginning on the day of i.c. MV infection (challenge) and were observed for the occurrence of clinical signs of encephalitis. The symptoms were graded on a scale from 0 to 4: 0, no signs; 1, abnormal

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posture, ruffled fur, slight weight loss; 2, convulsions, severe weight loss; 3, paresis, incontinence, lethargy; 4, death. For analysis of the neuropathological correlate to clinical disease, animals were killed when they exhibited grade 2+ symptoms or at variable intervals postinfection (p.i.). The brain and spinal cord were fixed in buffered paraformaldehyde, paraffin embedded, cut, and stained with hematoxylin and eosin.

Depletion of CD8$^+$ T lymphocytes. In order to define which lymphocyte subpopulation restricts virus spread in brain tissue, CD8$^+$ T cells were depleted from animals by an approach similar to that described previously (23). Starting 1 day before MV infection, rats were injected i.p. on 3 consecutive days with 100 μl of mouse ascites containing antibodies directed against the rat CD8 molecule (OX8, immunoglobulin G1 [IgG1] subclass). The efficacy of the antibody treatment was determined retrospectively by fluorescence-activated cell sorting (FACS) analysis in MVN-immunized and MV-infected rats at the end of the experiment, when the animals died or were killed. Superficial cervical lymph nodes were prepared as single-cell suspensions and stained with antibodies against rat T-cell surface molecules, particularly R73 (against α/β T-cell receptor [TCR]), W3/25 and OX35 (both CD4 specific), OX8 (CD8 specific), and control OX12 (κ chain of immunoglobulin) as well as a monoclonal antibody directed against the S protein of JHM coronavirus (obtained from Helmut Wege, Würzburg, Germany).

Determination of MV-specific immune response. For determination of humoral immunity, serum samples were taken by tail vein puncture before MV infection and at various intervals thereafter. The titers of antiviral antibodies were determined by an enzyme-linked immunosorbent assay (ELISA) and the MV neutralization test based on plaque reduction as described before (17). The presence of cytotoxic antibodies was tested by a ^{51}Cr release assay with persistently MV-infected lung fibroblasts as described previously (13). The specificity of the antiviral antibodies for MV structural proteins was further analyzed by immunostaining of MV that was separated by PAGE prior to Western transfer onto nitrocellulose membranes or by their ability to immunoprecipitate [^{35}S]methionine-labeled MV-infected Vero cell lysates.

The MV-specific cellular immune response in the presence of purified measles virions or N protein was analyzed in cell preparations of lymph nodes from different animals before and after MV infection in a lymphoproliferation assay as described before (16). Briefly, single-cell suspensions (1×10^5 to 3×10^5) from spleen and the mesenterial and cervical lymph nodes were incubated in microtiter plates with optimal concentrations of antigen (UV-inactivated MV, purified MV N protein, and tuberculin protein as a control) for 72 h with an 18-h pulse of 0.2 μCi of [^{3}H]deoxythymidine ([^{3}H]dT) per well. The lymphoproliferative response (LPR) was determined by the ratio of thymidine incorporation in stimulated versus unstimulated cells and expressed as the stimulation index. When polyclonal lymphocyte populations were assayed, stimulation indices above 2 were considered specific.

CD4$^+$ T-cell lines were isolated from bulk populations by alternating cycles of expansion in interleukin-2 (IL-2)-containing medium and of restimulation with purified MV N protein as described before (15). The specificity was analyzed in the LPR assay, and the surface phenotype of the cell lines was determined after the third in vitro restimulation by FACS analysis. CD4$^+$ MV N-specific T-cell lines were adoptively transferred during the third or fourth in vitro restimulation-expansion cycle.

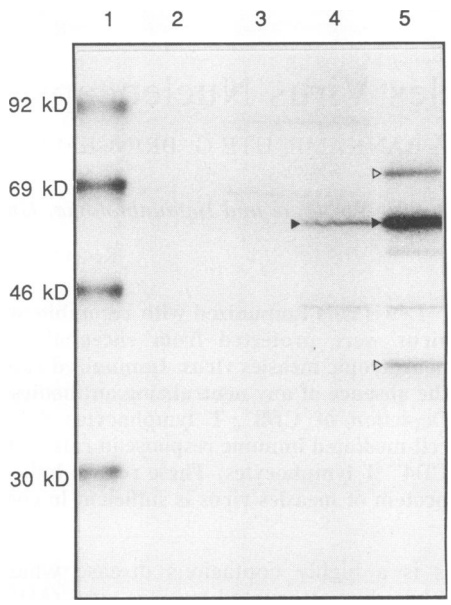

FIG. 1. Immunoprecipitation of [^{35}S]methionine-labeled Vero cell lysates with VV-wt-absorbed human serum from a patient with SSPE. Lysates were prepared from Vero cells (lane 2) or Vero cells infected with VV-wt (lane 3), VV-MVN (lane 4), or MV (lane 5). Molecular mass markers are in lane 1. Solid triangles mark the position of MV N protein at 60 kDa; open triangles show the H (78 kDa) and F_1 (41 kDa) proteins. Additional bands below 60 kDa represent major degradation products of MV N. The faint bands above 64 kDa in lanes 3 and 4 are nonspecific background.

MV isolation from brain material. MV replication in the CNS of the animals was assessed by cocultivation of fresh brain material with Vero cells as described before (17). Negative results were accepted after 3 weeks with three to four subcultivations.

RESULTS AND DISCUSSION

The entire coding sequence of the *N* gene of MV strain CAM/R40 was inserted into the thymidine kinase gene of VV-wt strain Copenhagen. Vero cells infected with the recombinant VV-MVN exhibited typical VV plaque morphology and revealed MV N protein in their cytoplasm by indirect immunofluorescence when incubated with MV-specific polyclonal or monoclonal antibodies against the MV N protein (not shown). Immunoprecipitation of ^{35}S-labeled VV-MVN-infected Vero cell lysates with serum from a patient with SSPE showed a band corresponding to a 60-kDa protein comigrating with the authentic MV N protein (Fig. 1).

The immunogenic potential of the recombinant N protein was analyzed by vaccination of Lewis rats with VV-MVN. N-specific T-cell LPR was demonstrable in the polyclonal lymphocyte cultures from spleen and mesenterial lymph nodes 5 days later, and MV-specific serum antibodies were detectable by ELISA with titers in the range of 1:200 to 1:1,600. The sera exhibited monospecificity for the MV N protein in Western blot analyses (Table 1). MV-neutralizing or cytotoxic antibodies were not detectable. Control rats immunized with VV-wt or mock-infected developed no MV-specific antiviral immune response.

TABLE 1. Immune response in Lewis rats following immunization with recombinant VV and infection with MV

Animals[a]	Immunization	Serum samples taken (day postimmunization)	ELISA titer[b]	NT titer[b]	MV protein specificity[c]					LPR[d] (stimulation index)
					H	P	N	F	M	
VV immunized	Mock	NA[e]	<50	<20	−	−	−	−	−	0.9 ± 0.4
	VV-wt	8–9	<50	<20	−	−	−	−	−	0.7 ± 0.4
	VV-MVN	8–9	200–1,600	<20	−	−	+	−	−	5.1 ± 0.8
VV immunized, MV challenged	Mock	6–14	50–200	<20	−	−	+	−	−	0.7 ± 0.5
	VV-wt	6–14	50–200	<20	−	−	+	−	−	1.2 ± 1.3
	VV-MVN	6–15	400–6,400	<20	−	−	+	−	−	4.7 ± 0.6

[a] Rats were immunized twice with either VV-wt or VV-MVN at intervals of 5 days. One group was challenged with neurotropic MV 3 to 4 days after the second booster immunization.
[b] The reciprocal ELISA and neutralization test (NT) titers shown were obtained in an ELISA with whole purified MV virions as coat antigens and in an MV NT (17).
[c] The specificity of MV antiviral antibodies was investigated by Western blot analysis.
[d] T-cell LPR is expressed as the stimulation index (ratio of incorporation of [^{3}H]dT in the presence and absence of purified MV N protein; mean ± standard deviation; three to five trials) (15).
[e] NA, Not applicable.

In order to investigate the protective effect of this immune response for measles CNS infection, weanling Lewis rats were i.p. vaccinated with VV-MVN or with VV-wt prior to i.c. infection with CAM/RBH neurotropic MV. The infectious dose used for challenge with MV was in the range of 0.5×10^4 to 8×10^4 $TCID_{50}$ per animal. All control rats exhibited an acute encephalitis with incubation periods of 12 ± 2 days and either died or were killed when they showed severe neurological symptoms. In contrast, animals vaccinated with VV-MVN did not exhibit symptoms during the 4-week observation period even with the highest MV challenge dose (Table 2). The humoral immune response that had been induced by immunization with VV-MVN was enhanced after the challenge infection with CAM/RBH, but MV-neutralizing antibodies were not detectable (Table 1).

The significance of the CMI response for virus spread and its consequences for the CNS MV infection were further investigated by attempts to isolate MV from fresh brain material by cocultivation with Vero cells. No infectious MV could be isolated from VV-MVN-vaccinated rats at any time point between days 3 and 21 p.i. (Table 2). From all controls, however, i.e., mock- and VV-wt-immunized rats, MV could be reisolated by cocultivation from day 4 p.i. onward, irrespective of the occurrence of clinical signs of encephalitis. These observations suggest that the MV infection is cleared within 72 h from the CNS of the VV-MVN-vaccinated rats.

This interpretation is supported by neuropathological investigations which revealed uniform disseminated encephalitis in all nonprotected rats, i.e., animals that were immunized with VV-wt or left untreated. Severe inflammatory and degenerative lesions involved both grey and white matter of the cerebral hemispheres, basal ganglia, and, to a lesser degree, brain stem. In contrast, approximately 50% of the VV-MVN-immunized rats had no detectable neuropathologic lesions (Table 2). The histopathological analysis of brain samples taken 3 to 4 weeks after MV infection revealed residual noninflammatory lesions (glial nodules, gliotic scars, no mononuclear cell infiltrates) in only 40% of cases. In brain sections obtained up to approximately 15 days p.i., only minor focal lesions predominantly near the site of injection in the left-brain hemisphere and occasionally in the frontal region, including the olfactory bulb, were seen. Such changes consisted of glial nodules, occasional neuronophagia, and scanty perivascular lymphomonocytic infiltrates with $CD4^+$ and $CD8^+$ T lymphocytes and macrophages.

TABLE 2. Protection of VV-MVN-immunized rats from MV-induced encephalitis[a]

Expt no.	Immunization	MV challenge dose (10^4 $TCID_{50}$)	No. of diseased rats/no. tested	MV isolated from CNS material (no. positive/no. of attempts)	Neuropathology		
					Time p.i.	No. of rats with CNS lesions/no. tested	Lesion type[b]
1	VV-wt	0.5	18/18	ND[c]	10–14 days	18/18	SDE
	VV-MVN	0.5	0/18	ND	4 wk	7/18	Residual
2	VV-wt	0.5	4/10[d]	9/9	3–14 days	9/10	SDE
	VV-MVN	0.5	0/17	0/17	3–14 days	12/17	MFE
	Mock	0.5	4/10[d]	10/10	3–13 days	10/10	SDE
3	VV-wt	0.5	6/6		20–23 days	6/6	SDE
	VV-MVN	0.5	0/6		20–23 days	0/6	None
		2	0/6		20–23 days	1/6	Residual
		8	0/8		20–23 days	5/8	Residual

[a] Weanling Lewis rats were immunized twice with VV constructs and infected 3 to 4 days later with the indicated dose of MV.
[b] SDE, Severe disseminated encephalitis; MFE, mild focal encephalitis; residual, residual lesions with a few microglial nodules and patches of gliosis in the absence of inflammatory cell infiltrates.
[c] ND, Not done.
[d] Six rats in each of these two groups were killed before they could exhibit clinical symptoms (day 4 to 10 p.i.).

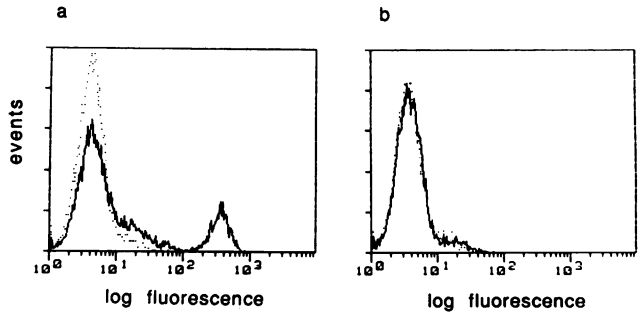

FIG. 2. Representative FACS profiles of the surface phenotype of superficial cervical lymphnode cells from a normal rat (a) and 6 days after depletion of $CD8^+$ T cells by OX8 antibody treatment (b). Dotted line represents control with a monoclonal antibody against JHM coronavirus glycoprotein; the solid line denotes $CD8^+$ cells. In panel a, 16.2% of the cells are $CD8^+$; in panel b, 0% are $CD8^+$. Concomitant with CD8 depletion, the relative frequency of $CD4^+$ cells increased from 45.9% in the normal rat to 61.7% in the CD8-depleted rat.

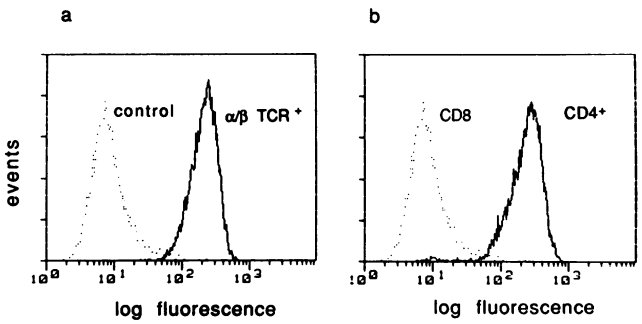

FIG. 3. FACS profile of the surface phenotype of MV N protein-specific T-cell line generated in vitro, determined on the day of transfer into recipient animals. (a) All T cells were α/β-TCR^+; (b) absence of $CD8^+$ T cells while all cells express the CD4 molecule on their surface.

These observations show that the immune response against the N protein of MV expressed by recombinant VV-MVN limits virus spread in the brain and focally restricts the neuropathologic lesions in rats that were infected i.c. with a neurotropic MV strain.

It has been reported previously that recombinant VV expressing the H and F glycoproteins of MV are capable of inducing protective immunity in mice (8). Since these animals exhibit high levels of virus-neutralizing and/or fusion-inhibiting antibodies, it appears likely that the humoral immune response contributes to or is even essential for the protection observed. Moreover, passive transfer of neutralizing antibodies directed against the hemagglutinin of MV modifies the experimental encephalitis in rats, while no such effect could be induced by antibodies with specificity for the MV internal proteins (16). In the present experiments, however, the cellular immune reaction conceivably plays an essential role, since neutralizing antibodies were not induced in VV-MVN-vaccinated rats (Table 1). Furthermore, the antinucleocapsid antibodies had no cytotoxic effect on MV-infected cells in a cytotoxic antibody assay (data not shown). These results suggest that, in vivo, activated functional N protein-specific T lymphocytes can effectively travel to MV-infected brain tissue and eliminate virus-infected cells.

In an attempt to find out whether activated $CD8^+$ lymphocytes are restricting virus spread in brain tissue, $CD8^+$ T cells were eliminated from VV-MVN-immunized rats by injection of monoclonal antibodies directed against the CD8 molecule. Within 24 h of OX8 treatment, $CD8^+$ T cells usually disappear in noninfected rats and cannot be found again for at least 35 days in the superficial cervical or mesenteric lymph nodes (Fig. 2). By retrospective analysis, 22 VV-MVN-immunized and MV-infected rats were found to be sucessfully and completely depleted of $CD8^+$ lymphocytes and were included in the experiment (Table 3). Of these animals, seven were killed before day 11 p.i. Of the remaining rats, while 12 showed no symptoms during the 3-week observation period, only 3 developed clinical disease between days 10 and 13 p.i. One died from fatal symptoms by day 11 p.i., while the other two rats recovered and were killed 14 and 16 days after MV infection. MV could not be isolated from the brain tissue of CD8-depleted VV-MVN-immunized rats between 4 and 21 days after MV infection except from two of the three rats that developed clinical symptoms. Histologic examination revealed residual noninflammatory lesions (3 weeks after MV infection) or minor focal encephalitic lesions in the two animals that had recovered from disease as well as in those without symptoms that were killed before day 14 p.i. The changes were not different from those in non-$CD8^+$-cell-depleted protected rats. These results show that $CD8^+$ MV-specific lymphocytes are not a prerequisite for protection from MV-induced encephalitis in rats.

Since depletion or functional inactivation of $CD4^+$ lymphocytes in the rat requires large amounts of antibodies

TABLE 3. Effect of $CD8^+$ T-cell depletion on MV-induced encephalitis in rats[a]

Immunization	No. of diseased rats/ no. tested	Incubation period (days)	MV isolated from brain tissue[b]	LPR (stimulation index)[c]	Histology[d]
None	8/8	8–13	+	1.3 ± 0.4	SDE
VV-wt	12/12	9–14	+	1.6 ± 0.6	SDE
VV-MVN[e]	3/22	10–13	−	4.8 ± 1.1	Residual/MFE

[a] Depletion of $CD8^+$ T cells was initiated in weanling rats aged 22 ± 1 days, 4 days after single-shot immunization with recombinant VV. Animals were infected with MV 1 day after $CD8^+$ T-cell depletion.

[b] Symbols: +, MV isolation positive within 3 to 7 days; −, no MV isolatable.

[c] See Table 1, footnote *d*.

[d] See Table 2, footnote *b*.

[e] Nineteen of the 22 rats in this group developed no clinical signs and were killed 4 to 21 days after MV infection. Three animals developed neurological symptoms between days 10 and 14 p.i.; two recovered completely and one died on day 11 p.i. MV was isolated from brain tissue of two of the three diseased VV-MVN-immunized rats on days 11 and 14 p.i. Nine rats investigated at 21 days p.i. had residual lesions. Two rats recovering from disease (days 14 and 16 p.i.) as well as 10 rats killed between days 4 and 14 p.i. had mild focal encephalitis. The dead rat showed severe disseminated lesions (day 11 p.i.).

TABLE 4. Adoptive transfer of CD4+ MV N protein-specific T cells[a]

Recipient rats	Specificity of transferred T cells	No. of diseased rats/ no. tested	MV isolated from brain tissue[b]	Neuropathology	
				Day p.i.	Lesion type
Uninfected	MV N protein	0/4	−	14	None
MV infected	MV N protein	0/8	−	14	Focal encephalitis
	Keyhold limpet hemocyanin	6/6	+	10–14	Severe generalized encephalitis

[a] Rats aged 3 to 4 weeks were infected i.c. with CAM/RBH 1 day prior to adoptive transfer via the tail vein of CD4+ MV N protein-specific (2×10^6 cells; 19,720 cpm; stimulation index, 63.5) or keyhold limpet hemocyanin-specific T-cell lines (3×10^6 cells; specific [^{3}H]dT incorporation, 27,690 cpm; stimulation index, 88.4). Both cell lines were R73+ W3/25+ OX8− in FACS analysis.

[b] See Table 3, footnote *b*.

against the CD4 molecule (11), a different approach was used to formally show that MV-specific CD4+ lymphocytes are restricting virus spread in brain tissue. MV N protein-specific T lymphocytes were isolated from VV-MVN-immunized rats. After three in vitro restimulations with purified N protein or inactivated MV virions, the T-cell lines were N protein specific, α/β-TCR+, CD4+, and CD8− (Fig. 3). Two million of these cells were adoptively transferred via the tail vein into rats 1 day after i.c. infection with the CAM/RBH strain of MV. None of the eight rats treated developed clinical symptoms, while the controls that received keyhole limpet hemocyanin (KLH)-specific T cells died within 10 to 14 days (Table 4). With these experiments, we show that CD4+ T lymphocytes specific for the MV N protein are sufficient to protect rats from encephalitis.

Our experiments indicate that immunization with VV expressing the internal N protein of MV protects rats from MV-induced encephalitis. Antiviral neutralizing antibodies or virus-specific CD8+ T cells are apparently not required to overcome the CNS infection. Clinical protection from systemic viral diseases following vaccination with VV recombinants has been described for many other viral systems in different animal species (19, 24, 25). Protective immune responses that helped to overcome fatal virus infections could be induced with glycoproteins as well as nucleocapsid protein. Further studies, particularly with influenza virus and lymphocytic choriomeningitis virus, demonstrated that the host major histocompatibility complex (MHC) defines which viral protein and which epitope is recognized by the cytotoxic T lymphocytes (9, 10, 27, 28). It will be of interest to find out whether similar MHC restrictions occur during MV infection in rat brain.

In our model, the CD4+ lymphocytes apparently function as effectors and restrict MV spread in the brain. A less likely possibility would be that mononuclear phagocytes either resident in the brain or recruited from the peripheral circulation are activated by cytokines secreted from the specific CD4+ lymphocytes to clear the MV from the brain tissue. However, due to the lack of an MV-specific cytotoxic assay in rats, we are not able at present to give a final answer to the question of whether the CD4+ T lymphocytes in the Lewis rat are cytotoxic, as was found for human peripheral blood lymphocytes (12).

The observation that an immune response to MV N protein controls MV infection of the CNS in the rat model raises the question of why the CMI response fails to eliminate MV in SSPE patients despite the fact that the MV N protein can always be found in infected cells of SSPE patients' brain tissue (14, 20). So far no specific immunological defects have been seen in SSPE patients which would explain the failure of the host defense mechanism in controlling MV infection. Maybe mutational events of MV genes as a consequence of viral persistence (3, 4) or an impairment of antigen processing and presentation (7) allows the CNS disease process to escape immune system surveillance. Other possible mechanisms include the downregulation of MHC class I and/or II molecules in infected cells (2) and the failure of those cells to express MHC antigens (6). The latter possibilities could render the major target cells for MV persistence in the human CNS unaccessible to the T-cell-mediated immune reactions. Certainly, the animal model will help to define the parameters by which CMI reactions block MV infection in brain tissue. With this information, it may be possible to gain more insight into the pathogenetic mechanisms involved in SSPE and other persistent viral infections of the CNS.

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