Localization of a Neutralization Site of Theiler's Murine Encephalomyelitis Viruses

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Theiler's murine encephalomyelitis viruses (TMEV) are picornaviruses that produce enteric and neurological diseases in mice. Subgroup TO strains of TMEV cause persistent infections with demyelination, while subgroup GDVII strains neither persist nor demyelinate. We produced neutralizing monoclonal antibodies (mAbs) to clarify the mechanisms of persistence and demyelination. Some of the neutralizing mAbs reacted with isolated VP1 on Western blots, while others were conformation specific. The neutralization site for the former TMEV mAbs was on the VP1 trypsin cleavage site of the intact virion. The neutralization site for the conformation-specific mAbs was distinct and was not affected by trypsin. Trypsin treatment of subgroup TO strains increased their infectivity for L cells, whereas the infectivity of subgroup GDVII strains was decreased by trypsin treatment. Subpopulations of virus in subgroup TO-infected tissue culture cells and in infected mouse brain homogenates contained VP1-cleaved virus; this VP1-cleaved virus gave rise to a large persistent fraction in neutralization tests when it was reacted with VP1-specific mAbs. These findings have implications regarding the pathogenesis of subgroup TO demyelinating disease. TMEV VP1 cleavage may be important for virus persistence because of disruption of a major neutralization epitope. The change in virus surface structure caused by VP1 cleavage may affect cell binding and lead to altered cytotropism. Immunocytes, which have been implicated in subgroup TO demyelination, may provide a source for proteases for VP1 cleavage.

Theiler's murine encephalomyelitis viruses (TMEV) are a group of murine picornaviruses that produce enteric and neurological diseases in mice. TMEV are divided into two subgroups on the basis of differences in biological behavior (15, 20), antigenicity (24), and RNase T₁ oligonucleotide two-dimensional maps (20, 21). Intracerebral (i.c.) inoculation of weanling mice with strains belonging to subgroup TO (which includes the TO and DA strains) produces a biphasic disease; there is an initial acute neuronal infection, and this is followed by a chronic demyelinating disease (13, 14). The mechanism that allows virus persistence in the face of large amounts of neutralizing antibody within the central nervous system (CNS) remains unclear (18). Also poorly understood is the pathogenesis of demyelination since both direct viral oligodendrocyte infection (28, 29, 32) and immune-mediated demyelination (4, 5, 14, 16, 29) have been implicated. Strains belonging to the second TMEV subgroup, subgroup GDVII (GDVII and FA strains), produce an acute neuronal infection in weanling mice with no demyelination or virus persistence (15).

We have produced a panel of TMEV monoclonal antibodies (mAbs) (24) in order to elucidate the mechanisms of demyelination and persistence of the subgroup TO strains. In this study we localized a TMEV major neutralization epitope to the trypsin cleavage site on VP1 of the intact virion. In this paper we present data which indicate that cleavage occurs both in tissue culture cells and in the mouse CNS. Our results suggest a hypothesis regarding the importance of VP1 cleavage in subgroup TO demyelinating disease.

MATERIALS AND METHODS

Virus. The passage history of, growth of, and infectivity assays used for the different TMEV strains have been described previously (15, 24, 30, 31). Virus grown in BHK-21 cells was used in all of the experiments except several neutralization tests in which suckling mouse brain-passed DA strain was used (31). For virus purification (30), a cytoplasmic lysate prepared in 1% Nonidet P-40 was adjusted to a final sodium dodecyl sulfate (SDS) concentration of 1%, pelleted through sucrose, and then banded on a cesium chloride equilibrium density gradient. Virus preparations and cytoplasmic lysates were treated in some cases with trypsin (catalog no. T-2395; Sigma Chemical Co., St. Louis, Mo.) and then with soybean trypsin inhibitor (Sigma) at a final concentration of 0.5 mg/ml for 10 min at 37°C (17, 19).

Neutralization tests. TMEV mAbs were prepared and characterized as described previously (Table 1) (24). Hyperimmune sera were obtained from demyelinated SJL/J mice which had been inoculated i.c. with DA strain, a member of subgroup TO, 3 months or more previously. Plaque reduction neutralization tests were performed as described by Roos et al. (29).

For suckling mouse neutralization tests, undiluted hybridoma supernatants, ascites, or 10⁻¹ dilutions of serum from DA demyelinated mice were incubated with 10-fold dilutions of DA strain at room temperature for 1 h and then inoculated i.c. or intraperitoneally (i.p.) into one or two litters of mice (Cumberland View Farm, Clinton, Tenn.) per virus dilution. The 50% lethal doses of virus remaining after reaction with the antibody were calculated at approximately weekly intervals for 1 month.

In weanling mouse neutralization tests, hybridoma supernatants or 10⁻¹ dilutions of hyperimmune serum were incu-

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TABLE 1. Summary of mAb reactivities

Antibody(ies)	Neutralization reactivity		Western blot VP1 reactivity
	Subgroup TO	Subgroup GDVII	
DAmAb1	+	—	—
DAmAb2	+	—	+
GDVII mAb1, mAb2	+	+	+
GDVII mAb3, mAb4, mAb5, mAb6	—	+	—

bated for 1 h at room temperature with 1,000 times an amount of virus which consistently produced demyelinating disease in 50% of weanling mice by 3 months postinfection (1,000 DD₅₀). The virus-antibody mixture was then inoculated i.c. into a group of five weanling SJL/J mice (Jackson Laboratory, Bar Harbor, Maine). In another experiment, groups of six to eight mice were inoculated i.p. on each of 2 days with 1-ml portions of hybridoma supernatants; the next day they were inoculated i.c. with 100 DD₅₀ of DA strain. The animals were observed for early neuronal or late demyelinating disease for the next 3 months. Early neuronal disease was considered present if flaccid paraplegia appeared in the first few weeks or if there was histological evidence of gray matter disease or both. Late demyelinating disease was evaluated by determining the appearance of spastic paraplegia after 3 weeks or the presence of histological evidence of white matter involvement or both.

Electrophoretic studies, Western immunoblots, and dot blots. Proteins from viral or control specimens were separated on 12.5% SDS-polyacrylamide gels in the presence or absence of 3 M urea and were stained with Coomassie blue or silver (31). For Western immunoblots, the separated proteins were transferred to nitrocellulose and processed for immunoreactivity with an immunoperoxidase detection system, as described previously (24). For dot blots, viral or control specimens were applied directly onto the nitrocellulose, allowed to dry, and processed as described above for Western blots.

RESULTS

Trypsin treatment of TMEV. As previously reported by two groups of workers (17, 19), trypsin treatment of infected cytoplasmic lysates of DA strain and other subgroup TO strains leads to cleavage of intact virion VP1 into a large piece having a molecular weight of approximately 31,500, which comigrates with VP2 on 12.5% SDS-polyacrylamide gels, and a small piece(s) having a molecular weight of approximately 2,000; this small piece may be lost during cesium chloride banding (Fig. 1, lanes A and B). At times we observed other minor cleavage products (see below).

There is dispute in the literature concerning whether GDVII and FA strains, two members of TMEV subgroup GDVII which neither demyelinate nor persist, also have a trypsin-cleavable intact virion VP1 (17, 19). We found that trypsin treatment of GDVII and FA cytoplasmic lysates followed by cesium chloride banding resulted in a VP1 cleavage pattern similar but not identical to the pattern observed with DA strain. Figure 1 (lanes C through F) shows an SDS-polyacrylamide gel containing purified capsid proteins obtained from untreated and trypsin-treated GDVII and FA strains. Lanes D and F show that trypsin-treated virus contained a cleaved VP1 running above VP2 at a molecular weight of approximately 32,000. A lighter stain-

ing band was also observed above VP3 at a molecular weight of about 26,000, and several additional even lighter bands were observed at times at molecular weights of about 29,000 and 28,000. The relatively light staining of these bands suggests that they represent cleavage products of VP0 or additional, less common products of VP1 cleavage. These bands were also observed occasionally in trypsin-treated DA strain preparations and were most apparent when the virus was treated with trypsin after purification on cesium chloride or sucrose gradients (data not shown).

In some strains of foot-and-mouth disease virus (FMDV), trypsin treatment of the virus causes a considerable loss of infectivity (1). For this reason we determined the titers of TMEV stocks in vitro before and after trypsin treatment. As Table 2 shows, trypsin treatment consistently increased the titers of subgroup TO strains on L cells. In contrast, trypsin treatment consistently decreased the titers of subgroup GDVII strains.

Neutralization epitope. Because neutralization sites of FMDV (7, 26) and poliovirus (9) lie on the VP1 trypsin cleavage sites of the intact virions, we tested whether the VP1 trypsin cleavage site on TMEV was a target for the VP1-specific neutralizing mAbs which we isolated. As previously shown (24), DA mAb2 and GDVII mAb1 and mAb2 neutralize all members of subgroup TO, while only the latter two mAbs neutralize members of subgroup GDVII (Table 1). These mAbs react against isolated denatured VP1, as dem-

FIG. 1. A 12.5% SDS-polyacrylamide gel containing untreated and trypsin-treated purified viral capsid proteins. Untreated and trypsin-treated TMEV cytoplasmic lysates were purified and trypsin treated as described in the text by using one (in the case of strains DA and GDVII) or two (in the case of strain FA) cesium chloride gradients and then were analyzed by SDS-polyacrylamide gel electrophoresis. Similar cleavage patterns were observed for all TMEV strains. Lane A, Untreated DA strain; lane B, trypsin-treated DA strain; lane C, untreated GDVII strain; lane D, trypsin-treated GDVII strain; lane E, untreated FA strain; lane F, trypsin-treated FA strain. The positions of the major cleaved fragment of VP1 are indicated by arrowheads. Several light-staining bands representing minor cleavage products were also present between VP2 and VP3.

TABLE 2. Effect of trypsin on TMEV infectivity

TMEV subgroup	Strain	Prepn	Titer ^a	
			Untreated	Trypsin treated
TO	DA	Tissue culture stock 1	7.0×10^6	2.5×10^7
	DA	Tissue culture stock 2	5.0×10^7	2.0×10^8
	TO	Tissue culture stock	4.5×10^6	2.7×10^7
	DA	Cytoplasmic lysate	1.4×10^9	4.1×10^9
	DA	Purified virus	1.6×10^9	5.5×10^9
GDVII	GDVII	Tissue culture stock 1	5.4×10^7	3.9×10^7
	GDVII	Tissue culture stock 2	1.5×10^{10}	1.2×10^9
	FA	Tissue culture stock	2.4×10^8	1.4×10^8
	GDVII	Cytoplasmic lysate	6.5×10^8	4.5×10^8

^a Expressed as plaque-forming units per milliliter on L cells.

onstrated by their ability to immunostain VP1 on Western blots of SDS-polyacrylamide gels in which purified virion capsid proteins were electrophoresed (Fig. 2, lanes B through D) (24). However, these mAbs failed to immunostain electrophoresed capsid proteins from DA virus that were trypsin treated during purification (Fig. 2, lanes H through J). Similarly, GDVII mAb1 and mAb2 immunostained GDVII strain VP1, but did not react with VP1 from GDVII virus that was trypsin treated during purification (data not shown).

These results suggested either that trypsin treatment disrupted a neutralization epitope or that the small piece(s) cleaved off VP1 during trypsin treatment of the cytoplasmic lysate contained the epitope but was lost during virus purification. To rule out the latter possibility, we dot blotted untreated and trypsin-treated TMEV directly onto nitrocellulose and then tested the immunoreactivity of the mAbs. The VP1-specific mAbs immunostained the untreated dot blotted lysate (Fig. 3, lanes b through d, j, and k, upper parts), but stained the treated lysate (lanes b through d, j, and k, lower parts) no differently than a negative control mAb (lanes e and l).

The dot blot experiment indicated that the mAbs did not react against any cleavage products of VP1 after trypsin treatment of the virion; i.e., the epitope was destroyed during trypsin treatment. A neutralization epitope presumably lies at the trypsin cleavage site of the intact virion VP1 either at the junction of the 31,500-dalton piece with a small 2,000-dalton piece or at the junction of another trypsin cleavage site within the 2,000-dalton piece. Although both DA strain and GDVII strain have a neutralization site at the trypsin cleavage site on VP1 of the intact virus, the epitopes on DA and GDVII strains are not identical since, as shown in Table 1, there is a one-way cross-reaction in the case of DA mAb2 (against subgroup TO strains but not subgroup GDVII strains) and there is a two-way cross-reaction with GDVII mAb1 and mAb2 (against both subgroup TO and subgroup GDVII strains). Conformation-specific TMEV mAbs (DA mAb1 and GDVII mAb3, mAb4, mAb5, and mAb6) are directed against a second, distinct neutralization site(s) since these mAbs react against both untreated and

trypsin-treated TMEV dot blots (Fig. 3, lanes a and f through i).

In vitro neutralization. TMEV mAbs and sera from DA demyelinated mice were tested in plaque reduction tests against uncleaved and VP1-cleaved viruses to further delineate the effect of trypsin treatment on the neutralization epitope. As expected, trypsin-treated purified DA strain was not neutralized by DA mAb2 or by GDVII mAb1 and mAb2, presumably because the epitope was cleaved by the treatment. DA mAb1 neutralized trypsin-treated DA and untreated DA strains with similar efficiencies, confirming the dot blot results and indicating that DA mAb1 has a different neutralization site than the VP1-specific mAbs. Similarly, VP1-specific GDVII mAb1 and mAb2 were not able to neutralize trypsin-treated GDVII and FA strains, while the one conformation-specific GDVII mAb tested neutralized the treated viruses effectively.

Since trypsin treatment disrupted the epitopes of several of the neutralizing mAbs, we wanted to determine whether sera taken from demyelinated mice had decreased antibody activity against trypsin-treated virus compared with uncleaved virus. Inefficient neutralization might be important in fostering virus persistence. Serum collected from a demyelinated mouse 5 weeks after DA strain inoculation had a neutralization titer of 1:800 against trypsin-treated strain DA, but a titer of more than 1:3,200 (the highest dilution tested) against untreated DA strain. Sera collected at later times (3, 5, and 9 months after inoculation) neutralized untreated and trypsin-treated virus to similar high titers. The delay in effective neutralization of VP1-cleaved virus by serum may be important in enabling the virus to establish a persistent infection.

Persistent fraction and the presence of VP1-cleaved TMEV in vitro. When we examined the results of neutralization tests by using VP1-specific mAbs against several DA virus stocks grown in BHK-21 cells, we noticed that there was a large nonneutralizable persistent fraction of virus left in the mAb-virus mixtures. As Table 3 shows, a 10^{-1} dilution of DA mAb2 left approximately 6 to 14% of the plaques found in virus-Hanks buffered saline solution controls, while DA mAb1 and DA hyperimmune serum left only 1% or less of the plaques found in controls. A large persistent fraction was also observed when DA strain passed in suckling mouse brain was neutralized with DA mAb2 or when DA-infected BHK-21 stocks were neutralized with GDVII VP1-specific mAbs.

We wondered whether the large persistent fraction might relate to the presence of a subpopulation of virus with a trypsin-cleaved VP1 in our tissue culture stocks of virus since this subpopulation would be resistant to the VP1-specific mAbs and leave a nonneutralizable fraction. Because the proteolytic cleavage of proteins in other virus infections depends on both the host cell type and the extent of viral cytopathic effects (12), we tested whether late harvest of the virus resulted in an increased persistent fraction and visible cleavage of virion VP1 on gels. The material from the late harvest contained a persistent fraction after reaction with DA VP1-specific mAb2 which was so large (45%)(Table 3) that it was almost nonneutralizable by this mAb (i.e., >50%). Purified DA virus from two separate preparations of cells harvested late after infection, at a time when there were extensive cytopathic effects, produced a visible band on gels that aligned with the VP1 trypsin cleavage product; resolution of this band and separation of it from VP2 were best accomplished by using silver-stained SDS-polyacrylamide-urea gels (Fig. 4, lanes A and B) (17).

FIG. 2. Immunoblot of mAbs and hyperimmune serum against untreated and trypsin-treated DA purified viral capsid proteins. Untreated (lanes A through E) and trypsin-treated (lanes F through J) DA-infected cytoplasmic lysates were purified, and the viral capsid proteins were then run on 12% SDS-polyacrylamide gels and transferred to nitrocellulose strips. The strips were overlaid with antibody and tested for immunoreactivity as described in the text (24). Lanes E and F were amido black-stained nitrocellulose strips showing that the VP1 of the untreated virus (lane E) was cleaved after trypsin treatment and comigrated with VP2 (lane F). Lanes A and G were overlaid with hyperimmune serum and show immunostaining of VP0, VP1, and VP2 of untreated virus (lane A) and the band containing VP2 and cleaved VP1 of treated virus (lane G). The VP1-specific mAbs (DA mAb2 and GDVII mAb1 and mAb2) immunostained VP1 of untreated DA strain (lanes B through D) but not trypsin-treated DA strain (lanes H through J). Clvd, Cleaved.

Virus grown for two passages in BHK-21 cells in the presence of trypsin inhibitor (0.5mg/ml) had a persistent fraction of 1% (Table 3), presumably due to a decrease in the population of cleaved virus.

Like DA strain, subgroup GDVII strains also showed evidence of VP1 cleavage of the intact virion in late harvests

of infected BHK-21 cells. Figure 4 shows that a silver-stained gel of capsid proteins from purified FA strain obtained from a late harvest of infected BHK-21 cells had evidence of a band at the same mobility as the VP1 cleavage product of trypsin-treated FA strain (lanes C and D).

When purified FA virus from this late harvest was reacted

FIG. 3. Dot blot of mAbs against untreated and treated TMEV. Untreated (upper wells) or trypsin-treated (lower wells) cytoplasmic lysates containing DA virus (lanes a through e) or GDVII virus (lanes f through l) were directly applied to nitrocellulose and allowed to dry. Antibody was then applied and tested for immunoreactivity as described in the text (24). DA mAb1, a conformation-specific mAb, stained untreated and trypsin-treated DA (lane a). DA mAb2, a VP1-specific mAb, stained untreated virus but not treated virus (lane b). GDVII mAb1 and mAb2, VP1-specific mAbs, stained untreated DA but not treated DA (lanes c and d) and GDVII (lanes j and k). GDVII mAb3, mAb4, mAb5, and mAb6, conformation-specific mAbs, stained untreated and trypsin-treated viruses (lanes f through i). A negative control mAb did not stain untreated or treated virus (lanes e and l). tryp, Trypsin.

TABLE 3. Persistent fraction in neutralization tests

DA virus prepn tested	Persistent fraction (%) ^a
Standard BHK-21 stock	6-14
Suckling mouse 10% brain homogenate	38
Purified, harvested from BHK-21 cells late in the growth cycle	45
BHK-21 stock grown with trypsin inhibitor	1

^a The value for the nonneutralizable persistent fraction at a 10^{-1} dilution of DA mAb2 against each virus preparation is expressed as a percentage of the plaques observed with virus and Hanks balanced salt solution. DA mAb1 and serum taken from a demyelinated mouse 3 months after inoculation had a 1 to 2% persistent fraction against the DA strain standard BHK-21 stock and DA strain harvested late in the growth cycle.

in neutralization tests with VP1-specific GDVII mAbs, there was a relatively small persistent fraction (5%) compared with that observed with DA strain, despite the visible subpopulation of VP1 cleavage. In addition, when the VP1-specific GDVII mAbs were reacted in neutralization tests with standard stocks of GDVII and FA strains, they did not leave a persistent fraction above the levels seen with conformation-specific mAbs (1%). Our explanation for these observations is that although a subpopulation of nonneutralizable VP1-cleaved GDVII or FA virus was present in the inoculum, the fact that VP1-cleaved GDVII and FA viruses are significantly less infectious than uncleaved viruses makes a persistent fraction less apparent than in the case of DA virus (in which VP1 cleavage enhances infectivity).

Mouse neutralization tests. Because the VP1-specific DA mAbs left a large persistent fraction in *in vitro* neutralization tests against TMEV, we tested the effect of inoculation of mAb-virus mixtures in mice. DA mAbs, control mAb, or serum was incubated with tissue culture stocks of DA virus and inoculated *i.c.* or *i.p.* into suckling mice. Suckling mice normally die of acute encephalomyelitis when they are inoculated with TMEV.

Figure 5 shows the 50% lethal doses calculated at different times after *i.c.* inoculation of the virus-antibody mixtures. DA mAb2 showed no neutralization compared with a control (negative) mAb; in fact, animals died sooner after inoculation with DA mAb2 than after inoculation with the control mAb-virus mixture. DA mAb1, a conformation-specific mAb, delayed early deaths slightly, but there was no significant neutralization at the end of 1 month compared with the control mAb. Serum from a DA demyelinated mouse delayed the early deaths and decreased the 50% lethal doses 10- to 100-fold at the end of 1 month compared with control mAb. When inoculated *i.p.*, DA mAb2 and mAb1, as well as the hyperimmune serum, caused substantial decreases in the 50% lethal dose (Fig. 6).

In weanling mouse neutralization tests, no clinical evidence of early neuronal disease was observed in any inoculated weanling. The incidence of late demyelinating disease in inoculated animals was similar in animals receiving virus with DA mAb1, with DA mAb2, or with control mAb. When histological sections obtained after 12 days or 3 months were compared, no substantial differences in the incidence of early gray matter or late white matter disease were apparent among the different groups.

DISCUSSION

We produced a panel of TMEV mAbs (24) to clarify differences between the two TMEV subgroups and to better

understand the mechanisms of virus persistence and demyelination. We delineated at least two separate TMEV neutralization sites. One of these sites is on the trypsin cleavage site of VP1 of the intact virion and is recognized by VP1-specific TMEV mAbs. The other site is conformation specific and is not affected by trypsin treatment of the virion. There are at least two distinct neutralization epitopes of the VP1-specific neutralization site since some of the VP1-specific mAbs react only with subgroup TO, while other mAbs react with both subgroups.

Our observations are consistent with findings obtained with other picornaviruses. More than one neutralization site has been described on poliovirus (8), rhinovirus, and FMDV. Of interest is the localization of the major neutralization site of FMDV at the trypsin-cleavable region of VP1 of the intact virion (7, 26). This site is a target for neutralizing mAbs directed against isolated VP1 and conformation-specific neutralizing mAbs (2, 7, 26). While this manuscript was in preparation, some strains of poliovirus were found to have a trypsin-cleavable region of VP1 which contains a major epitope for neutralizing mAbs (9).

Certain strains of FMDV show substantial losses in infectivity after trypsin treatment (1), while the Sabin strain of poliovirus type 1 shows no change in infectivity after trypsin treatment (9). We found that subgroup TO strains had increased infectivity for L cells after trypsin treatment of intact virions, while subgroup GDVII strains had decreased infectivity after treatment.

The delineation of the VP1 trypsin-cleavage site of the virion as a major neutralization epitope of TMEV suggested that this site may play a role in the biological activities of the virus and the pathogenesis of DA demyelinating disease. We initially tested whether TMEV had VP1 cleaved *in vitro*. We found that virions harvested late in the growth cycle from BHK-21 cells contain a subpopulation of virus with cleaved VP1 which could be visualized on gels. We suspect that a small amount of VP1 cleavage occurs even in virus harvested early, but that it is too small an amount to be detected on gels. Infected cells undergoing extensive cytopathic effects presumably release a trypsin-like lysosomal enzyme which cleaves the virion VP1. As expected, DA virus produced a large persistent fraction in neutralization tests when it was reacted against VP1-specific mAbs, presumably because a trypsin-like protease cleaved the epitope of the mAbs in some of the virus. Virus harvested early or virus grown in the presence of trypsin inhibitors had a smaller persistent fraction. To our knowledge, the cleavage of a neutralization epitope by cellular proteases is a previously unrecognized cause of a large persistent fraction (10, 11).

It is likely that a certain amount of VP1 cleavage of virus occurs within the CNS since a large persistent fraction was seen in neutralization tests of infected suckling mouse brain homogenates by a VP1-specific mAb. In addition, in past experiments analyzing viral proteins directly from infected brains, a broad VP2 band was frequently observed on silver-stained SDS-polyacrylamide gels of purified capsid proteins (31). At that time Roos and Whitelaw commented that "in some gels, VP2 appeared broadened or as a doublet for reasons that are not clear" (31). In retrospect, the broad band and doublet presumably represented cleaved VP1 that comigrated with or near VP2.

The presence in the mouse CNS of VP1-cleaved virus, along with the disruption of a major neutralization epitope, provides a mechanism by which the virus could partially avoid the immune response. Our studies have indicated that serum taken from demyelinated mice relatively early after

FIG. 4. Silver-stained polyacrylamide gels of untreated and trypsin-treated purified TMEV capsid proteins. Untreated DA strain (lane A) and FA strain (lane C) cytoplasmic lysates from BHK-21 cells harvested late in the virus growth cycle and trypsin-treated DA strain (lane B) and FA strain (lane D) from standard BHK-21 infected preparations were purified, and the viral capsid proteins were analyzed on 12.5% SDS-polyacrylamide gels with (lanes A and B) or without (lanes C and D) 3 M urea. One band (arrowheads) in the untreated TMEV lanes (lanes A and C) was aligned with the VP1 cleavage product of trypsin-treated TMEV (lanes B and D). The lighter-staining bands observed in untreated and treated virus may be additional proteolytic products. A small amount of residual uncleaved VP1 was present in the trypsin-treated TMEV (lanes B and D). Clvd, Cleaved.

infection showed less effective neutralization against VP1-cleaved virus than against uncleaved virus, although serum taken later after infection contained high levels of neutralizing antibody to both untreated and trypsin-treated virus. This delay in effective neutralization may permit oligodendrocyte infection and the beginning of a restricted cell infection (3a). Despite the presence of serum neutralizing antibody, it is likely that cleavage of a major neutralization epitope tends to foster persistence and delay effective neutralization within the restricted confines of the CNS compartment. In other areas of the body, such as the gut, protection from virus may depend more on local immunoglobulins than on serum antibody levels (25).

Our *in vivo* tests demonstrated that DA mAb2 does not neutralize DA strain after *i.c.* inoculation of the virus-antibody mixture in suckling and weanling mice. In another experimental demyelinating disease with a biphasic course, strain JHM murine hepatitis virus 4 infection of mice, neutralizing mAbs decreased the degree of early neuronal infection, but had no effect on late demyelination (3). In the case of DA mAb2, we suspect that the large nonneutralized fraction of DA virus is not adequately cleared by the host immune system. We have no certain answer as to why DA mAb1, a conformation-specific mAb, showed ineffective neutralization after *i.c.* inoculation of virus-antibody mixtures. After *i.p.* inoculation of a virus-antibody mixture, both mAbs effectively protected suckling mice, presumably because the host animal had sufficient time to clear the nonneutralized virus systemically before its entry into the CNS. The ineffectiveness of TMEV immune serum *i.c.* in *in vivo* neutralization tests and its effectiveness in *i.p.* tests were reported years ago (34).

Proteolytic cleavage of the virion VP1 may also affect

tissue tropism by changing the surface structure of the virus and thereby its binding to cell receptors. Proteases are known to play a key role in determining virus expression, surface structure, infectivity, and host range in other virus infections (e.g., paramyxovirus infections). Protease treatment of FMDV with cleavage of VP1 drastically affects the ability of the virus to bind to certain cells. Our finding of enhanced infectivity with VP1-cleaved subgroup TO strains and decreased infectivity with VP1-cleaved subgroup GDVII strains indicates that virus-cell binding is significantly influenced by VP1 proteolytic cleavage. The observation that the hemagglutination titer of subgroup GDVII strains at 20°C increases after trypsin treatment (22; unpublished data) also demonstrates the change in virus-cell binding after protease treatment.

It is possible that there are different receptors for uncleaved DA virus and VP1-cleaved DA virus; a receptor on neurons might accept virus with an intact or cleaved VP1 for binding, while a receptor on oligodendrocytes might require VP1-cleaved virus for binding. The characteristic biphasic disease caused by DA strain may be caused by initial infection of neurons by uncleaved virus; after proteolytic cleavage, the virus could infect oligodendrocytes and produce demyelination. Preliminary results suggest that the VP1-cleaved virus is less neuronotropic than the uncleaved virus (unpublished data); we await results pertaining to the incidence of late demyelination. Of interest is a recent report concerning intratypic variants of coxsackievirus B3, which demonstrated that cells may have one or both of two different receptors for a particular picornavirus and that this cell property affects virus tropism and disease expression (27).

One may ask why the subgroup GDVII strains do not

FIG. 5. Suckling mouse neutralization tests. Undiluted hybridoma supernatants, ascites, or 10^{-1} dilutions of serum from DA demyelinated mice were incubated with 10-fold dilutions of DA virus at room temperature for 1 h and then inoculated i.c. into one or two litters of mice per virus dilution. The 50% lethal doses (LD50) of virus remaining after reaction with the antibody were calculated at approximately weekly intervals for 1 month after inoculation. Ab, Antibody.

persist and demyelinate since they, like the subgroup TO strains, have a trypsin-cleavable VP1. We suspect that the different effects of VP1 cleavage on the infectivities of the two subgroups may be an important factor; i.e., subgroup GDVII strains lose infectivity in vitro, while subgroup TO

strains gain infectivity after trypsin cleavage of VP1. There may also be a difference in tissue tropism between VP1-cleaved DA and GDVII strains; for example, oligodendrocytes may have a receptor for VP1-cleaved DA strain but not VP1-cleaved GDVII strain. Studies have shown that VP1

FIG. 6. Suckling mouse neutralization test. The methods used were the same as those described in the legend to Fig. 5, except that the mice were inoculated i.p. LD50, 50% lethal dose; Ab, Antibody.

cleavage of GDVII strain, but not DA strain, influences the hemagglutination titer (22; unpublished data). These results suggest different kinds of virus-cell binding among members of the two subgroups.

What is the source of proteases which can cleave the virion VP1 within the CNS? Trypsin-like proteases are known to exist in a variety of tissues, including brain (6, 33). In addition, protease cleavage could occur in macrophages; these cells, which contain a rich supply of proteases, are recruited into the CNS during DA strain infection and are known to become infected (4, 5). This interrelationship between the immune system and DA strain could explain reports emphasizing both immune factors and viral oligodendrocyte lysis in the pathogenesis of DA demyelination. Immuncytes could be critical to DA demyelination, not by exerting a direct or indirect immunopathological effect, but by acting as a source of proteases to cleave the virion; the subsequent change in virus surface structure could promote virus persistence and alter cytotropism. The importance of macrophages in changing virus expression has been emphasized in another experimental viral demyelinating disease, visna (23).

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