JOURNAL OF VIROLOGY, Sept. 1988, p. 3527–3529
0022-538X/88/093527-03$02.00/0

Vol. 62, No. 9

Trypsin-Sensitive Neutralization Site on VP1 of Theiler's Murine Encephalomyelitis Viruses

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We generated Theiler's murine encephalomyelitis virus mutants resistant to several neutralizing monoclonal antibodies (MAbs) having their epitopes near a trypsin cleavage site of VP1. Neutralization and Western blot (immunoblot) studies suggest that two of the MAbs have identical epitopes that partly overlap the epitope of a third MAb. Sequencing of RNA of the mutants localized the epitopes to a site near the carboxyl end of VP1. The limited diversity of nucleotide changes seen in the mutants and the immunodominance of the site suggest that the carboxyl end of VP1 may have an important function.

Theiler's murine encephalomyelitis viruses (TMEV) are picornaviruses closely related to cardioviruses (5, 8, 9) that can be classified into two subgroups on the bases of their biological activity (3, 4) and reactivity with neutralizing monoclonal antibodies (MAbs) (6). The TO (or DA) subgroup strains produce a persistent demyelinating infection in mice, while GDVII subgroup strains cause acute polioencephalomyelitis (3, 4). A major goal of TMEV research is to define the genes and gene products that determine the differences in the biological behavior of the strains.

We previously prepared TMEV-neutralizing MAbs to localize epitopes critical in determining various biological activities of the subgroups (6). We identified three MAbs (DA MAb2, GDVII MAb1, and GDVII MAb2), each of which had a neutralization site located near the trypsin cleavage site at the carboxyl end of VP1 of the intact virion; we suggested that this epitope was important in DA persistence and demyelination (5, 7). Previous studies (6, 7) showed that DA MAb2 neutralizes and immunostains VP1 only in TO subgroup strains, while GDVII MAbs1 and 2 neutralize and immunostain VP1 of all TMEV subgroup strains, suggesting that the epitope for DA MAb2 is distinct from that of GDVII MAbs1 and 2. In this study, we generated DA and GDVII mutant viruses resistant to these three neutralizing MAbs, identified the mutations, and localized the neutralization site.

Five or more mutants resistant to each of the MAbs were isolated by growing DA or GDVII virus in the presence of a particular MAb and trypsin inhibitor (0.5 mg/ml; Sigma T9253, type 11-0) on BHK-21 cells for four to seven passages and then growing the putative mutants under an agar overlay in the presence of the MAb and trypsin inhibitor on L cells. We used trypsin inhibitor to prevent VP1 cleavage from cellular proteases released during viral cytopathic effect.

DA virus mutants generated against GDVII MAb2, as well as GDVII virus mutants generated against GDVII MAb1, showed no reactivity when tested with either GDVII MAb2 or GDVII MAb1 by dot blots, Western blots (immunoblots), or neutralization test, suggesting that GDVII MAbs1 and 2 have the same neutralization site (data not shown). Therefore, these MAbs will be referred to generally as GDVII MAb1/2 in this paper when epitopes are discussed. On the other hand, DA MAb2 reacted with DA virus escape mutants resistant to GDVII MAb2, presumably because DA MAb2 has a different epitope than GDVII MAb1/2. The difference in the epitopes was confirmed by other experiments that showed that DA virus mutants resistant to DA MAb2 were not immunostained or neutralized by DA MAb2 but were still reactive with GDVII MAbs1 and 2.

In contrast to the tests mentioned above, other Western blots, involving mouse serum, demonstrated an overlap of the DA MAb2 and GDVII MAb1/2 epitopes. Figure 1 shows a Western blot of DA virus mutants resistant to GDVII MAb2. As expected, GDVII MAb2 immunostained VP1 of wild-type DA virus (lane 1B) but not of the mutant virus (lane 1A); DA MAb2, with a different neutralization site, reacted with VP1 of both the mutant and the wild type (lanes 2A and 2B). Of interest were experiments with an individual antiserum and two separate antiserum pools randomly collected from DA-virus-demyelinated mice 3 months after infection. Antiserum from demyelinated mice, as noted previously (7), usually immunostains DA wild-type VP1 and VP2, and occasionally VP0 and VP3, depending on the virus stock employed and the characteristics of the particular

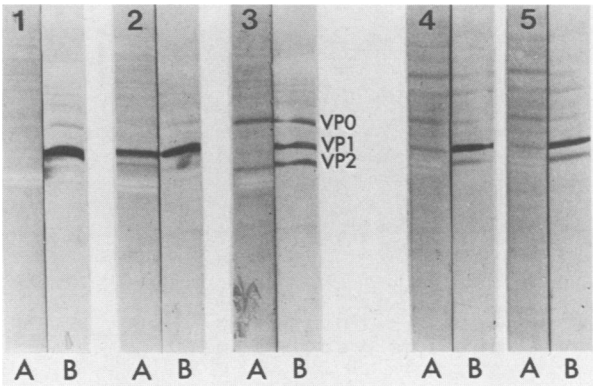

FIG. 1. Western immunoblot of neutralizing MAb and antisera reacted against mutants and wild type. Lanes: A, representative DA escape mutant virus against GDVII MAb2; B, DA wild-type virus. Panels: 1, GDVII MAb2; 2, DA MAb2; 3, serum pool 1 from DA-infected demyelinated mice, 4, individual serum from DA-infected demyelinated mouse; 5, serum pool 2 from DA-infected demyelinated mice.

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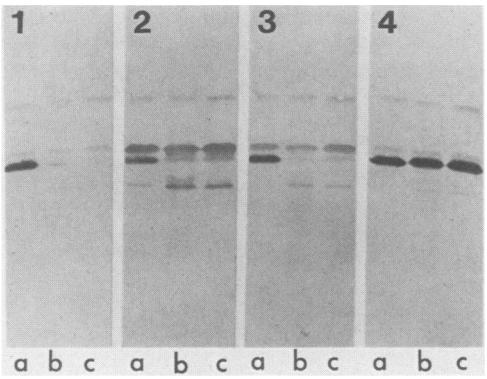

FIG. 2. Western immunoblot of neutralizing MAb and antisera reacted against mutants and wild type. Lanes: a, DA wild-type virus; b and c, representative DA escape mutant viruses resistant to DA MAb2. Panels: 1, DA MAb2, 2, individual serum 1 from DA-infected demyelinated mouse; 3, individual serum 2 from DA-infected demyelinated mouse; 4, GDVII MAb2.

mouse serum. In our experiments, we were surprised to observe that the DA-infected mouse sera failed to immunostain, or only minimally immunostained, VP1 of the DA mutant virus (Fig. 1, lanes 3A, 4A, and 5A), although they reacted with wild-type VP1 (Fig. 1, lanes 3B, 4B, and 5B). This study suggests that polyclonal antiserum is directed against an immunodominant site on denatured VP1 of DA virus which is associated with the epitope of GDVII MAb2. These observations became difficult to interpret, however, when we compared them with results from analogous experiments using DA virus mutants resistant to DA MAb2. Figure 2 shows findings very similar to that described above with respect to the immunoreaction of DA-virus-demyelinated mouse sera; i.e., the sera failed to immunostain, or only minimally immunostained, denatured VP1 of the DA escape mutants. Our interpretation of this blot is that the antiserum is directed against an immunodominant site on denatured VP1 of DA virus that is associated with the epitope of DA MAb2.

Since infected mouse sera reacted with one site on denatured VP1 of DA virus that is associated with the epitopes for both GDVII MAb1/2 and DA MAb2, the experiments described above suggest that GDVII MAb1/2 and DA MAb2 have overlapping (immunodominant) epitopes. However, the different reactivities of the MAb to the virus subgroups and the previously described cross-neutralization studies suggest that the epitopes are distinct. The epitopes of DA MAb2 and GDVII MAb1/2 presumably partly overlap and constitute one neutralization site.

To clarify the neutralization sites of these MAbs more definitively, we directly sequenced the complete VP1 coding regions of the RNA of escape mutants by a dideoxynucleotide primer extension method (1). As predicted (5), the only amino acid changes were near the carboxyl end of VP1 (Fig. 3). All six DA virus mutants resistant to DA MAb2 changed a G to a T 23 nucleotides upstream from the end of the coding area of VP1, thereby changing residue 268 of DA virus VP1 from valine to phenylalanine. All five DA virus mutants resistant to GDVII MAb2 and all five GDVII virus mutants resistant to GDVII MAb1 changed an A to a T 17 nucleotides upstream from the end of the coding area for VP1, thereby changing residues 270 of DA virus VP1 and 272 of GDVII virus VP1 from isoleucine to phenylalanine; although this phenylalanine residue in DA is aligned in position with the residue in GDVII, the numbering differs because of a small deletion upstream in DA virus VP1 (8).

The sequencing studies described above allow us to make certain statements regarding the boundaries of the epitopes and neutralization sites of these MAbs (Fig. 3). The uniformity of the mutations in TMEV mutants resistant to GDVII MAb1 and GDVII MAb2 confirmed immunoblot and cross-neutralization data that suggest that GDVII MAbs1 and 2 have identical neutralization sites. The similarity in location of the mutations in GDVII MAb1/2 escape mutants to those of the mutations in mutants resistant to DA MAb2 suggests that epitopes of DA MAb2 and GDVII MAb1/2 are located within one neutralization site. The earlier-noted finding that DA-virus-infected mouse serum has an epitope(s) that is disrupted in TMEV mutants resistant to either DA MAb2 or GDVII MAb1/2 indicates that this epitope(s) must at least include DA VP1 residue 268 (GDVII VP1 residue 270) and DA VP1 residue 270 (GDVII VP1 residue 272). Since DA MAb2, in contrast to GDVII MAb1/2, reacts only with TO

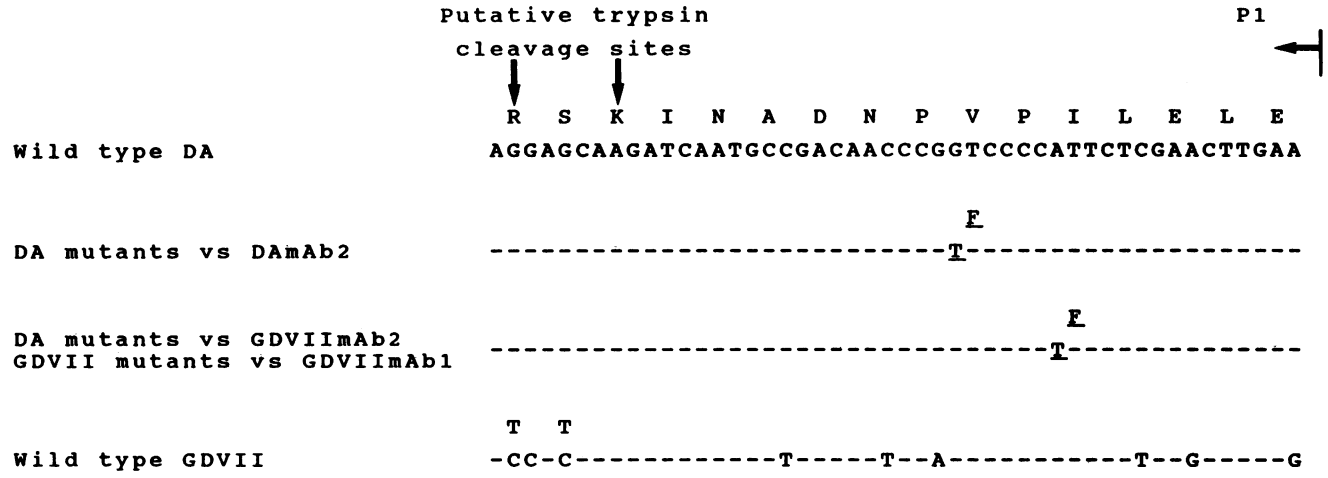

FIG. 3. Mutations found in TMEV escape mutants with trypsin-sensitive epitopes on VP1. The nucleotide sequence of the RNA corresponding to the carboxyl end of VP1 of DA wild-type virus is noted, with the deduced amino acid single-letter code written above the nucleotide triplets (Ohara et al., in press). ---, Nucleotide sequences identical to those of DA wild type; P1, end of area coding for the carboxyl end of VP1. Nucleotide and amino acid differences are noted for escape mutant viruses (underlined) and GDVII wild-type virus.

subgroup strains, we assume that the neutralization site for DA MAb2 contains some sequences found in DA but not in GDVII. DA and GDVII virus have identical sequences from the carboxyl end of VP1 upstream to DA VP1 residue 260, so the epitope for DA MAb2 must extend upstream from VP1 residue 268 at least as far as residue 260. The epitope for GDVII MAb1/2 does not extend as far upstream as this VP1 residue, since these MAbs neutralize all TMEV strains.

Putative trypsin cleavage sites are present at residues 259 and 261 of DA and residue 263 of GDVII (Fig. 3). Trypsin treatment of these residues would cleave off the neutralization site from the rest of VP1, rendering the virus resistant to neutralization or binding by DA MAb2 or GDVII MAb1 or MAb2. We found that all the escape mutants generated against the three MAbs were still sensitive to trypsin cleavage, leaving uncertainty as to whether the trypsin cleavage site is within or only near the neutralization site. Identification of the precise trypsin cleavage site and a better clarification of its relationship to the neutralization site must await site-directed mutagenesis of infectious TMEV cDNA.

One interesting finding of our sequencing studies was the limited diversity of nucleotide changes seen among escape mutants. We do not feel that this finding is an artifact of our isolation technique, causing repeated selection of the same mutant, for the following reasons. (i) Somewhat similar isolation techniques have been used to generate a diverse group of mutants in other virus systems (2). (ii) The same mutation was present both in DA virus escape mutants generated against GDVII MAb2 and in GDVII virus escape mutants generated against GDVII MAb1. (iii) Escape mutants generated from other picornaviruses, foot-and-mouth disease virus (B. Baxt, V. Vakharia, A. J. Franke, D. O. Morgan, and D. M. Moore, Abstr. ICN-UCI Int. Conf. Virol. 1988, p. 6) and hepatitis A virus (L.-H. Ping, R. W. Jansen, J. T. Stapleton, J. Cohen, and S. M. Lemon, Abstr. ICN-UCI Int. Conf. Virol. 1988, p. 25), have occasionally shown a similarly limited diversity in mutations seen. The limited diversity of nucleotide changes seen in TMEV escape mutants suggests that these nucleotides may have an important biological role. In the case of foot-and-mouth disease virus, trypsin cleavage disrupts cellular binding of the virion. To clarify the importance of the trypsin cleavage site in TMEV, the effect of escape mutants on in vivo demyelination is presently under investigation, as is the role of the carboxyl end of VP1 in cell binding.

We thank M. Ferguson for helpful suggestions, H. Lipton and D. Pevear for unpublished sequence information, D. Steiner for the preparation of primers, and M. Witt for expert secretarial assistance.

The work was supported by Public Health Service grant 2P01NS21442-04 from the National Institutes of Health and by a grant from the National Multiple Sclerosis Society.

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