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Antigenic Site II of the Rabies Virus Glycoprotein: Structure and Role in Viral Virulence

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Twelve monoclonal antibodies neutralizing the CVS strain of rabies virus were used to characterize antigenic site II of the viral glycoprotein. Nineteen antigenic mutants resistant to neutralization by some of these antibodies were selected; some continued to normally or partially bind the antibody, whereas others did not. Mutations conferring resistance to neutralization by site II-specific monoclonal antibodies were localized into two clusters, the first between amino acids 34 and 42 (seven groups of mutants) and the second at amino acids 198 and 200 (three groups of mutants). Two intermediate mutations were identified at positions 147 and 184. Four mutations resulted in reduced pathogenicity after intramuscular inoculation of the virus in adult mice. One of the mutants, M23, was 300 times and the others were 10 to 30 times less pathogenic than CVS. In three cases the attenuated phenotype was related to an important modification of antigenic site II, whereas the other known antigenic sites were unchanged.

Rabies virus is composed of a nucleocapsid containing a negative-strand RNA molecule (around 12,000 nucleotides long) wrapped into three protein species (L, M1, and N) and surrounded by a lipid bilayer associated with two protein species (M2, which is internal, and G, which is a glycosylated transmembrane). In addition to the five viral proteins, some contaminants of cellular origin such as actin (24) are included in the virion. The glycoprotein (which is the only protein external to the virion) plays an important role in viral pathogenesis: it is responsible for the recognition of specific cell surface receptors, thus controlling at least one aspect of viral tropism. It induces the synthesis of neutralizing antibodies (32), and after presentation by infected cells it is recognized by cytotoxic T cells (6).

Antigenic characteristics of the protein have been studied by using mutants resistant to neutralization by monoclonal antibodies (MAbs) selected as described by Seif et al. (28). One major site recognized by around 70% of the available MAbs has been described by Lafon et al. (20) (site II). The presence of another site (site III) recognized by 20% of the MAbs and several minor sites has also been demonstrated (20).

We have previously shown that site III is linear, extending from amino acids 330 to 340 (28). Some mutants affected in site III have lost virulence for adult animals such as mice, foxes, and various kinds of rodents (7, 8, 26). Further investigations located the mutation at amino acid 333, an arginine replaced by either glutamine, glycine, or isoleucine (12, 28). This mutation completely modified the host range spectrum of the virus (18).

The above results and others concerning influenza virus (5), reovirus (17, 30), coronavirus (9, 14), and mumps virus (22) suggested that antigenic sites on external proteins of virions are crucial regions which interact not only with antibodies but also with other host molecules such as cellular receptors. It is therefore of special interest to study the effect on pathogenicity of mutations modifying a major (or minor) antigenic site of a viral external protein. We therefore decided to undertake a systematic study of rabies antigenic mutants of site II. The isolation, characterization, and pathogenicity of such mutants are described in this report.

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MATERIALS AND METHODS

Cells. BHK-21 hamster kidney cells and CER (chicken embryo-related) hamster cells were grown in Eagle minimal essential medium supplemented with 10% calf serum. Hybridomas were grown in Dulbecco modified Eagle medium with 10% fetal calf serum, 2 mM L-glutamine, 10 mM sodium pyruvate, 0.1 mM hypoxanthine, 0.4 μM aminopterine, and 16 mM thymidine. The cells were maintained in a 5% CO_2 atmosphere.

MAbs. Twelve neutralizing antiglycoprotein MAbs specific for antigenic site II were used in this study. Three were obtained from T. J. Wiktor (Wistar Institute) (MAbs 231.22, 719.3, and 101.1), and two were obtained from M. Lafon (Institut Pasteur, Paris) (MAbs PVE and PVK). MAbs 7B1, 19C5, 24A1, 25A1, 25A2, 25B4, and 45C5 were produced with BALB/c mice immunized with UV-inactivated strain CVS virus and characterized in our laboratory as previously described (28).

Viruses. The CVS (challenge virus standard) strain of rabies virus was originally obtained from P. Atanasiu (Institut Pasteur). Mutants were selected for their resistance to neutralization by a specific MAb as described by Seif et al. (28). Mutants M23 and P3 were selected from two different clones of CVS with MAbs 25A1 and 25B4, respectively. The others were selected from a 5-fluorouracil-mutagenized stock. K mutants were selected with MAb 101.1, J mutants were selected with MAb 719.3, and mutants A3 and A17 were selected with MAb 231.22. Titers and susceptibility to neutralization were determined by plaque assay on CER cell monolayers as previously described (4).

Stocks were prepared by infecting BHK-21 cells at a multiplicity of 0.1 PFU per cell. Infection proceeded for 60 h at 33°C in Eagle medium with 2% calf serum. Purified virions were obtained as follows. Virions were pelleted through a 13-ml cushion of 25% glycerol in TNE (10 mM Tris chloride [pH 7.5], 1 mM EDTA, 50 mM NaCl) in an SW28 rotor for 1 h at 27,000 rpm. The pellets were suspended in TD (0.8 mM Tris chloride [pH 7.4], 150 mM NaCl, 5 mM KCl, 0.7 mM

Na_2HPO_4)–10 mM EDTA and further purified by centrifugation in a sucrose gradient (10 to 40% [wt/vol] in TD). The yield of purified virions was determined by the method of Bradford (3).

Fixation of MAbs on live virus. Viruses were concentrated by centrifugation through a cushion of 25% glycerol as described above. Pellets were suspended in 100 μl of 150 mM NaCl and then incubated with 10 μl of ascitic fluids for 1 h at room temperature. The mixture was centrifuged again through a glycerol pad (25% in TNE). The pellets were suspended in Laemmli buffer (19) and loaded on a 10% sodium dodecyl sulfate–polyacrylamide gel.

Nucleotide sequence analysis. The genomic RNA was obtained from 150 to 200 μg of purified virion, after digestion with proteinase K, followed by a phenolic extraction as previously described (28). The dideoxy chain-terminator method (27) was used for sequencing. Reverse transcription was performed directly on the genomic RNA by using synthetic oligonucleotides to prime the reaction. The five primers used had the sequences 5′ ATGGTXCCTGA PurGTT 3′, 5′ AGAGGCAGAGACCTA 3′, 5′ GATTACAC CATTTGGAT 3′, 5′ GTCCCAGGGTTTGGAAA 3′, and 5′ GCACCCCCTGGCTGA 3′; they were synthesized at the Pasteur Institute, except the third one (a generous gift of D. H. L. Bishop).

The sequencing was done by the method of Seif et al. (28) with the following modifications: the RNA was dissolved in 4.5 μl of water, and 1 μl of primer (1 μg/μl) and 1 μl of 25 mM CH_3HgOH were added. This solution was incubated at room temperature for 10 min. Then 2 μl of 175 mM β-mercaptoethanol was added and mixed with 2 μl of 1 M Tris chloride (pH 8.8), 1 μl of 200 mM $MgCl_2$, 3 μl of 1 M KCl, 2 μl of 3′ deoxynucleotide triphosphate mixture (1 mM dGTP, 1 mM dTTP, 1 mM dCTP, 0.1 mM dATP), 3 μCi of [α-^{32}P]dATP (>3,000 Ci/mmol) in 3 μl, and 20 U of reverse transcriptase. Then it was immediately dispensed in 4.5-μl fractions into four tubes, each containing 1 μl of a different chain terminator (100 μM ddGTP, 100 μM ddTTP, 100 μM ddCTP, 10 μM ddATP). The four polymerization mixtures were incubated at 37°C for 15 min, and then 6 μl of deionized formamide–10 mM EDTA–0.02% xylene cyanole–0.02% bromophenol blue was added. Portions of 1 to 1.5 μl were heated at 100°C for 5 min and applied to an 8 or 6% polyacrylamide slab gel (55 by 19 by 0.02 cm; Macrophore; LKB Produkter AB, Bromma, Sweden). Gels were run at 2,500 V for various lengths of time (1 h 30 min to 6 h). At the end of the run the gel was transferred on a Whatman 3MM paper, covered with Saran Wrap, and autoradiographed with a Kodak X-Omat AR or S film at −70°C with a Dupont Cronex Lightning-Plus intensifying screen.

Pathogenicity test. Five 6-week-old OF1 female mice (Iffa Credo) were inoculated with 50 μl of serial 10-fold dilutions of the virus in the masseter muscle. The exact dose injected was controlled by plaque assay on CER cells. The number of mice surviving at each dilution was recorded for 14 days, at which time the dose-effect curve was established.

Immunosorbent assay. An enzyme-linked immunosorbent assay was performed with alkaline phosphatase-conjugated goat anti-mouse immunoglobulins (Cooper Biomedical, Inc., West Chester, Pa.). About 300 ng of virus was dispensed into each well of a microtiter plate. The antigen was diluted in phosphate-buffered saline (10 mM phosphate [pH 7], 150 mM NaCl) and allowed to dry overnight at 37°C. The binding sites of the plate were then saturated with 10% horse serum in phosphate-buffered saline. Between each successive step three washes were performed. All washes were done with 1% horse serum in phosphate-buffered saline. MAbs were incubated with the antigen for 90 min at 37°C, alkaline-phosphatase-labeled anti-mouse immunoglobulin was added, and the mixture was incubated for 1 h at 37°C. Finally paranitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) in 1 M Tris chloride (pH 8) was added. Optical density was measured at 410 nm with a Mini Reader Spectrophotometer (Dynatech Laboratories, Santa Monica, Calif.).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Electrophoresis of proteins was performed as previously described (19). Slab gels were stained with Coomassie brillant blue or by the silver staining method (25).

RESULTS

Isolation and characterization of site II-specific antigenic mutants. Twelve neutralizing MAbs recognizing site II of the viral glycoprotein were used in this study. They have been classified as site II specific because they failed to neutralize representative site II mutants but retained their capability to neutralize mutants at other sites. Five of them were used to select antigenic mutants, and then their resistance to the other antibodies was determined. A mutant was considered fully resistant when its titer in the presence of antibody was at least 50% of that observed without antibody. The mutant was considered slightly resistant when the titer after incubation with antibody was between 50 and 10% of the control. Below 10% of the control, the mutant was considered sensitive. All class II mutants were still neutralized by the specific MAbs for the antigenic site III of the CVS strain (data not shown); no overlapping of antigenic sites II and III was observed. All mutants were at least resistant to the MAb used for their selection, even though two of them were only slightly resistant: K18 had 10% resistance to MAb 101.1, and M23 had 30% resistance to MAb 25A1.

The mutants were classified into 12 different groups according to their pattern of resistance to the 12 MAbs (Fig. 1). Resistance varied from being restricted to a single MAb (K14) to including almost every site II-specific MAb (A17 and J25). Seven groups were represented by a single mutant. Five groups contained two or more mutants. In this case two or three representatives were retained for further studies.

Fixation of MAbs on the mutants. The fixation of MAbs on the mutants and on CVS was studied with the enzyme-linked immunosorbent assay, using the same amount of dried virus as the antigen. As expected, the fixation of a given MAb on mutants which were still neutralized was similar to that observed on CVS. Partial neutralization of a mutant usually correlated with a normal fixation of the MAb; the only exception was the weak fixation of MAbs 231.22 and 24A1 on mutant P3 (Fig. 1).

For mutants fully resistant to neutralization all possible situations were observed: either the mutants continued to fix the MAb normally (for instance, MAb 45C5 with mutants of groups 4, 5, and 11), there was no fixation at all (45C5 with mutants of group 2), or the fixation was intermediate (45C5 with mutants of groups 3 and 10). Similarly, for a given antibody, all of these situations could be found (Fig. 1).

To determine whether the fixation occurred on live virus the following experiment was performed. Virus, purified and concentrated by a rapid procedure which preserves infectivity, was incubated with antibody and sedimented through a glycerol pad. Pellets were suspended in sodium dodecyl sulfate buffer and analyzed on polyacrylamide gels. As a control the virus was incubated with an antinucleocapsid MAb (21A6) which would fix only on disrupted virions. In

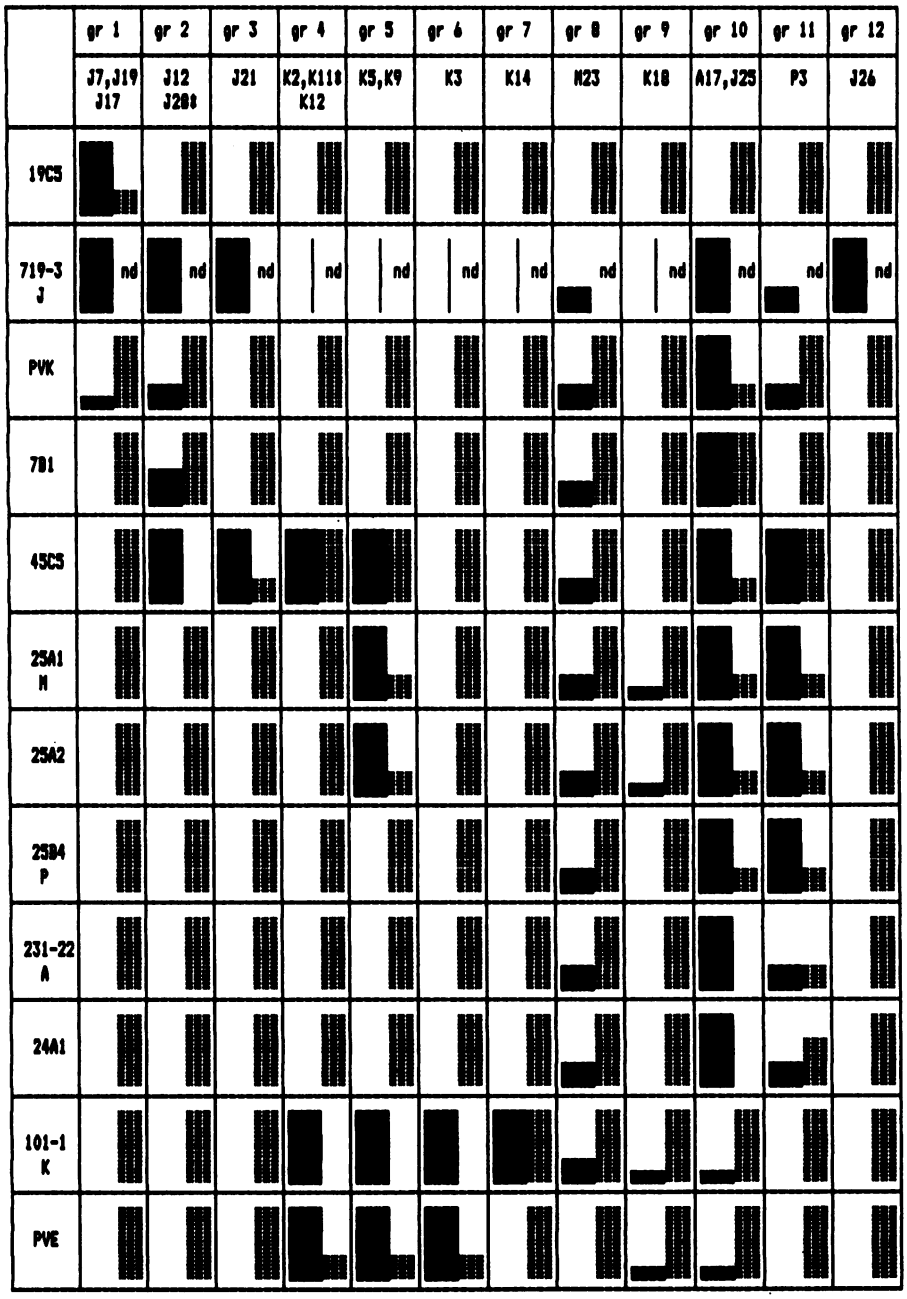

FIG. 1. Classification of antigenic mutants selected with site II-specific neutralizing MAbs. In each square are presented both the resistance to the neutralization by the antibody calculated by plaque assay as described in Seif et al. (28) (■) and the fixation of the antibody on the mutant determined by ELISA (■). nd, Not done.

this latter case, no fixation was observed, which indicated that most of the pelleted virions were indeed intact (Fig. 2). The fixation of MAb 45C5 on CVS, A17, and K2 was similar, although both mutants were resistant to neutralization. By comparison only partial fixation of MAb 45C5 on A17 was found in the enzyme-linked immunosorbent assay (Fig. 1).

The intensity of light and heavy immunoglobulin bands was compared with that of viral proteins on Coomassie blue-stained gels (data not shown). The results suggested that one molecule of immunoglobulin was fixed on each glycoprotein of the virus.

Experiments with MAbs 7B1 and PVK were more difficult to quantify because their heavy and light chains migrated closer to N and M_2. The fixation of PVK on CVS and K2 was similar; it was notably reduced on A17 (which was resistant to this antibody). The fixation of 7B1 was similar on the three viruses (Fig. 2).

With the exception of MAb 45C5 with A17, results obtained with dried or live viruses were very similar. Both methods indicate that resistance to neutalization is not necessarily correlated with a lack of fixation of a given MAb.

Electrophoretic pattern of the mutant. It has been previously shown that an amino acid substitution at position 198 results in complete loss of the GII form of the glycoprotein (34). We found the same result for group 11 and only a partial loss for group 10, both affected at lysine 198. As an example

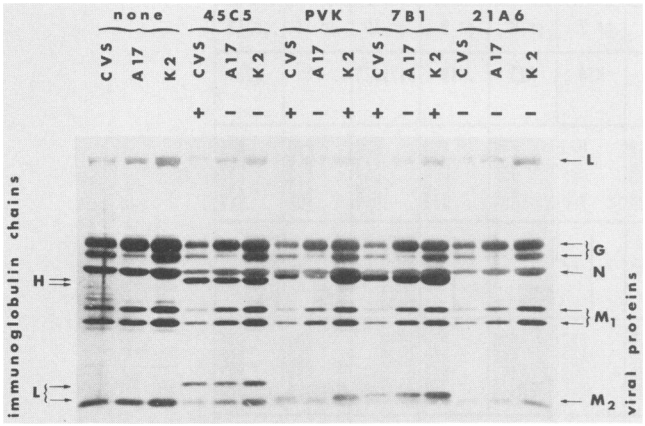

FIG. 2. Fixation of MAbs on live virions. After incubation with or without MAbs, live purified virions were sedimented through a glycerol pad (see Materials and Methods) to separate the virus-MAb complexes from nonadsorbed immunoglobulins. Proteins from the pellet were electrophoresed in a 10% polyacrylamide Laemmli gel. The neutralizing power of the MAb was determined as described in the legend to Fig. 1. Lanes: +, neutralization; −, no neutralization.

the electrophoretic profile of A17 is shown in Fig. 2. Mutants of the other groups had the two forms of the glycoprotein in normal amounts (compare K2 and CVS in Fig. 2).

Localization of the mutation of class II antigenic mutants. The nucleotide sequence of CVS parental and mutant glycoproteins has been partially or totally established by the dideoxy chain-termination method. The deduced amino acid sequence of the parental glycoprotein is shown in Fig. 3. Three differences appeared between this sequence and that published by Yelverton et al. (35): at position 36, an isoleucine instead of a threonine; at position 122, a leucine, instead of a valine, like in the ERA strain; and at position 156, a glycine instead of a serine, like in the PV strain (1, 31).

For each antigenic site II mutant, a length of the gene was sequenced; we found only one base substitution which in any case resulted in an amino acid change (Table 1). The substitutions were located within two major clusters. The first cluster corresponds to seven groups of mutants having amino acid substitutions between positions 34 and 42. The second cluster corresponds to three groups of mutants having a substitution at amino acid 198 or 200. The two remaining mutants had mutations located in position 147 (M23) or 184 (K18). Mutations in positions 147 (M23) and 198 (A17, J25, P3) conferred partial or total resistance to most MAbs recognizing site II, although the sensitivity to MAbs recognizing sites I and III was unchanged (data not shown). In this last position a change to glutamic acid modified site II more deeply than a change to methionine. At three other positions, 36, 40, and 42, amino acid residues had undergone mutations to two different amino acids, which also resulted in a different panel of resistance. At position 36, one of the mutations observed gave rise to the amino acid detected in another CVS strain (35) and in the ERA strain (1). In the first cluster (amino acids 34 through 42) mutants selected with MAbs 719.3 and 101.1 were not randomly distributed; mutations conferring resistance to 719.3 were located in positions 34 and 36, and mutations conferring resistance to 101.1 were located in positions 40 and 42. A single mutation at position 34 conferred resistance to MAb 19C5. It is therefore likely that the epitope recognized by this antibody is distant from those recognized by the other site II-specific MAbs.

Of the 12 substitutions observed, 6 resulted in a charge modification, the protein being in all instances more acidic: a positively charged amino acid was changed for a neutral or even an acidic amino acid, or a neutral amino acid was changed to a glutamic acid. Five substitutions resulted in shifts from hydrophobic to hydrophilic or vice versa.

Pathogenicity of class II mutants. Some class II mutants have already been found to be pathogenic for adult mice after intracerebral inoculation (8). To detect any difference between the site II mutants and the CVS parental strain the pathogenicity of representative site II mutants was studied after intramuscular inoculation. Serial dilutions of the virus were inoculated into five adult mice, and the survival of animals was followed (Fig. 4). By this route of inoculation, the 50% lethal dose was equal to 350 PFU for CVS; for J19, A17, P3, and J25, respectively, it was equal to 3.5×10^3, 8.9×10^3, 9.3×10^3, and 10.7×10^3 PFU (i.e., 10 to 30 times more), and for M23 it was equal to 9.3×10^4 PFU (300 times more). Representatives of other site II groups of mutants were as pathogenic as the parental CVS strain (K2 in Fig. 3).

DISCUSSION

We studied the structure of the main antigenic site of the rabies glycoprotein (site II) by using a collection of mutants resistant to neutralization by several MAbs directed to this site. Amino acid substitutions of representative mutants

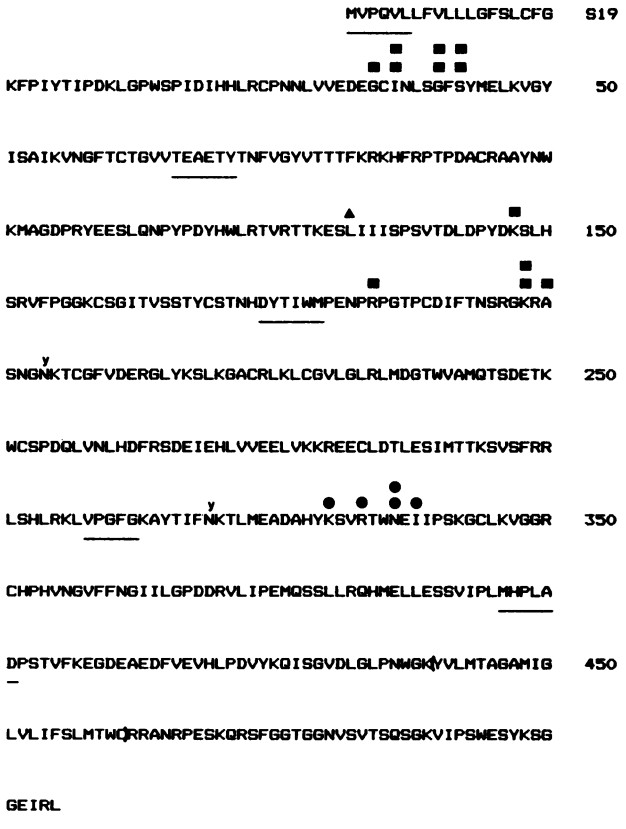

FIG. 3. Amino acid sequence of the CVS glycoprotein (in a one-letter code). The sequence was deduced from the nucleotide sequence determined in our laboratory. The signal peptide sequence comes from Yelverton et al. (35). Symbols: ■, mutations affecting antigenic site II; ●, mutations affecting site III (28); ▲, temperature-sensitive mutation (30a). The membrane-anchoring region is shown within parentheses. The positions of the primers are underlined, and glycosylation sites (y) are indicated.

TABLE 1. Localization of mutations conferring resistance to neutralization by site II MAbs[a]

Group	Mutant	Sequenced region (amino acid nos.)	Position	Nucleotide substitution	Amino acid change
1	J7	5–63, 80–289, 318–365	34	GGA——GAA	Gly to Glu
	J17	15–162, 183–240, 319–378	34	GGA——GAA	Gly to Glu
	J19	4–54, 98–146, 182–226	34	GGA——GAA	Gly to Glu
2	J12	15–73, 183–276	36	ATC——ACC	Ile to Thr
	J28	15–73, 183–276	36	ATC——ACC	Ile to Thr
3	J21	4–54, 98–146, 182–226	36	ATC——GTC	Ile to Val
4	K2	1–74, 183–271, 317–415	40	GGG——GTG	Gly to Val
	K11	13–73	40	GGG——GTG	Gly to Val
	K12	3–70	40	GGG——GTG	Gly to Val
5	K5	15–75	40	GGG——GAG	Gly to Glu
	K9	5–73, 79–287, 315–365	40	GGG——GAG	Gly to Glu
6	K3	13–73	42	TCC——CCC	Ser to Pro
7	K14	5–73, 183–290	42	TCC——TTC	Ser to Phe
8	M23	5–415	147	AAA——CAA	Lys to Gln
9	K18	3–70, 75–272, 319–357	184	AGA——GGA	Arg to Gly
10	A17	183–290	198	AAG——GAG	Lys to Glu
	J25	183–290	198	AAG——GAG	Lys to Glu
11	P3	183–240	198	AAG——ATG	Lys to Met
12	J26	189–239	200	GCA——GTA	Ala to Val

[a] Sequences were determined by the dideoxy chain termination method with five oligonucleotide primers corresponding to the ectodomain of the protein.

have been located between positions 34 and 42 and positions 198 and 200 and in two intermediate positions (147 and 184). Our results show that with most MAbs it should be possible to select mutants in both clusters. A similar situation has been observed for influenza virus, in which different regions of the polypeptide chain occupied positions close to each other in the three-dimensional structure of the hemagglutinin to give one antigenic site (5). Analysis of the tertiary structure of this molecule showed that site C is constituted by two distant regions joined by a disulfide bond (33). In the case of rabies virus Dietzschold et al., analyzing unreduced cyanogen bromide fragments of the glycoprotein, have shown that the peptide containing amino acids 34 through 42 was joined by a disulfide bridge to the one containing amino acids 198 through 200 (11). There are two cysteines in the first fragment, one in position 24 and the other in position 35. This last cysteine, even though located in the middle of the first cluster, was conserved in all of the mutants so far examined. We can therefore postulate that antigenic site II results from a folding which brings close together two separate regions of the viral glycoprotein. At this point of our work, valuable information would arise from three-dimensional analysis of the protein; therefore, as a preliminary step to the crystallographic approach, we are presently cloning the glycoprotein gene in an expression vector.

As with antigenic mutants of herpes simplex virus (15), measles virus (29), Newcastle disease virus (16), poliovirus (2, 10), and rabies virus (20), resistance to neutralization is not always accompanied by a lack of fixation of the corresponding MAb. In several instances, the MAb was still able to fix on the non-neutralizable mutants as efficiently as on the neutralizable CVS strain, and this fixation could reach a maximal value of one molecule of immunoglobulin per molecule of glycoprotein. One can therefore wonder what neutralization means; the fixation of antibodies may induce some change in the virion which could prevent one or the other step of the viral cycle (for a review, see reference 13). We are currently investigating which of these steps is blocked in the case of rabies and the possible modifications of the virus induced by the fixation of antibodies.

We found that five mutants were substantially less pathogenic than the CVS parental strain (by a factor of 10 or 100).

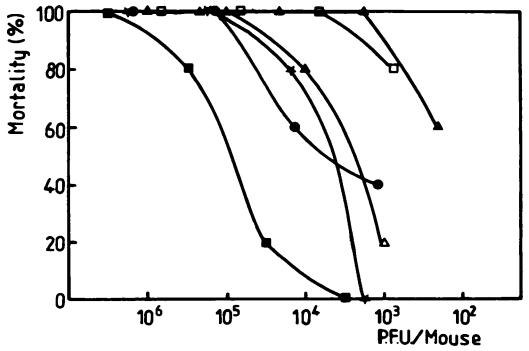

FIG. 4. Pathogenicity of antigenic mutants resistant to neutralization by site II MAbs. Groups of five adult mice were injected in the masseter muscle with 50 μl of increasing dilutions of the mutants. The viral titers were determined by plaque assay in CER cells. Symbols: ▲, CVS; □, K2; △, J19; ★, J25; ●, P3; ■, M23.

In mutant J17 the mutation is at one extremity of the first cluster, but site II is deeply modified in the four others.

The structure of antigenic site II is very different from that of site III, which expands linearly from amino acids 330 to 340 (28). This region was previously implicated in the recognition of specific receptors present on nerve endings (18). The replacement of arginine 333 within this site by a glutamine abolishes the capability of the virus to penetrate parasympathetic or retinopetal ocular nerve endings while preserving its capability to enter trigeminal endings. We therefore postulated that different receptors were present at those termini and that the viral mutation prevented the recognition of some but not all of them. Of course we cannot infer from the above results whether a different region of the glycoprotein is implicated in the recognition of trigeminal receptors.

Lentz et al. had presented a comparison of the amino acid sequence of rabies virus glycoprotein with the long neurotoxin from snakes of the Elapidor family (21). These neurotoxins are polypeptides that bind to the nicotinic acetylcholine receptor and then block the fixation of acetylcholine. The greatest similarity between the virus glycoprotein and neurotoxin occurred with the neurotoxin residues that bound to the acetylcholine receptor and residues 189 through 200 of the glycoprotein. In this region, amino acid 198 is one of the few residues conserved among all of the neurotoxins and the glycoprotein. Lentz et al. concluded that the nicotinic acetylcholine receptor could be the rabies receptor, which is probably not correct. As already pointed out there seem to be several receptors for rabies, and at least some of them interact with site III (and not site II) of the viral glycoprotein.

The fact that some of site II antigenic mutants, especially those affected in amino acid 198, are less pathogenic for adult animals is certainly interesting. It could reveal another type of interaction between the virus and the host, either at the beginning or at the end of the infection, when the virus has invaded the nervous system of the host and is disseminating in some non-nervous specialized cells (23). As expected antigenic mutants with modified pathogenicity are promising tools to study the multiple aspects of viral virulence.

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LITERATURE CITED

1. **Anilionis, A., W. H. Wunner, and P. J. Curtis.** 1981. Structure of the glycoprotein gene of rabies virus. Nature (London) **294**:275–278.
2. **Blondel, B., R. Crainic, O. Fichot, G. Dufraisse, A. Candrea, D. Diamond, M. Girard, and F. Horaud.** 1986. Mutations conferring resistance to neutralization with monoclonal antibodies in type 1 poliovirus can be located outside or inside the antibody-binding site. J. Virol. **57**:81–90.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Bussereau, F., A. Flamand, and D. Pèse-Part.** 1982. Reproducible plaquing system for rabies virus in CER cells. J. Virol. Methods **4**:277–282.
5. **Caton, A. J., G. G. Brownlee, J. W. Yewdell, and W. Gerhard.** 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell **31**:417–427.
6. **Celis, E., R. W. Miller, T. J. Wiktor, B. Dietzschold, and H. Koprowski.** 1986. Isolation and characterization of human T cell lines and clones reactive to rabies virus: antigen specificity and production of interferon. J. Immunol. **136**:692–697.
7. **Coulon, P., P. Rollin, M. Aubert, and A. Flamand.** 1982. Molecular basis of rabies virus virulence. I. Selection of avirulent mutants of the CVS strain with anti-G monoclonal antibodies. J. Gen. Virol. **61**:97–100.
8. **Coulon, P., P. E. Rollin, and A. Flamand.** 1983. Molecular basis of rabies virus virulence. II. Identification of a site on the CVS glycoprotein associated with virulence. J. Gen. Virol. **64**: 693–696.
9. **Dalziel, R. G., P. W. Lampert, P. J. Talbot, and M. J. Buchmeier.** 1986. Site-specific alteration of murine hepatitis virus type 4 peplomer glycoprotein E2 results in reduced neurovirulence. J. Virol. **59**:463–471.
10. **Diamond, D. C., B. A. Jameson, J. Bonin, M. Kohara, S. Abe, H. Itoh, T. Komatsu, M. Arita, S. Kuge, A. Nomoto, A. D. M. E. Osterhaus, R. Crainic, and E. Wimmer.** 1985. Antigenic variation and resistance to neutralization in poliovirus type 1. Science **229**:1090–1093.
11. **Dietzschold, B., T. J. Wiktor, R. I. MacFarlan, and A. Varrichio.** 1982. Antigenic structure of rabies virus glycoprotein: ordering and immunological characterization of the large CNBr cleavage fragments. J. Virol. **44**:595–602.
12. **Dietzschold, B., W. H. Wunner, T. J. Wiktor, A. D. Lopes, M. Lafon, C. L. Smith, and H. Koprowski.** 1983. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. Proc. Natl. Acad. Sci. USA **80**:70–74.
13. **Dimmock, N. J.** 1987. Multiple mechanisms of neutralization of animal viruses. Trends Biochem. Sci. **12**:70–75.
14. **Fleming, J. O., M. D. Trousdale, F. A. K. El-Zaatari, S. A. Stohlman, and L. P. Weiner.** 1986. Pathogenicity of antigenic variants of murine coronavirus JHM selected with monoclonal antibodies. J. Virol. **58**:869–875.
15. **Holland, T. C., S. D. Marlin, M. Levine, and J. Glorioso.** 1983. Antigenic variants of herpes simplex virus selected with glycoprotein-specific monoclonal antibodies. J. Virol. **45**:672–682.
16. **Iorio, R. M., and M. A. Bratt.** 1985. Selection of unique antigenic variants of Newcastle disease virus with neutralizing monoclonal antibodies and anti-immunoglobulin. Proc. Natl. Acad. Sci. USA **82**:7106–7110.
17. **Kaye, K. M., D. R. Spriggs, R. Bassel-Duby, B. N. Fields, and K. L. Tyler.** 1986. Genetic basis for altered pathogenesis of an immune-selected antigenic variant of reovirus type 3 (Dearing). J. Virol. **59**:90–97.
18. **Kucera, P., M. Dolivo, P. Coulon, and A. Flamand.** 1985. Pathways of the early propagation of virulent and avirulent rabies strains from the eye to the brain. J. Virol. **55**:158–162.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **Lafon, M., T. J. Wiktor, and R. I. Macfarlan.** 1983. Antigenic sites on the CVS rabies virus glycoprotein: analysis with monoclonal antibodies. J. Gen. Virol. **64**:843–851.
21. **Lentz, T. L., P. T. Wilson, E. Hawrot, and D. W. Speicher.** 1984. Amino acid sequence similarity between rabies virus glycoprotein and snake venom curaremimetic neurotoxins. Science **226**:847–848.
22. **Löve, A., R. Rydbeck, K. Kristensson, C. Orvell, and E. Norrby.** 1985. Hemagglutinin-neuraminidase glycoprotein as a determinant of pathogenicity in mumps virus hamster encephalitis: analysis of mutants selected with monoclonal antibodies. J. Virol. **53**:67–74.
23. **Murphy, F. A., A. K. Harrison, W. C. Winn, and S. P. Bauer.** 1973. Comparative pathogenesis of rabies and rabies-like viruses. Infection of the central nervous system and centrifugal

spread of virus to peripheral tissues. Lab. Invest. **29**:1–16.
24. **Naito, S., and S. Matsumoto.** 1978. Identification of cellular actin within rabies virus. Virology **91**:151–163.
25. **Oakley, B. R., D. R. Kirsch, and N. R. Morris.** 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. **105**:361–363.
26. **Pépin, M., J. Blancou, M. F. A. Aubert, J. Barrat, P. Coulon, and A. Flamand.** 1985. Oral immunization against rabies with an avirulent mutant of the CVS strain: evaluation of its efficacy in fox (Vulpes vulpes) and its infectivity in seven other species. Ann. Virol. **136E**:65–73.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
28. **Seif, I., P. Coulon, P. E. Rollin, and A. Flamand.** 1985. Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. J. Virol. **53**:926–934.
29. **Sheshberadaran, H., and E. Norrby.** 1986. Characterization of epitopes of measles virus hemagglutinin. Virology **152**:58–65.
30. **Spriggs, D. R., and B. N. Fields.** 1982. Attenuated reovirus type 3 strain generated by selection of haemagglutinin antigenic variants. Nature (London) **297**:68–70.
30a. **Tidke, R., C. Prehaud, P. Coulon, J. Blancou, and A. Flamand.** 1987. Characterization of a double avirulent mutant of rabies virus and its potency as a vaccine, live or inactivated. Vaccine **5**:229–233.
31. **Tordo, N., O. Poch, A. Ermine, G. Keith, and F. Rougeon.** 1986. Walking along the rabies genome: is the large G-L intergenic region a remnant gene? Proc. Natl. Acad. Sci. USA **83**:3914–3918.
32. **Wiktor, T. J., E. György, H. D. Schlumberger, H. D. Sokol, and H. Koprowski.** 1973. Antigenic properties of rabies virus components. J. Immunol. **110**:269–276.
33. **Wiley, D. C., I. A. Wilson, and J. J. Skehel.** 1981. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature (London) **289**:373–378.
34. **Wunner, W. H., B. Dietzschold, C. L. Smith, M. Lafon, and E. Golub.** 1985. Antigenic variants of CVS rabies virus with altered glycosylation sites. Virology **140**:1–12.
35. **Yelverton, E., S. Norton, J. F. Obijeski, and D. V. Goeddel.** 1983. Rabies virus glycoprotein analogs: biosynthesis in *Escherichia coli*. Science **219**:614–620.