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Rabies Virulence: Effect on Pathogenicity and Sequence Characterization of Rabies Virus Mutations Affecting Antigenic Site III of the Glycoprotein

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Using four neutralizing monoclonal antibodies which presumably bind to the same antigenic site on the CVS glycoprotein (antigenic site III as defined by cross-neutralization tests), we isolated 58 mutants of the CVS strain of rabies virus. These mutants were highly resistant to the selecting antibodies and grew efficiently in cell cultures. We classified them into five groups on the basis of the pattern of resistance to the four antibodies. We determined pathogenicities of the mutants for adult mice by intracerebral inoculation. Group 2 mutants were nonpathogenic or had attenuated pathogenicity. On the contrary, mutants from the other groups were pathogenic, causing paralysis and death as does CVS. We determined the nucleotide alterations of representative mutants from each group by using the dideoxy method of RNA sequencing. In the glycoproteins of eight nonpathogenic or attenuated mutants, we identified an amino acid substitution at position 333. Arginine 333 was replaced by either glutamine or glycine. In the glycoprotein of eight pathogenic mutants, we identified an amino acid substitution at lysine 330, asparagine 336, or isoleucine 338. Thus, although all substitutions affected neutralization and were located close to each other in the glycoprotein sequence, only substitutions at position 333 affected pathogenicity.

Rabies virus particles, usually transmitted by animal bites, migrate to the brain and cause an acute and fatal disease associated with intense viral replication in the central nervous system. Injection of rabies virions into mouse or rat brains leads to the development of the same symptoms. A better understanding of the role of the viral components in virulence may suggest new approaches to the treatment and prevention of rabies.

We previously reported that the high pathogenicity of the CVS strain of rabies virus was affected by certain mutations in the viral glycoprotein (8, 9). Our experiments showed that 9 of 10 mutants selected by collecting these viral clones resistant to the two neutralizing anti-glycoprotein antibodies 194-2 and 248-8 were either nonpathogenic or highly attenuated (at least when tested in adult immunocompetent animals) (9). Using the same system, Dietzschold et al. (10) confirmed this relationship between pathogenicity and the resistance to both antibodies (194-2 and 248-8). Analyzing four mutants, they found that the relationship corresponded to an amino acid substitution at position 333 in the glycoprotein, arginine 333 being replaced by either isoleucine (in three mutants of the ERA strain) or glutamine (in one mutant of the CVS strain).

Recently, 88 CVS glycoprotein mutants, selected with 12 neutralizing anti-glycoprotein monoclonal antibodies, were assembled into three independent classes by Lafon et al. (19), on the basis of similarities found between the mutants in resistance patterns for the 12 antibodies (cross-neutralization tests). The three classes of mutants may correspond to three antigenic sites of the CVS glycoprotein (I, II, and III). Antibodies 194-2 and 248-8, mentioned above, neutralize all mutants from the three classes, except some class III mutants; these antibodies are among the class III-specific monoclonal antibodies. Therefore, all nonpathogenic CVS mutants are class III mutants. On the other hand, in a previous report (9), we showed that class III mutants resistant to 194-2 but not to 248-8, or resistant to 248-8 but not to 194-2, were pathogenic in adult mice.

Since we were basically interested in the molecular basis of rabies virulence, a detailed analysis of the glycoprotein of class III CVS mutants was undertaken in our laboratory to answer the following questions. Is the loss of pathogenicity always associated with the replacement of arginine 333? What are the sequence differences between the glycoproteins of nonpathogenic mutants, attenuated mutants, and pathogenic mutants? What is the structural complexity of the glycoprotein region(s) influencing the neutralization by the class III-specific monoclonal antibodies? Are some of the mutations that cause resistance to these antibodies also causing temperature-sensitive growth?

In this report, we describe (i) the selection of class III mutants, (ii) the distribution of these mutants into groups, according to their precise pattern of resistance to several class III-specific monoclonal antibodies, (iii) the growth of the mutants in cell cultures, (iv) the pathogenicity of the mutants for adult mice, and (v) glycoprotein sequences of representative mutants from each group. We found that although all of these mutants had one amino acid substitution at or around position 333 in the glycoprotein. Only substitutions at position 333 affected pathogenicity.

MATERIALS AND METHODS

Cells. BHK-21 baby hamster kidney cells and CER (chick embryo related) hamster cells were grown in Eagle minimal essential medium (MEM) supplemented with 10% calf serum. The mouse myeloma Sp2/O was grown in Dulbecco modified MEM with 10% fetal calf serum, 2 mM L-glutamine, and 10 mM sodium pyruvate. Hybridomas were grown in the same medium with 2% 50× HAT (hypoxanthine-aminopterin-thymidine; Flow Laboratories, Rockville, Md.). All

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cells were maintained in a 5% CO_2 atmosphere. The hybridomas used for mutant virus selection were grown in the peritoneal cavity of pristane-primed BALB/c mice.

Viruses. The CVS (challenge virus standard) strain of rabies virus was originally obtained from P. Atanasiu (Institut Pasteur). Mutants were derived from seven plaque-purified CVS subclones (b to h) and from a 5-fluoro-uracil (5-FU)-mutagenized CVS stock (4). Titers were determined by plaque assay on CER cell monolayers as previously described (5). Plaques were counted after 5 days of infection at 33°C. Stocks were prepared at 33°C in MEM with 0.1% bovine serum albumin, by infection of BHK-21 cells at a multiplicity of 0.1 PFU per cell. For RNA-sequencing experiments, 15×10^7 BHK-21 cells were infected at a multiplicity of 1 PFU per cell. Infection proceeded for 60 h at 33°C. Virions were purified by centrifugation in a sucrose gradient (10 to 40%). The yield of purified virions was determined by a colorimetric test for quantitation of protein (2). Usually, 200 μg was obtained.

Monoclonal antibodies. Neutralizing anti-glycoprotein monoclonal antibodies 194-2, 248-8, and 507-1 were obtained from T. J. Wiktor (Wistar Institute) as ascitic fluids (16). Seven anti-CVS monoclonal antibodies were produced and characterized in our laboratory. Mice were immunized by two intraperitoneal injections of 100 μg of UV-inactivated CVS, with 3 months intervening. Spleen cells from these mice were fused with Sp2/O myeloma cells, by the method of Fazekas de St. Groth and Scheidegger (13). The specificity of the hybridoma supernatants was determined by enzyme-linked immunosorbent assay (ELISA), using the nucleocapsid and the virion glycoprotein as antigens. Hybridoma 21B4, which secreted immunoglobulins specific for the glycoprotein, was cloned by limiting dilution and injected into mice for production of ascitic fluid.

Nucleocapsid. Purification of the nucleocapsids was performed by the method of Roux and Waldvogel (21). BHK-21 cells (2.5×10^8) were infected with CVS at a multiplicity of 1 PFU per cell. After 24 h, the cells were washed twice in 8 mM Tris chloride (pH 7.4)–150 mM NaCl–5 mM KCl–0.7 mM Na_2HPO_4 (TBS). They were pelleted at $2,000 \times g$ for 10 min. The pellet was suspended in 3 ml of 50 mM Tris chloride (pH 8.0)–10 mM NaCl–0.5% Nonidet P-40. The suspension was centrifuged at $3,000 \times g$ for 15 min at 4°C. The supernatant was recovered, adjusted to 6 mM EDTA, and loaded onto a 2.5-ml sucrose solution (5% sucrose, 20 mM Tris chloride [pH 7.5], 50 mM NaCl, and 2 mM EDTA) layered onto a 7-ml CsCl gradient (20 to 40% CsCl, 20 mM Tris chloride [pH 7.5], 50 mM NaCl, and 2 mM EDTA). After centrifugation at 30,000 rpm for 18 h at 10°C in a Beckman SW41 rotor, a band of nucleocapsids was clearly visible (density, 1,304). The band was recovered, dialyzed, and diluted 1:100 in TBS with 1 mM EDTA.

Glycoprotein. Glycoprotein was isolated from purified virions incubated for 30 min at room temperature with 500 μl of 10 mM Tris chloride (pH 7.5)–5 mM EDTA–5% Triton X-100. The suspension was adjusted to 150 mM $CaCl_2$, layered onto 3 ml of a 20% glycerol solution, and centrifuged at 35,000 rpm for 1 h in a Beckman SW50.1 rotor. The supernatant contained the glycoprotein and traces of the M1 and M2 proteins. It was diluted 1:30 in TBS with 1 mM EDTA.

ELISA. An ELISA was performed (11), with alkaline phosphatase-conjugated goat immunoglobulins specific for the mouse immunoglobulins A (IgA), IgG, and IgM (Cappel Laboratories, West Chester, Pa.). The diluted antigen (nucleocapsid or glycoprotein) was dispensed into the wells of a microtiter plate (50 μl per well) and dried overnight at 37°C. The plate was rinsed three times in phosphate-buffered saline (7.4 mM $NaHPO_4$, 2.4 mM KH_2PO_4) with 10% horse serum. Each hybridoma supernatant to be tested was added to two wells (50 μl per well). After incubation at 37°C for 90 min in a humidified atmosphere, the wells were washed three times with phosphate-buffered saline containing 1% horse serum. The alkaline phosphatase-conjugated anti-mouse immunoglobulins were diluted 1:200 in phosphate-buffered saline containing 0.2% sodium azide, and 150 μl was added to each well. After incubation at 37°C for 1 h, the wells were washed three times with phosphate-buffered saline containing 1% horse serum. Finally, 250 μl of 1 M Tris chloride (pH 8.0) and 1.33 mM *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) was added to each well. The absorbance in each well was determined at 410 nm in a spectrophotometer (Mini Reader; Dynatech Laboratories, Santa Monica, Calif.).

Selection of mutants. Various dilutions of CVS virus, in 100 μl of MEM plus 0.1% bovine serum albumin, were incubated for 30 min with 10 μl of a diluted ascitic fluid (neutralizing anti-glycoprotein monoclonal antibody); ascitic fluids 194-2, 248-8, and 21B4 were diluted 1:10 in MEM-bovine serum albumin and ascitic fluid 507-1 was diluted 1:100. The mixtures were plated onto monolayers of CER cells in petri dishes. After incubation at 33°C for 5 days, well-separated plaques were picked. Small stocks were prepared in BHK-21 cells at 33°C. Then, ca. 1,000 PFU of each clone, in 100 μl of MEM-bovine serum albumin was incubated with 10 μl of the selecting antibody (194-2, 248-8, and 21B4 diluted 1:100 and 507-1 diluted 1:1000). The mixtures were plated onto CER cells. Clones giving more than 900 plaques (90% of the titer) were considered fully resistant to their selecting antibody. These clones were tested for resistance to the remaining three antibodies. Clones giving more than 500 plaques (50% of the titer) were considered highly resistant. Clones giving less than 10 plaques (1% of the titer) were considered sensitive.

Pathogenicity test. A 1-PFU dose of CVS virus injected intracerebrally into mice kills 50% of the mice in 7 days, whereas higher doses kill 100%. For each mutant virus, a dose of 1,000 PFU in 30 μl of TBS was injected intracerebrally into at least five adult Swiss mice. Surviving mice were kept under observation for 30 days and then challenged intracerebrally with 40 PFU of CVS. They survived the challenge.

Sequencing procedure. The oligonucleotide primer was generously supplied by D. H. L. Bishop. 2′-deoxynucleoside 5′-triphosphates and 2′,3′-dideoxynucleoside 5′-triphosphates were obtained from P-L Biochemicals (Milwaukee, Wis.). ^{32}P-labeled 2′-deoxyadenosine 5′-triphosphate was obtained from the Radiochemical Centre (Amersham Corp., London, England) or New England Nuclear Corp. (Boston, Mass.). Methylmercuric hydroxide was purchased from Alfa (Danvers, Mass.). The chemicals for polyacrylamide gels were purchased from Bio-Rad Laboratories (Richmond, Calif.). Most of the other chemicals and proteinase K were obtained from E. Merck AG (Darmstadt, Federal Republic of Germany). The reverse transcriptase of avian myeloblastosis virus was purchased from Stehelin (Basel, Switzerland).

We used the following working solutions: Tris chloride (1 M, pH 8.8, 20°C), NaCl (5 M), KCl (1 M), EDTA (0.5 M), $MgCl_2$ (0.2 M), sodium dodecyl sulfate (10% [wt/vol]), methylmercuric hydroxide (50 mM), β-mercaptoethanol (350 mM), phenol (containing 0.1% 8-hydroxyquinoline; ex-

tracted and equilibrated with an equal volume of 100 mM Tris chloride [pH 8.8] and 0.2% β-mercaptoethanol), ether (saturated with water), stop solution (formamide deionized by ion-exchange resin, 10 mM EDTA, 0.02% xylene cyanole, 0.02% bromophenol blue), primer (dGTCCCAGGGTTTG GAAA, 1 mg/ml), proteinase K (20 mg/ml), avian myeloblastosis virus reverse transcriptase (12 U/μl), rabies virions (1 mg/ml in TBS), dGTP (1 mM), dATP (1 mM), dTTP (1 mM), dCTP (1 mM), ddGTP (50 μM), ddATP (50 μM), ddTTP (50 μM), ddCTP (50 μM), [α-^{32}P]dATP (10 mCi/ml, 300 or 3,000 Ci/mmol), and polyacrylamide (8% acrylamide, 0.27% bisacrylamide, 7 M urea, 90 mM Tris borate (pH 8.3), 2 mM EDTA, 0.1% Temed, 0.08% ammonium persulfate).

In a test tube, 200 μl of virions, 30 μl of Tris chloride, 6 μl of EDTA, 60 μl of sodium dodecyl sulfate, and 3 μl of proteinase K were mixed. The mixture was incubated at 37°C for 30 min, mixed with phenol by vortexing for 5 min, clarified with ether, adjusted to 0.1 M NaCl, added to 2.5 volumes of ethanol, and stored at −70°C for 2 h. The nucleic acids were recovered by centrifugation at 12,000 × *g* for 10 min. The pellet was dissolved in 9 μl of water, 0.5 μl of primer, and 1 μl of CH_3HgOH. This solution was incubated at room temperature for 10 min, supplemented with 2 μl of β-mercaptoethanol, incubated at room temperature for 5 min, and added to the following mixture: 4 μl of Tris chloride, 6 μl of KCl, 2 μl of $MgCl_2$, 2 μl of dGTP, 2 μl of dATP, 2 μl of dTTP, 2 μl of dCTP, 4 μl of [α-^{32}P]dATP, and 2 μl of reverse transcriptase. Then, it was dispensed in 9-μl volumes into four tubes containing 2 μl of a different chain terminator (ddGTP, ddATP, ddTTP, or ddCTP). The four polymerization mixtures were incubated at 37°C for 15 min, and then 15 μl of stop solution was added to each tube. Portions (3 μl) were heated at 100°C for 10 min and applied to polyacrylamide slab gels (40 by 20 by 0.04 cm). Gels were run at 23 mA for various lengths of time (3 to 17 h), covered with Saran Wrap, and autoradiographed at −70°C for 10 h with a Kodak X-Omat AR film and a Dupont Cronex Lightning-Plus intensifying screen.

RESULTS

Production of a class III-specific monoclonal antibody. To increase the collection of class III-specific monoclonal antibodies, we produced seven anti-CVS hybridomas by fusion of mouse myeloma cells with spleen cells from CVS virus-immunized mice. The specificity of the seven corresponding monoclonal antibodies was determined by ELISA. Six antibodies recognized the viral glycoprotein, whereas the remaining antibody bound to the viral nucleocapsid. Next, we studied the effect of the six anti-glycoprotein antibodies on the plaquing efficiency of CVS. Four of them neutralized CVS. We then tested the neutralizing effect of these four antibodies on one class I mutant of CVS, three class II mutants, and two class III mutants (Table 1). Monoclonal antibodies 25A1, 25A2, and 25B4 failed to neutralize class II mutant A17 and thus were classified as class II-specific antibodies. The fourth monoclonal antibody, 21B4, failed to neutralize class III mutant C14. Therefore, 21B4 appeared as a potentially useful new class III-specific monoclonal antibody.

Selection of class III mutants. Plaque-purified CVS clones or a 5-FU-mutagenized CVS stock were incubated with one class III-specific monoclonal antibody (194-2, 248-8, or 507-1 from the Wistar Institute collection, or 21B4) and then used to infect cultures of CER hamster cells. Viruses from each plaque that appeared at 33°C on the monolayer of CER cells were grown in BHK-21 hamster cells to give virus stocks which were then tested for their ability to resist to the antibody used for selection. We thus isolated 200 resistant mutants displaying different degrees of resistance to the selecting antibody. The average frequency of the mutants varied from 10^{-4} (CVS clones) to 10^{-3} (5-FU CVS stock).

TABLE 1. Neutralizing effect of anti-glycoprotein monoclonal antibodies[a]

New antibodies	Class I	Class II			Class III	
	D65 (509-6)	A17 (220-8, 231-22)	G48 (220-8, 719-3)	K34 (220-8, 101-1)	C14 (248-8, 507-1)	Av01 (248-8, 194-2)
25A1	++	−	++	++	++	++
25A2	++	−	++	−	++	++
25B4	++	−	++	++	++	++
21B4	++	++	++	++	−	++

[a] Representative mutants from each cross-neutralization class are shown. The neutralizing antibodies that fail to neutralize the mutant are shown in parentheses. ++, Neutralization; −, no neutralization.

Growth of class III mutants in cell culture. We examined the replication cycle of our class III mutants in CER and BHK-21 cells by measuring (i) the titer of the mutant stocks, (ii) the size of the plaques produced on a monolayer of CER cells, (iii) the effect of temperature on plating efficiency, and in some cases (iv) the intensity and the velocity of the virion band obtained by centrifugation in a sucrose gradient. By these criteria, the majority of the mutants carried out cycles of infection in CER and BHK-21 cells which were not detectably different from those of the parent virus.

Other class III mutants gave tiny plaques on CER cell monolayers and poor yields in BHK-21 cell cultures. These mutants defective for growth in vitro were not considered any further, since mutants growing efficiently in cell cultures are preferable for specific study of defects in neuropathogenicity.

A single class III mutant, C7, was clearly temperature sensitive. It was unable to give plaques at 38.5°C, although the titer of the stock was normal at 33°C. This mutant had been selected at 33°C from the 5-FU-mutagenized CVS stock with monoclonal antibody 507-1. The synthesis of the viral proteins and the genomic replication were apparently normal at the nonpermissive temperature. Therefore, the *ts* lesion was suspected to be in genomic sequences not required for transcription, translation, and replication, for example in sequences coding for the glycoprotein or the matrix protein M2.

Resistance patterns of class III mutants. We opted for the study of 58 mutants fully resistant to their selecting antibody and growing efficiently in cell cultures. These mutants (Fig. 1) were tested against the four class III-specific monoclonal antibodies: 194-2, 248-8, 507-1, and 21B4. We identified five groups of mutants: group 1 mutants resistant only to 194-2, group 2 mutants resistant to 194-2 and 248-8, group 3 mutants resistant to 507-1 and slightly resistant (10%) to 248-8 and 21B4, group 4 mutants resistant to 248-8, 507-1, and 21B4, and group 5 mutants resistant to all four antibodies.

We tried to find mutants resistant only to 248-8, 507-1, or 21B4 by partially determining the resistance pattern of numerous class III mutants not listed in Fig. 1. We found a mutant resistant to 21B4 and sensitive to 248-8 and 507-1, but in this report, we will not further detail the properties of the mutant because it grew very poorly in vitro. We did not find mutants resistant only to 248-8 or 507-1.

Parent virus	Selecting monoclonal antibody	Number of mutants in group				
		Group 1	Group 2	Group 3	Group 4	Group 5
CVS: 5-FU stock	194-2	7	7			1
"	248-8		2		9	
"	507-1			2	13	
CVS: clone b	248-8		1			1
" c	248-8		2		1	1
" d	248-8		2			
" e	248-8		2			
" f	248-8		2			
" g	248-8		4			
" h	21B4				1	

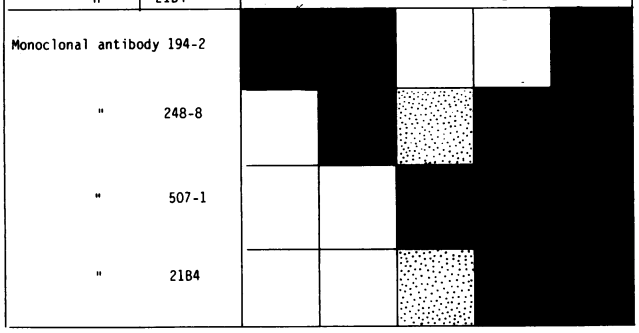

FIG. 1. Selection and classification of class III mutants. Fully resistant mutants were selected with one class III-specific antibody and then were characterized as being sensitive (white squares) or slightly resistant (stippled squares) or highly resistant (black squares) to the remaining three class III-specific antibodies. On this basis, mutants were distributed into five groups.

One of the two group 3 mutants is the temperature-sensitive mutant C7. We explored the possibility that the temperature-sensitive phenotype is caused by the mutation responsible for the pattern of resistance: we selected 12 independent C7 revertants which were no longer temperature sensitive (C7-R1, C7-R2, and so on) and tested them against the four class III-specific antibodies. All of them, unfortunately, retained the resistance pattern of C7.

Pathogenicity of class III mutants. The neuropathogenicity of the 58 mutants classified in Fig. 1 was evaluated by injecting 1,000 PFU of mutant virus into brains of adult Swiss mice. The inoculation of the mutants from group 1, 3, 4, or 5 caused paralysis and death within 7 days, like the inoculation of CVS. Therefore, these mutants were regarded as virulent. On the contrary, two mutants from group 2 were markedly less pathogenic, only rarely causing paralysis and death (attenuated mutants At01 and At02). The remaining 19 mutants of group 2 did not cause any noticeable disease (avirulent mutants Av01, Av02, and so on).

We wondered what effect on pathogenicity a spontaneous additional mutation modifying the pattern of resistance of Av01 could have. We thus selected from an Av01 stock mutants resistant to monoclonal antibody 507-1. We isolated several mutants and studied one of them, Av01-C2, in particular. This mutant was fully resistant to 507-1, 194-2, and 248-8 and highly sensitive to 21B4. It was nonpathogenic for adult mice.

We also tested the pathogenicity of the temperature-sensitive mutant C7. Like most of the temperature-sensitive rabies mutants tested until now (3), C7 was pathogenic, probably because the temperature of the mouse is relatively permissive. The temperature-resistant revertant C7-R7 was also pathogenic.

Because we wondered what the effect would be of a pathogenic revertant spontaneously appearing in a nonpathogenic mutant stock, we examined, by intracerebral injection, the effect of various doses of CVS (ranging from 0.1 to 1,000 PFU) mixed with 1,000 PFU of Av01. These experiments showed that the specific pathogenicity of CVS (50% lethal dose, 1 PFU) was not affected by the addition of a high dose of a nonpathogenic mutant to the inoculum. Therefore, a pathogenic true or pseudo-revertant, even present at a very low frequency in the nonpathogenic mutant inoculum, could have a pathogenic dominant effect. Thus, the possible presence of pathogenic revertants should be kept in mind while interpreting our preliminary tests of pathogenicity.

Amino acid substitutions of class III mutants. Yelverton et al. (28) have obtained the amino acid sequence of the CVS glycoprotein by deducing it from a copy DNA sequence encoding the protein (Fig. 2). Besides, Dietzschold et al. identified the mutational alteration of a nonpathogenic CVS mutant selected with monoclonal antibody 194-2 by establishing the sequence of a lysine-containing tryptic peptide specific of the mutant glycoprotein (10). Their sequence analysis showed that glutamine replaces arginine at position 333 of the mature glycoprotein.

By ion-exchange cochromatography, we compared the [^{3}H]arginine-containing tryptic peptides of the Av010 glycoprotein with the [^{14}C]arginine-containing tryptic peptides of the CVS glycoprotein. We found that the chromato-

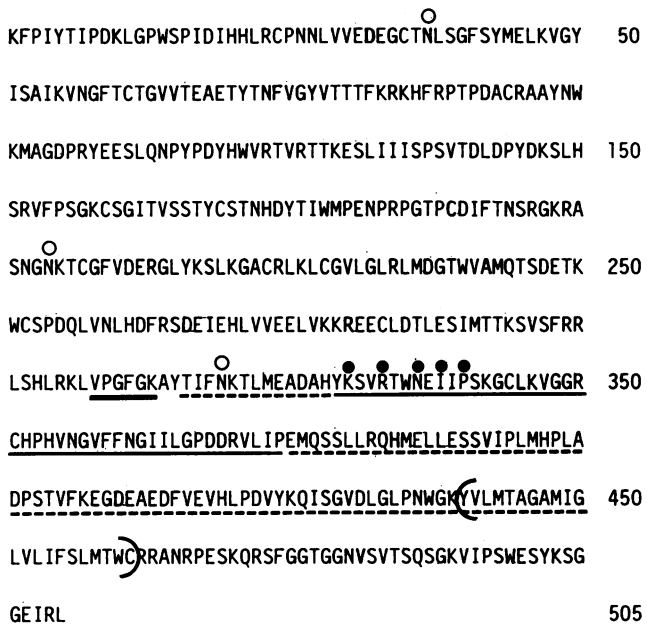

FIG. 2. Partial comparison between the CVS glycoprotein and mutant glycoproteins. The complete sequence of the mature CVS glycoprotein is taken from Yelverton et al. Amino acid residues are symbolized as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. The open circles designate three putative glycosylation sites (NXT or NXS). The semicircles enclose the putative membrane-anchoring region. The amino acid sequence V P G F G K, underlined by a bar, corresponds to the synthetic oligonucleotide we used to prime the sequencing of the glycoprotein gene of CVS and various mutants. The solid and dashed lines indicate the region sequenced for all viruses and regions sequenced for some viruses, respectively (Table 2). The closed circles mark amino acid residues replaced in mutant glycoproteins.

TABLE 2. Characterization of class III mutants

Group	Mutant	Parent virus[a]	Pathogenicity for adult mice[b]	Temp dependence	Sequenced region (amino acid no.)	Mutation		
						Amino acid no.	Amino acid change	Codon change
1	F69	5-FU	P		323–391	330	Lys to Thr	AAG to ACG
2	Av01	5-FU	NP		321–450	333	Arg to Gln	CGG to CAG
	Av04	5-FU	NP		327–374	333	Arg to Gln	CGG to CAG
	Av010	b	NP		320–421	333	Arg to Gly	CGG to GGG
	Av011	c	NP		323–405	333	Arg to Gln	CGG to CAG
	Av012	d	NP		315–374	333	Arg to Gln	CGG to CAG
	Av013	c	NP		323–408	333	Arg to Gln	CGG to CAG
	At01	5-FU	At		321–450	333	Arg to Gln	CGG to CAG
	At02	5-FU	At		318–438	333	Arg to Gln	CGG to CAG
3	C7	5-FU	P	Ts	317–399	336	Asn to Thr	AAT to ACT
	C7-R7	C7	P		317–400	336	Asn to Thr	AAT to ACT
	C10	5-FU	P		316–392	336	Asn to Ile	AAT to ATT
4	B1506-11	c	P		323–410	336	Asn to Asp	AAT to GAT
	L46	h	P		329–392	336	Asn to Asp	AAT to GAT
5	F67	5-FU	P		323–409	338	Ile to Thr	ATC to ACC
	B702	b	P		328–407	338	Ile to Thr	ATC to ACC
	B1510	c	P		320–392	338	Ile to Thr	ATC to ACC
Double mutant	Av01-C2	Av01	NP		326–395	333 340	Arg to Gln Pro to His	CGG to CAG CCC to CAC

[a] The parent virus was a clone of CVS (b, c, d, or h), a 5-FU-treated CVS stock, C7, or Av01.
[b] P, Pathogenic; NP, nonpathogenic; At, attenuated pathogenicity.

graphic profiles were identical, except that a peak was missing in the Av010 profile (data not shown). This suggested to us that the mutation in Av010 could be similar to the mutation in the virus studied by Dietzschold et al. Therefore, we chose to analyze the sequence of our mutant glycoproteins primarily in the region of arginine 333. To this effect, we determined the corresponding genomic RNA sequence.

A synthetic oligonucleotide (a gift of D. H. L. Bishop) having the sequence 5′-GTCCCAGGGTTTGGAAA-3′ was used to prime, on the genomic RNA, four reactions of reverse transcription which differed by the added chain-terminating nucleotide (ddGTP, ddATP, ddTTP, or ddCTP; dideoxy-sequencing according to Sanger et al. [23]). Numerous publications have reported valuable modifications to the original procedure. Above, we have given our own modification which consists of using the labeled nucleotide ([α-^{32}P]dATP + cold dATP) at as high a concentration as each cold nucleotide (dGTP, dTTP, and dCTP at 50 μM). In these conditions, optimal for the RNA-dependent DNA polymerase (reverse transcriptase), there were much fewer artifact bands in the sequence ladders than with procedures relying on the use of a high nucleotide concentration only for a final chase.

First, we established the genomic sequence of our CVS virus in the region encoding amino acids 315 to 456 of the mature glycoprotein (Fig. 2; data not shown). Then, we established the sequences of 18 mutants and compared them with the CVS sequence (Table 2). We found only one base substitution per mutant (except for the double mutant Av01-C2), and all base substitutions resulted in amino acid substitutions. The base substitutions occurred at seven positions in a sequence of eleven codons: AAG-TCA-GTC-CGG-ACC-TGG-AAT-GAG-ATC-ATC-CCC (Fig. 3 and Table 2). There were no ambiguities in sequencing gels and no traces of the parental base at the position of each substitution, except in the F67 ladders where the parental base was also present. Three similarly ambiguous cases were noted by Dietzschold et al. (10), when they sequenced the glycoprotein gene of three nonpathogenic mutants of the ERA strain. Dietzchold et al. systematically detected both arginine and isoleucine at position 333.

Interestingly and strikingly, all attenuated and nonpathogenic mutants lost arginine 333. This arginine was replaced by glutamine in the two attenuated mutants and by either glutamine or glycine in the six nonpathogenic mutants. We could not detect any difference between the three mutants Av01, At01, and At02 in the sequences we compared. Moreover, comparisons of the glycoprotein tryptic peptides, by reverse-phase high-performance liquid chromatography, did not reveal any difference in other regions of the protein (A. Diallo and F. Lafay, personal communication).

The nonpathogenic mutant Av01-C2, isolated from an Av01 stock incubated with monoclonal antibody 507-1, had retained the Av01 amino acid substitution at position 333 and, in addition, showed another amino acid substitution, proline to histidine at position 340.

None of the sequenced pathogenic mutants had a substitution at the crucial 333 position. They had an amino acid substitution at position 330, 336, or 338. Lysine 330 was changed into threonine, asparagine 336 was changed into aspartic acid, threonine, or isoleucine, and isoleucine 338 was changed into threonine.

The pathogenic temperature-sensitive mutants C7 and its no longer temperature-sensitive revertant C7-R7 had no other amino acid substitution besides the one at position 336 (asparagine replaced by threonine). Moreover, preliminary comparisons of the glycoprotein tryptic peptides of CVS, C7, and C7-R7, by reverse-phase high-performance liquid chromatography did not reveal any other mutational alteration (A. Diallo and F. Lafay, personal communication).

We summarize the effects of the single-amino acid substitutions on the neutralizing power of the four antibodies (Fig. 4). In at least one case (the substitutions at position 336), the nature of the substitution, and not only its position, plays a substantial role. At position 340, we did not represent the substitution Pro to His of Av01-C2 since Av01-C2 is a double mutant. Nevertheless, we must point out that the resistance pattern of Av01-C2 emphasizes the functional differences between antibody 21B4 and the other three antibodies:

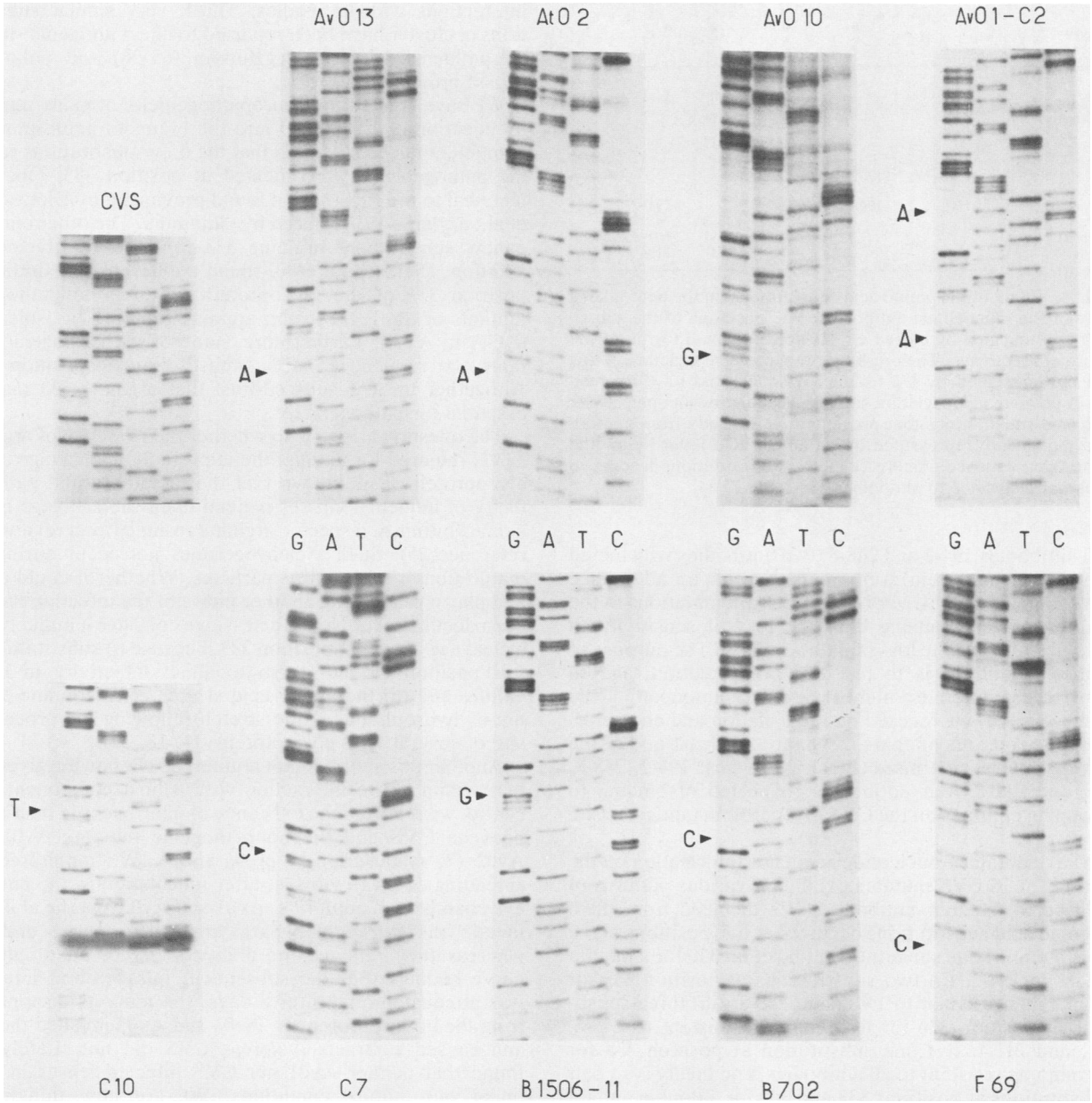

FIG. 3. Nucleotide sequence analysis of the glycoprotein gene from CVS and representative class III mutants. The lanes tracks of each gel correspond to ddGTP-, ddATP-, ddTTP-, and ddCTP-terminated reactions of reverse transcription. The derived plus-strand CVS sequence is (from bottom to top): 5'-TCACTACAAGTCAGTCCGGACCTGGAATGAGATCATCCCCTCAAAAGGGTGTTTGAAA-3'. Base changes are indicated by arrowheads. A wide band across all the tracks of the gel was obtained when the [α-^{32}P]dATP was purchased from New England Nuclear Corp. (for example, in the C10 gel).

mutant Av01-C2 was resistant to 194-2, 248-8, and 507-1, but not to 21B4.

To have an idea on the native secondary structure of the CVS glycoprotein, we used the algorithm of Garnier et al. (17) which identifies α-helices, β-strands, and turns. Putative elements of structure obtained by this method are represented in Fig. 4: a possible α-helix from leucine 322 to tyrosine 329, unresolved structural motifs from lysine 330 to isoleucine 339, and finally a typical turn from proline 340 to glycine 343. None of the substitutions seems to modify this putative secondary structure.

We also performed tests to measure the ability of the four monoclonal antibodies to bind to the mutant virions (by ELISA), because loss of neutralizing activity does not necessarily mean lack of binding activity. Preliminary results indicated that the correlation between neutralization and binding to the virions was not complete. For example, monoclonal antibody 21B4 did not bind to L46 (mutant resistant to 21B4) but did bind to B702 (mutant resistant to 21B4). We think it would be interesting to examine thoroughly the binding properties of the antibodies.

DISCUSSION

Recently, we reported that most mutants of the CVS strain of rabies virus that were resistant to the neutralizing mono-

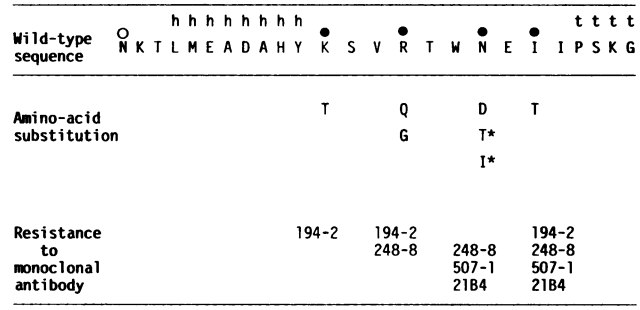

FIG. 4. Effect of the amino acid substitutions on the neutralizing activity of the monoclonal antibodies. The positions of the substitutions are indicated by closed circles above a small region of the glycoprotein sequence. The presumptive secondary structure of this region is represented by h's (α-helix residues) and t's (turn residues). A putative glycosylation site is designated by an open circle. Amino acid substitutions that prevented an antibody from neutralizing a mutant are represented by an amino acid letter below the glycoprotein sequence. Asterisks indicate substitutions leading to only partial resistance to antibodies 248-8 and 21B4.

clonal antibodies 194-2 and 248-8 (two antibodies which bind to the CVS glycoprotein) were nonpathogenic for adult mice (9). We concluded that, very likely, certain mutations in the glycoprotein were causing both the loss of sensitivity to 194-2 and 248-8 and the loss of pathogenicity. The purpose of the present study was to test this interpretation, and to obtain and characterize a number of different nonpathogenic mutants. Above, we focused on the isolation and characterization of numerous mutants resistant to at least one of the following neutralizing monoclonal antibodies: 194-2, 248-8, 507-1, and 21B4, four antibodies suspected of binding to overlapping epitopes on the CVS glycoprotein (antigenic site III).

We have obtained nucleotide sequence information on the mutations of 18 CVS mutants exhibiting various patterns of resistance to the four antibodies. We deduced from them that amino acid substitutions occurred at five positions in the glycoprotein: (i) one substitution at position 330 for a mutant resistant to 194-2, (ii) two substitutions at position 333 for eight mutants resistant to 194-2 and 248-8, (iii) three substitutions at position 336 for five mutants resistant to 248-8, 507-1, and 21B4, (iv) one substitution at position 338 for three mutants resistant to all antibodies, and finally (v) a pair of substitutions at positions 333 and 340 for a double mutant resistant to 194-2, 248-8, and 507-1. Although we sequenced only in part the glycoprotein gene (Fig. 2), a comparison of the tryptic peptides of representative mutant glycoproteins by reverse-phase high-performance liquid chromatography suggested the absence of mutational alterations in the other regions of the protein (A. Diallo and F. Lafay, personal communication). We therefore postulate that most of the mutations causing the loss of sensitivity to 194-2, 248-8, 507-1, and 21B4 are single amino acid substitutions located within a small region of the glycoprotein sequence. One hypothesis is that this small region comprises some of the amino acids that interact directly with the combining sites of the monoclonal antibodies. At least three arguments are in favor of this interpretation. First, we carried out experiments which showed that some of the substitutions prevented the antibodies from binding to the resistant virions; however, the effect of the substitutions on binding may be indirect. Second, lysine 330, arginine 333, and asparagine 336 are hydrophilic residues likely to be accessible for interactions with antibodies. Third, very similar substitutions in cluster have been reported to affect antigenic sites of the influenza virus hemagglutinin (6, 26) and poliovirus capsid protein 1 (12).

We have studied the neuropathogenicity of many mutants by injection of 1,000 PFU into the brains of adult immunocompetent mice. We found that the only substitutions reducing pathogenicity were located at position 333. One was identical to the substitution found previously by Dietzschold et al.: arginine 333 replaced by glutamine. The other one was a new substitution: arginine 333 replaced by glycine. In addition, Dietzschold et al. found a different substitution at position 333 of the glycoprotein of three nonpathogenic mutants of the ERA strain: arginine replaced by isoleucine (AGA to ATA). Furthermore, none of the pathogenic mutants we sequenced had a substitution at position 333. Altogether these results suggest that arginine 333 may be essential for pathogenicity.

The question arises as to whether the presence of arginine 333 is required for an hypothetic proteolytic cleavage of the glycoprotein. It is known that the infectivity and pathogenicity of influenza viruses depend upon the cleavage of the hemagglutinin at a specific arginine residue (for a review, see reference 25). Such a cleavage does not occur during the maturation of rabies virus particles. Whether it could occur and play a role during another phase of the infection has not been documented. Nevertheless, we consider it unlikely that a cleavage occurs at position 333, because (i) substitutions at this position did not seem to affect infectivity in tissue culture and (ii) the amino acid stretch after arginine 333 is not as hydrophobic as the stretch following the processing site of several viral glycoproteins (1, 18, 20).

Another possibility is that arginine 333 lies on the glycoprotein within a site interacting with a host component. Recently, we reported the existence of differences in host range between CVS and the nonpathogenic mutants Av01 and Av02 (7; manuscript in preparation). We monitored the spreading of these viruses, after inoculation in the anterior eye chambers of adult rats. Av01 and Av02 lost the ability to invade the intraocular parasympathetic nerve endings, whereas they retained the ability to infect the trigeminal nerve endings (but the subsequent intracerebral infection was attenuated, and after 8 days, the mutants disappeared from the brain). Moreover, Av01 and Av02 invaded the lens and caused cataracts, whereas CVS did not. Lately, we found that neither Av01 nor CVS infected lenses in vitro under our culture conditions. We are now thinking of selecting, in vivo, mutants which are able to efficiently invade lenses.

We also reported that the production of interferon and the natural killer cell activity are greatly stimulated in Av01- and Av02-inoculated animals (15).

What are the genomic differences between our attenuated mutants At01 and At02, and a nonpathogenic mutant like Av01? We could not find any difference between their glycoproteins, neither by partial nucleotide sequencing nor by tryptic peptide mapping. Since we showed, by reconstitution experiments, that CVS, even present at a low frequency in a nonpathogenic mutant inoculum, had a pathogenic dominant effect, we speculate that the attenuated phenotype of At01 and At02 is caused by a pathogenic or attenuated subpopulation. We are testing this hypothesis.

Incidentally, revertants may also appear postinjection if a sufficient replication of the mutant occurs in vivo. In the future, it may be informative to study the pathogenicity and the glycoprotein sequence of viruses isolated from the brains

of mice killed by injection of a mutant. Besides, this process may be a source of interesting pseudorevertants (second-site revertants).

Studies have indicated that certain mutations in the vesicular stomatitis virus glycoprotein alter its conformation and prevent the protein from migrating to the cell surface at elevated temperatures (a step which is essential for the assembly of mature virions) (29). We wondered whether mutations preventing the virus from being neutralized by a monoclonal antibody could also have this deleterious temperature-dependent effect. We tested the temperature dependence (at 33°C and 38.5°C) of numerous CVS mutants resistant to neutralizing anti-glycoprotein monoclonal antibodies (mutants from classes I, II, and III). We found only one mutant to be temperature sensitive for growth in cell culture, a class III mutant, C7. The 12 independent temperature-resistant revertants we selected retained the pattern of resistance of C7 to the four class III-specific antibodies. The revertant we sequenced, C7-R7, retained the amino acid substitution of C7 at position 336 (asparaginine to threonine). Comparisons between the tryptic peptide maps of CVS, C7, and C7-R7 did not suggest the presence of any other mutation (A. Diallo, personal communication). Furthermore, the frequency of *ts* mutants in the 5-FU-mutagenized stock from which C7 originates is high (3%) (22). Altogether, these results suggest that neither the temperature-sensitive phenotype nor the revertant phenotype was caused by a mutation in the glycoprotein of C7.

It seems reasonable to presume that chances are low for a variable antigenic site at the surface of a virion to be extensively involved in pathogenicity. Domains of a surface viral protein that are essential for the virus are expected to be less variable and less antigenic. For example, the receptor-binding site of the influenza hemagglutinin seems to be located in a poorly antigenic pocket (26, 27). It is interesting that in the case of our nonpathogenic rabies mutants and in the case of certain attenuated reovirus mutants (14, 24), a highly antigenic site of a surface viral protein seems to have an important role in pathogenicity. However, many of our site III mutants are pathogenic, which suggests that only a part of site III is involved in pathogenicity. At present, only arginine 333 is implicated. In the future, we may find new nonpathogenic mutants by selecting mutants exhibiting partial, instead of full, resistance to the neutralizing anti-glycoprotein monoclonal antibodies.

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