INTRODUCTION

Rabies virus contains a negative stranded RNA genome and belongs to the family Rhabdoviridae. The genome codes for five structural proteins, of which one, the glycoprotein, is the only external protein of the virion. It is a protein of 505 amino acids with a signal peptide cleared in the endoplasmic reticulum, an ectodomain of 439 amino acids, a transmembrane region of 22 amino acids and a cytoplasmic portion of 44 amino acids. The glycoprotein of the CVS strain, the laboratory strain used in all our experiments, has two sites of glycosylation at positions 204 and 319. Because of its position in the virion, the glycoprotein plays an important role during the cycle of infection. The first is the recognition of target cells which determines the tropism of the virus. While many cell lines are permissive for rabies virus, in animals the virus infects neurones almost exclusively. This means that it recognizes a specific receptor(s) at the surface of these cells and that this recognition is mediated by the glycoprotein. Another important role of the glycoprotein is the stimulation of the immune system. In addition to its role in cell-mediated immunity, the glycoprotein stimulates the synthesis of circulating antibodies and most of these antibodies neutralize the infectivity of the virus. The development of hybridoma technology made possible the use of the neutralizing power of anti-glycoprotein monoclonal antibodies (Mab) to isolate antigenic mutants which resist neutralization. The study of these mutants, associated with an analysis of their reactivity patterns with Mabs, provides an indication as to the location of antigenic sites at the surface of the glycoprotein. Furthermore, this methodology has been widely used for the study of the antigenicity of viral proteins.

The first anti-rabies Mabs were produced by Wiktor at the Wistar Institute (Wiktor & Koprowski 1978) and the first analyses of antigenic mutants isolated from the CVS strain resisting neutralization by antiglycoprotein Mabs was conducted by Lafon using antigenic mutants, most of which were isolated in our laboratory (Lafon, Wiktor & MacFarlan 1983). After testing the neutralizing activity of each Mab with each antigenic mutant, it was possible to compose clusters of Mabs which were classified as being active at three distinct antigenic sites (Lafon et al. 1983). Because this study was performed with a small number of Mabs (around 20), it was not possible to quantify the relative importance of these sites, although it was clear that site I is not a major antigenic region.

MOLECULAR CHARACTERIZATION OF ANTIGENIC SITES

Site II

Antigenic mutants resisting neutralization by site II-specific Mabs were sequenced by the dideoxy nucleotide method. The entire nucleotide sequence of the ectodomain was determined for only a limited number of mutants; for the others, only part of the sequence was investigated. Nevertheless, in each case, only one nucleotide substitution was detected in the mutant sequence, when compared to parent CVS, and this substitution corresponded to an amino acid change in the glycoprotein in each case. The substitutions were located in two regions:

- Between amino acids 34 and 42.
- Between amino acids 198 and 203.

Two individual mutations were detected outside these two regions (Prehaud, Coulon, Lafay, Thiers & Flamand 1988) (Fig. 1a). The primary sequences of these two regions are separated in the primary structure but they are close together in the three
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dimensional structure. The assumption that a disulfide bridge could keep these two regions close together had previously been suggested by Dietzschold, Wiktor, MacFarlan & Varrichio (1982) who had studied glycoprotein fragments generated after cleavage with cyanogen bromide. This disulfide bridge would link the cysteine 35 (which is not affected by antigenic mutations) and either cysteins 189 or 207 (Dietzschold et al. 1982).

Experiments which examined the binding of Mabs with the antigenic mutants by using enzyme immunoassay showed that in most cases there is a correlation between the neutralizing activity of the Mab and its binding to the antigenic mutant (Prehaud et al. 1986). Nevertheless, there are several examples of binding of the antibody in the complete absence of neutralization (vide infra).

Because we are interested in understanding the molecular mechanisms of the pathogenicity of rabies virus, site II antigenic mutants were systematically inoculated into adult mice in order to determine their pathogenicity. In most cases there was no reduction in virulence with the exception of three cases where the LD50 decreased by a 30-fold factor, i.e. slight attenuation. One mutant had an amino acid substitution at position 34 and the two others at position 198 (Prehaud et al. 1988) (Fig. 1a).

The region just before amino acid 200 has been shown to be homologous to the toxic loop of several snake-venom neurotoxins which are antagonists of acetylcholine (Lentz, Wilson, Hawrot & Speicher 1984). In view of this strong homology, it has been suggested that the acetylcholine receptor could be the receptor for rabies virus at the surface of the neurones (Lentz 1985).

Our results show that a substitution of the lysine at position 198, which is one of the six or seven conserved amino acid residues between the neurotoxins and rabies glycoprotein, affects virulence, but this effect is not dramatic since we have only a 30-fold reduction of the LD50. Thus, the acetylcholine receptor is probably not the only receptor for rabies virus.

### Site III

Using the same principle as for the study of site II, antigenic mutants have been isolated and clustered according to their resistance to site III-specific Mabs. Contrary to the situation with some site II mutants which may bind antibody despite complete resistance to neutralization by the same Mab, none of the antigenic site III mutants binds site III-specific Mabs if those Mabs do not neutralize infectivity.

The results of sequencing experiments indicated that the amino acid substitutions were all located in the same region, between amino acids 330 and 338 (Seif, Coulon, Rollin & Flamand 1985) (Fig. 1b). In spite of an apparently linear structure, site III is probably a conformational site because site III-specific Mabs are unable to bind a peptide containing the 330–338 region. In addition, these antibodies do not produce western blots even when the glycoprotein is denatured in the absence of β-mercaptoethanol, while some site II-specific antibodies are able to bind the protein in these mild denaturing conditions.

Mutations in positions 331, 336 and 338 have no effect on the virulence of the virus. One of the two

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**FIG. 1** Localization of antigenic mutations in the sequence of the CVS glycoprotein; (a) on antigenic site II; (b) on antigenic site III and minor site a.
mutations in position 330 results in a 30-fold attenuation (substitution of lysine with asparagine) (Fig. 1b). Mutations at position 333 resulted in complete apathogenicity with one exception. If lysine replaces the arginine, the mutant is slightly attenuated in adult mice following intracerebral inoculation. It should be noted from the SADBern strain, which has the same amino acid sequence as the CVS strain at site III, that a mutant with a lysine in position 333 instead of the arginine is not pathogenic in adult mice by the intramuscular route. The substitution of arginine 333 by an amino acid other than lysine results in an apathogenic mutant (Dietzschold, Wunner, Wiktor, Lopes, Lafon, Smith & Koprowski 1983; Tuffereau et al. 1989). These mutants are avirulent for adult animals (with the exception of skunks) whatever the route and the dose of the inoculum but are pathogenic for suckling mice. To date, eight different mutants at position 333 have been isolated (Fig. 1b). Other substitutions have not been tested because site-directed mutagenesis is not yet possible on negative strand RNA viruses.

Avirulent mutants are important for two reasons:

- They are good tools in basic research for the understanding of the mechanisms of virulence (Lafay, Coulon, Astic, Saucier, Riche, Holley & Flamand 1991).

- More practically, they are good inducers of the immune system and consequently it is possible to construct vaccinal strains with avirulent mutations.

This has been applied to the SADBern strain, a vaccinal strain used during the first campaigns involving oral vaccination of foxes in Western Europe, in which avirulent mutations have been introduced in order to eliminate the residual pathogenicity of SADBern for non-target species. The resulting strain, called SAG-2, is under study and the first results on its efficacy will be presented elsewhere.

From avirulent mutants produced from the CVS strain, it has been possible, in some cases, to isolate pathogenic revertants after one passage in sucking mouse brain followed by one passage in adult mice. Around 40 independent pathogenic revertants have been isolated from four different avirulent mutants. With two exceptions, the virus recovered had arginine at position 333. In the two exceptions lysine was detected at that position (Tuffereau, Leblois, Benejean, Coulon, Lafay & Flamand 1989). Here again, in the SAD context, the result is different. It is much more difficult to isolate pathogenic revertants from avirulent mutants of the SAD strain, probably because these revertants have no selective advantage over the avirulent mutant in sucking mice. This result implies that the recovery of a pathogenic phenotype cannot be the consequence of a second mutation in another region of the glycoprotein which corrects the effect of the mutation at position 333. As a consequence, it is likely that arginine 333 itself is engaged in the interaction with the viral receptor.

**RELATIVE IMPORTANCE OF THE ANTIGENIC SITES**

These results show that the two major antigenic sites concern only small discrete regions at the surface of the CVS glycoprotein. In the absence of detailed knowledge of the three-dimensional structure of the protein, there is no idea of the number of amino acids which interact with the Mabs. Nevertheless, it is important to emphasize that an amino acid associated with an antigenic site can also interact with a receptor.

Over the years this laboratory has produced several hundred different hybridomas affording the opportunity to estimate the relative importance of the antigenic sites. Two hundred and sixty-six neutralizing Mabs have been tested for their capacity to neutralize representative mutants of sites II and III. The results show that 73% of the Mabs are site II-specific and 24% are site III-specific (Benmansour, Leblois, Coulon, Tuffereau, Gaudin, Flamand & Lafay 1991). Thus, 97% of the Mabs recognize two immunodominant sites (Fig. 2). Among the remaining antibodies, six define what we call the minor site a. This site is located very close to site III since antigenic mutants of the minor site have their amino acid substitution in positions 342 and 343 (Benmansour et al. 1991). In spite of their closeness, there is no cross reactivity between antibodies directed against site III and minor site a. These two sites are independent probably because of the proline in position 340 which induces a turn in the peptide chain (Fig. 1b).

The so-called site I is defined by only one Mab, so it should be considered as an epitope. A mutant resistant to neutralization by this antibody was isolated and the antigenic mutation was located in position 231 (Benmansour et al. 1991).

![Fig. 2 Relative importance of antigenic sites in the CVS glycoprotein. The Mabs were classified from their capacity to neutralize representative sites II and III antigenic mutants previously characterized in Prehaud et al. (1988) and Saif et al. (1985)](image-url)
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The antigenic sites are considered to be conformational because the antibodies that define them do not recognize the glycoprotein in western blots. On the other hand, the few Mabs which do produce western blots are usually non-neutralizing. Thus few antibodies share the two properties of neutralizing activity and recognition of a linear epitope. There are three exceptions, 6-15C4 (Bunschoten, Gore, Claassen, Uytdehaag, Dietzschold, Wunner & Osterhaus 1989) and 1D1 and 8C3 selected in our laboratory (Benmansour et al. 1991). Escape mutants were selected with 6-15C4 and 1D1. The mutant RV-6-15C4 has an amino acid substitution at position 264 of the glycoprotein (Dietzschold, Gore, Marchadier, Bunschoten, Otvos, Wunner, Ertl, Osterhaus & Koprowski 1990) as do two mutants resistant to 1D1. Two other mutants have an amino acid substitution in position 392 where the valine is replaced by an alanine or a glycine. These four mutants are still neutralized by 8C3. In order to establish precisely where the Mabs bind on the glycoprotein, we have developed an expression system in yeast of randomly generated peptides of the CVS glycoprotein. The glycoprotein sequences are inserted behind the promoter and the signal peptide of the mating type, which is a secreted hormone of the yeast. This system allows the excretion of the peptides. The yeast clones are cultivated directly on nitrocellulose sheets and the clones secreting peptides are identified by immunodetection with a polyclonal antiserum.

When a given Mab reacts with a peptide, it is clear that the antigenic binding site is included in this peptide. The converse (i.e. no reaction) may be the consequence of the complete or only a partial absence of the binding site. For 1D1, boundaries of its binding site are located between amino acids 255 and 276 (Fig. 3). This result was not unexpected since it was previously shown that Mab 6-15C4 binds a synthetic peptide comprising amino acids 253 to 275 (Dietzschold et al. 1990) and these two antibodies allowed the selection of antigenic mutants at the same position. Amino acids shared by the peptides reacting with Mab 8C3 are between positions 222 and 260 (Fig. 3). Since peptides starting at amino acid 228 or 244 did not react with 8C3, it is likely that the precise binding site is closer to the left portion of the shared amino acid sequence. This hypothesis is in agreement with the fact that all the 1D1 escape mutants are neutralized by 8C3. Experiments are in progress to select 8C3 escape mutants.

Two conclusions can be made:

- Amino acid substitutions outside the antibody binding site can confer neutralization resistance, since 1D1 binds on the region 250–275: two out of four of its neutralizing resistant mutants have a substitution at position 392.
- After immunization, ant glycoprotein Mabs that produce western blots are in a minority (in our collection, 12 out of several hundreds), and among them, again a minority are neutralizing (two out of 12).

**CONCLUSION**

All the data presented here concerns the CVS strain of rabies virus. The comparison of the amino acid sequences of the glycoproteins from various laboratory strains (including CVS, ERA, SAD B19, HEP, PV) and those of the glycoprotein from street rabies

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**Fig. 3** Binding of Mabs 1D1 and 8C3 on CVS glycoprotein peptides. The peptides were synthesized in yeast, excreted and blotted on nitrocellulose filter. The reaction was demonstrated with an anti-mouse IgG peroxidase conjugated. Intensity of the reaction was estimated by comparison with the binding of a mouse anti-CVS antiserum.
virus strains indicates that there is good conservation of antigenic sites II and III among these different strains of virus (Benmansour et al. 1991; Bai, Warner & Fekadu 1993). Nevertheless, a number of neutralizing Mabs differentiate between the strains, indicating that all the amino acids that define sites II and III are not yet identified.

REFERENCES


