Antigenic Sites on the CVS Rabies Virus Glycoprotein: Analysis with Monoclonal Antibodies

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(Accepted 1 December 1982)

SUMMARY

Antigenic variation in the glycoprotein of rabies (CVS-11) virus was studied. Neutralization-resistant variant viruses were isolated in vitro at high frequency ($10^{-4}$ to $10^{-5}$) in the presence of anti-glycoprotein monoclonal antibody. Analysis of these variants identified at least three functionally independent antigenic sites, based on the grouping of variants that were no longer neutralized by one or more of a panel of 24 monoclonal antibodies. Competition radioimmunoassay suggested that one of these three antigenic sites was topologically distinct, with the other two in close proximity. In addition, it was shown that most (but not all) neutralization-resistant variants failed to bind the relevant monoclonal antibody. Viruses with altered antigenicity were shown to accumulate in virus stocks following several passages in vitro in the absence of antibody. In addition, variants were isolated in vivo following treatment of mice with monoclonal antibody.

INTRODUCTION

Until recently all strains of rabies virus were considered to be antigenically similar, on the basis of conventional immunological tests (Wiktor & Clark, 1973). However, antigenic differences among strains of fixed or street rabies viruses were easily detected using monoclonal antibody specific for the rabies virus glycoprotein (Wiktor et al., 1980; Flamand et al., 1980; Wiktor & Koprowski, 1980), which is the only viral antigen with protective activity (Wiktor et al., 1973).

In a previous report, we described antigenic variants of the CVS-11 strain of rabies that were selected after treatment of cloned virus populations with monoclonal antibodies directed against the viral glycoprotein (Wiktor & Koprowski, 1980). By definition, these variants resisted neutralization by the antibody used for their selection. Furthermore, animals that were immunized with vaccines prepared from these variant viruses were not fully protected against challenge with the parental CVS-11 virus (Wiktor & Koprowski, 1980). This suggested that antigenic variation among street viruses might explain occasional failures in post-exposure vaccination of patients with rabies (Wiktor & Koprowski, 1982). Indeed, when mice were vaccinated with standard rabies vaccine and then challenged with street viruses isolated from fatal cases of human rabies, some instances of vaccine failure were observed. Vaccine failure correlated with the degree of antigenic disparity between the vaccine and challenge viruses, as revealed by analysis with monoclonal antibodies (Wiktor & Koprowski, 1980).

In order to understand antigenic variation of rabies virus, and to develop a logical basis for the selection of vaccine strains, which currently are derived from a limited number of isolates (Clark & Wiktor, 1972; Wiktor & Koprowski, 1982), it is necessary to know something of the antigenic structure of the rabies virus glycoprotein. In this report we describe a functional antigenic map of the CVS-11 glycoprotein obtained by a comparative analysis of antigenic variants selected in the presence of monoclonal antibodies. In addition, we show that these variants arise with high frequency and can also be isolated in vivo.
METHODS

Virus strains. A cloned CVS-11 strain of fixed rabies virus was propagated in BHK-21 cell culture monolayers as described by Wiktor (1973).

Monoclonal anti-glycoprotein antibodies. Hybridomas that secrete monoclonal antibodies specific to rabies virus proteins were produced by the fusion of P3X63Ag8 or 653 cells with splenocytes of BALB/c mice immunized with several strains of rabies virus (Wiktor & Koprowski, 1978). In the present study, we used 24 monoclonal antibodies specific for rabies virus glycoprotein (G) and capable of neutralizing CVS-11.

Ascitic fluids were obtained from BALB/c mice primed with 0.3 ml Pristane (Aldrich Chemical Co.) injected intraperitoneally (i.p.), and then inoculated 2 weeks later with $5 \times 10^6$ hybridoma cells. Tumours and ascitic fluid developed in approximately 2 weeks. The ascitic fluid was collected, separated from cells, and purified by affinity chromatography on a Protein A-Sepharose 4B column (Pharmacia) according to the method of Ey et al. (1978). The concentration of protein was calculated from the absorbance of the solutions at 280 nm, assuming an extinction coefficient (1% w/v, 1 cm) of 14.

Selection of variants in vitro. Twelve monoclonal antibodies capable of neutralizing the CVS-11 rabies virus were used for selection of variants as previously described (Wiktor & Koprowski, 1980). Serial tenfold dilutions of virus were prepared and mixed with either undiluted antibody-containing hybridoma tissue culture medium or ascitic fluid diluted 1/100. After 1 h of incubation at 37 °C, 0.1 ml of virus-antibody mixture was added to monolayers of CER cells grown in wells of Costar 6 cluster plates. Following adsorption for 1 h at 37 °C, 2 ml of nutrient agarose alone or nutrient agarose containing the corresponding monoclonal antibody was poured onto the cell layers. Plates were incubated for 4 to 5 days at 35 °C in a 5% CO$_2$ atmosphere and then scored for virus plaques, which were easily visible without staining. At the highest virus dilution still forming visible plaques, well-separated plaques were picked up with Pasteur pipettes and dispersed in 5 ml of medium containing $1 \times 10^6$ freshly trypsinized BHK-21 cells in T-25 Falcon plastic tissue culture flasks. Infected cells were transferred from the T-25 flasks in 0.5 ml amounts into wells of four-chamber Lab-Tek tissue cultures slides in order to monitor infection. The T-25 flasks and Lab-Tek slides were incubated for 3 days and the presence of infected cells in the parallel Lab-Tek cultures confirmed by the fluorescent antibody staining technique (Wiktor & Koprowski, 1978). The virus recovered from T-25 flasks was then tested for neutralization with the antibody used for its selection.

Neutralization-resistant variants (RV) are identified by the number of the monoclonal antibody used for selection, and a letter if more than one reactivity pattern was observed (Fig. 1). In the initial analysis, several clones of each class of variant were tested (Fig. 1). In further experiments, a single representative clone was used.

Selection of variants in vivo. Young adult ICR mice were inoculated i.p. with 0.2 ml of undiluted ascitic fluid containing monoclonal antibody 101-1 (neutralization titre 1/200000). After 24 h, mice were injected intracerebrally (i.c.) with CVS-11 virus at various dilutions. When mice became paralysed, the virus was isolated from their brain tissue and tested for the presence of variants. Variants were also isolated from brain tissue of mice injected i.c. with dilutions of virus following incubation for 1 h in the same ascitic fluid diluted 1/100.

Virus neutralization tests. The neutralizing effect of monoclonal antibodies on the parent CVS-11 virus and on CVS-11 variants was evaluated by determining the virus neutralization index as described by Wiktor & Koprowski (1978). An index of at least 2.5 log$_{10}$ units was considered evidence of neutralization.

Radioimmunoassay (RIA). For preparation of immunoadsorbents, virus-containing tissue culture fluid was concentrated by high-speed centrifugation, purified in a sucrose gradient as described by Dietzschold et al. (1979) and inactivated by u.v. irradiation. The protein concentration of purified virus was determined by the spectrophotocolorimetric method (Bramhall et al., 1969). Antigen (100 to 200 ng) was dried on soft plastic 96-well plates (disposable polyvinyl; Dynatech) and the plates washed with phosphate-buffered saline (PBS) containing 10% gamma horse serum. Hybridoma medium, either undiluted or in serial threefold dilutions, was added to each well (25 µl/well). Plates were incubated for 1 h at 37 °C, then washed three times with PBS. To each well was added 25 µl of $^{125}$I-labelled goat anti-mouse antibodies (Fab goat anti-mouse IgG and IgM) heavy and light chains, 30 000 cts/min, sp. act. 0.5 mCi/mg (Kierkegaard and Perry Co., Gaithersburg, Md. U.S.A.). The plates were again incubated at 37 °C for 1 h and washed three times with PBS. The bottoms of the wells were then cut with an incandescent wire, and radioactivity in the wells measured in a gamma counter.

Competitive RIA. Purified monoclonal antibodies were radiolabeled using the Iodo-Gen technique (Fraker & Speck, 1978). Briefly, 10 µg of antibody was incubated with 500 µCi Na$^{125}$I (Amersham) for 10 to 15 min at room temperature in a tube coated with 1 µg of Iodo-Gen (Pierce Chemical Co.). The free iodine was removed by passing the radiolabelled protein through a 5 ml Sepharose G25 pre-packed column (Pharmacia). Competitive binding RIA was performed as described by Lubeck & Gerhard (1981). The viral immunoadsorbent, 200 ng of purified CVS-11, was dried overnight (25 µl/well) onto polyvinyl microtiter plates at 37 °C. Plates were then washed and incubated for 1 h with PBS containing 1% bovine serum albumin. Twenty-five µl of $^{125}$I-labelled antibody ($5 \times 10^4$ cts/min) plus 25 µl of unlabelled antibody at various concentrations were then added. Plates were incubated for 1 h at 37 °C and washed three times with PBS. The radioactivity bound in each well was determined in a gamma counter. The assay was performed in triplicate, and the percentage competition was...
**Antigenic sites on rabies virus glycoprotein**

<table>
<thead>
<tr>
<th>Variant</th>
<th>Group</th>
<th>Number in group</th>
<th>Monoclonal antibody</th>
<th>Antigenic site</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV 509-6</td>
<td>4</td>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>RV 231-22</td>
<td>14</td>
<td></td>
<td></td>
<td>IIA</td>
</tr>
<tr>
<td>RV 220-8</td>
<td>1</td>
<td></td>
<td></td>
<td>IIB</td>
</tr>
<tr>
<td>RV 101-1</td>
<td>A 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV 162-3</td>
<td>A 5</td>
<td></td>
<td></td>
<td>IIC</td>
</tr>
<tr>
<td></td>
<td>B 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV 719-3</td>
<td>A 2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>B 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV 226-11</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV 240-3</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV 194-2</td>
<td>2</td>
<td></td>
<td></td>
<td>IIIA</td>
</tr>
<tr>
<td>RV 248-8</td>
<td>A 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV 718-4</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV 507-1</td>
<td>A 16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C 1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Parent virus CVS-11

Fig. 1. Functional antigenic map of CVS-11 glycoprotein. Neutralization-resistant variants (RV) were selected using monoclonal antibodies, then tested for susceptibility ( Asterisk) or resistance to neutralization by the monoclonal antibody used in selection of the variant (Circle) and by another antibody of the panel (Diamond).

Calculated by the formula: \( \frac{(L - C)}{L} \times 100 \) where \( L \) is the radioactivity of labelled antibody bound in the absence of competitor and \( C \) is the radioactivity bound in the presence of competitor. Counter background was subtracted from both \( L \) and \( C \).

**RESULTS**

Selection and characterization of variants in vitro

Twelve monoclonal antibodies were used to select 88 neutralization-resistant variants of CVS-11 virus expressing antigenically altered glycoprotein. Thirty-four variants were selected from an untreated CVS-11 stock at The Wistar Institute. The remaining 54 variants were selected at the University of Orsay, France, from the same virus following treatment with the mutagen 5-fluorouracil (Coulon et al., 1982). In both cases the selection frequency was between \( 10^{-4} \) and \( 10^{-5} \) as previously reported (Wiktor & Koprowski, 1980).

These variants could be grouped by their pattern of reactivity in cross-neutralization tests with a panel of monoclonal antibodies. If a variant lost reactivity to a number of antibodies simultaneously, then the epitopes recognized by those antibodies were considered to be functionally linked. Conversely, if selection using one antibody resulted in a number of variants of which none was resistant to neutralization by a second antibody, then the epitopes recognized by the two antibodies were considered independent. An antigen map constructed on these principles is shown in Fig. 1. Three major antigenic sites (I, II and III) are defined by this
Selection of CVS-11 antigenic variants

<table>
<thead>
<tr>
<th>Monoclonal antibody*</th>
<th>Antigenic site recognized†</th>
<th>Frequency of variants (log_{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td>101-1</td>
<td>II</td>
<td>-4.5</td>
</tr>
<tr>
<td>162-3</td>
<td>II</td>
<td>-5.2</td>
</tr>
<tr>
<td>220-8</td>
<td>II</td>
<td>-4.5</td>
</tr>
<tr>
<td>248-8</td>
<td>III</td>
<td>-4.2</td>
</tr>
<tr>
<td>101-1 + 162-3</td>
<td>II + II</td>
<td>-5.5</td>
</tr>
<tr>
<td>101-1 + 220-8</td>
<td>II + II</td>
<td>-5.5</td>
</tr>
<tr>
<td>101-1 + 248-8</td>
<td>II + III</td>
<td>&lt; -7.0‡</td>
</tr>
</tbody>
</table>

* Monoclonal antibodies were used to select variants as described in Methods.
† Antigenic sites as defined by cross-neutralization mapping (see Fig. 1).
‡ No variants isolated.

The group of variants designated RV 509-6 includes four members which are neutralized by all antibodies, except that used for selection (509-6), and defines antigenic site I. Antigenic site II consists of at least three subregions (IIA, IIB, and IIC). Because antibody 220-8 failed to neutralize certain of the variants defining each of these subregions (those selected by antibodies 231-22, IIA; 101-1 and 162-3, IIB; and 719-3, IIC) the existence of a complex antigenic site II is indicated. In some instances, selection using a single antibody resulted in the selection of several groups of variants, each of which differed in their reaction with the panel of monoclonal antibodies. For example, selection using antibody 101-1 resulted in variant groups RV 101-1 A, RV 101-1 B, and RV 101-1 C (Fig. 1). In addition, although the variants within each group showed identical patterns of reactivity, they may not be identical since different mutations could produce the same phenotypic changes in antigenicity. Antibodies 101-1 and 162-3 selected variants showing an identical pattern of neutralization and yet the specificity of these antibodies is not identical, as they can discriminate between strains of street virus (Wiktor & Koprowski, 1982).

Antigenic site III is also complex, consisting of subregion IIIA which is defined by variants selected using antibodies 194-2 and 248-8, and subregion IIIB which is defined by variants selected using antibodies 718-4, 507-1 and 248-8. In this case the reaction pattern of antibody 248-8 indicates that subregions IIIA and IIIB are functionally linked (antigenic site III).

With an additional, recently produced panel of 12 antibodies capable of neutralizing the CVS-11 virus, the above described map was confirmed. Eight antibodies recognized variants of site II and two antibodies recognized variants of site III (data not shown). Two antibodies were unable to distinguish between CVS-11 and any of the variants tested. This could indicate the existence of an additional antigenic site, but we were unsuccessful in selecting additional variants with these antibodies.

Further evidence that antigenic sites II and III are distinct was obtained when mixtures of two antibodies were used for selection of variants. As shown in Table 1, the frequency of variants selected with each of the four monoclonal antibodies used alone was from $10^{-4.2}$ to $10^{-5.2}$. When antibodies delineating the same site were used together, such as 101-1 and 162-3, the frequency of variants was of the same order ($10^{-5.5}$). In contrast, no variants could be isolated when monoclonal antibodies delineating distinct antigenic sites, such as 101-1 and 248-8, were mixed. If the two epitopes change independently, the expected frequency of variants with mutations in both epitopes should equal the product of the individual variant frequencies ($10^{-8.5}$). Efforts to select variants in the presence of two antibodies recognizing epitopes located on two distinct antigenic sites were unsuccessful, even when we used CVS-11 concentrated 50-fold by high-speed centrifugation ($2 \times 10^9$ to $3 \times 10^9$ p.f.u./ml).

Analysis of CVS-11 variants in RIA

The antigen map (Fig. 1) was constructed by neutralization analysis. We therefore tested whether antibodies that neutralized the parental CVS-11 strain might still bind to glycoprotein from a variant resistant to neutralization. Purified, u.v.-inactivated preparations of CVS-11 and variant viruses were used as immunoadsorbents in an RIA. Representative results are shown in Fig. 2 and 3. In general, the results in RIA correlated well with those in the neutralization test.
Antigenic sites on rabies virus glycoprotein

Fig. 2. Binding of monoclonal antibodies to neutralization-resistant variants. Monoclonal antibodies 101-1 (a), 231-22 (b), 220-8 (c) and 240-3 (d) were tested in an indirect RIA for capacity to bind to neutralization-resistant variants. Antigens: CVS-11 (●); RV 101-1A (○); RV 231-22 (▲); RV 220-8 (□); RV 226-11 (△); RV 240-3 (■).

Fig. 3. Binding of monoclonal antibody 719-3 to neutralization-resistant variants. Monoclonal antibody 719-3 was tested in an indirect RIA for capacity to bind to neutralization-resistant variants. Antigens: CVS-11 (●); RV 719-3 A (○); RV 719-3 B (▲); RV 719-3 C (□).
Table 2. Topological distribution of antigenic sites

<table>
<thead>
<tr>
<th>125I-labelled antibody</th>
<th>509-6</th>
<th>231-22</th>
<th>101-1</th>
<th>719-3</th>
<th>613-2</th>
<th>194-2</th>
<th>248-8</th>
<th>507-1</th>
</tr>
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<tbody>
<tr>
<td>509-6</td>
<td>100</td>
<td>96</td>
<td>96</td>
<td>15</td>
<td>10</td>
<td>10</td>
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<td>101-1</td>
<td>96</td>
<td>78</td>
<td>67</td>
<td>75</td>
<td>60</td>
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<tr>
<td>719-3</td>
<td>21</td>
<td>21</td>
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<td>14</td>
<td>14</td>
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<td>14</td>
<td>14</td>
</tr>
<tr>
<td>507-1</td>
<td>2</td>
<td>2</td>
<td>21</td>
<td>67</td>
<td>67</td>
<td>67</td>
<td>67</td>
<td>67</td>
</tr>
</tbody>
</table>

* Results shown are with 200 ng CVS-11 immunoadsorbent and 250 ng unlabelled antibody.
† Antigenic site as shown in Fig. 1.
‡ Percentage competition (see Methods). Homologous combinations are shown in bold type.

For example, monoclonal antibody 101-1 did not bind (Fig. 2a) or neutralize (Fig. 1) an RV 101-1 A variant. The same antibody, however, both bound and neutralized CVS-11, as well as the variants RV 231-22 and RV 220-8. There was also good agreement between RIA and neutralization for antibodies 231-22, 220-8 and 240-3 (Fig. 2b, c and d). In contrast, antibody 719-3 bound the variants RV 719-3 A, B and C, but did not neutralize them (Fig. 3). Thus, binding and neutralization are not necessarily linked phenomena.

**Competition RIA**

Four IgG monoclonal antibodies (509-6, 101-1, 719-3 and 507-1) were purified and radiolabelled as described in Methods. In order to obtain information concerning the topological relationship of antigenic sites I, II and III, we tested whether unlabelled homologous or heterologous antibody could compete for binding sites on the CVS-11 glycoprotein against these labelled antibodies. Table 2 shows that in each case competition was greatest in homologous combinations. Antibody 509-6 (site I) was efficiently blocked only by itself, and unlabelled 509-6 did not compete with antibodies delineating antigenic sites II or III. This indicates the topological independence of site I. Similarly, unlabelled antibodies delineating site III competed only with labelled antibody 507-1 (site IIIB). There was, however, some competition against 507-1 by unlabelled antibodies delineating site II. This might be due to a lower avidity of antibody 507-1 or a particular spatial arrangement of these epitopes, although we have no evidence that this is the case. A close proximity of epitopes within site II was suggested, as unlabelled antibodies recognizing sites IIA, B and C competed well with labelled 101-1 (site IIIB) and 719-3 (site IIC). The competition of antibodies 194-2 (site IIIA) and 248-8 (site IIIB) with labelled 719-3 may well be overestimated, as they are of μ and α isotype respectively. All other antibodies are of γ isotype.

**Changes in antigenic composition of CVS-11 virus glycoprotein during serial propagation in tissue culture**

Because the results indicated the presence of a relatively high frequency of variants in the virus stocks, it was of interest to investigate whether antigenic changes would occur with the same frequency during prolonged passage of virus in BHK-21 cell culture and after several cloning steps. The CVS-11 virus was received in our laboratory in 1967 (passage level 0), and has since been subcultured 19 times, including five clonings accomplished by the endpoint plaque formation technique (Wiktor, 1973). Hybridomas secreting anti-glycoprotein antibodies were generated from the fusion of splenocytes from mice immunized with CVS-11 virus at passage level 18. Twelve antibodies neutralized CVS-11 at passage level 18. The original stock virus (Wiktor et al., 1980) was analysed in the neutralization tests with the panel of monoclonal antibodies used for variant selection. All but two antibodies (220-8 and 240-3) neutralized the original stock. When virus stocks at various passage levels were tested, the epitopes recognized by antibodies 220-8 and 240-3 became detectable (neutralization index, 2-5 and 2-0) at the 7th passage level and after three clonings (Table 3). The neutralization index increased at the 12th
Table 3. Antigenic variation of CVS-11 virus following propagation and cloning in tissue culture

<table>
<thead>
<tr>
<th>Passage level</th>
<th>Number of clonings</th>
<th>Neutralization index (log₁₀) with monoclonal antibody*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>220-8</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>7</td>
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<td>2.5</td>
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<tr>
<td>12</td>
<td>4</td>
<td>4.0</td>
</tr>
<tr>
<td>19</td>
<td>5</td>
<td>4.0</td>
</tr>
</tbody>
</table>

* A neutralization index of more than 2.5 was considered significant.
† NT, Not tested.

Table 4. Isolation of variants in vivo

<table>
<thead>
<tr>
<th>Treatment of mice†</th>
<th>Challenge virus‡</th>
<th>Virus dilution*</th>
<th>Isolation of variants I</th>
<th>Isolation of variants II</th>
<th>Isolation of variants III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody 101-1</td>
<td>CVS-11</td>
<td>1/2 (7.5 × 10⁵ p.f.u.)</td>
<td>4/4</td>
<td>2/2</td>
<td>4/4</td>
</tr>
<tr>
<td>Nil</td>
<td>CVS-11</td>
<td>1/10 (1.5 × 10⁵ p.f.u.)</td>
<td>4/4</td>
<td>0/4</td>
<td>1/4</td>
</tr>
<tr>
<td>Antibody 101-1</td>
<td>CVS-11</td>
<td>1/50 (0.3 × 10⁵ p.f.u.)</td>
<td>4/4</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Nil</td>
<td>CVS-11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Initial titre of CVS-11 virus was 5 × 10⁷ p.f.u./ml.
† Mice were given antibody 101-1 24 h prior to infection. Titre of circulating neutralizing antibody at time of infection was 1/16000.
‡ Mice were injected i.c. with 0.03 ml of CVS-11 virus or CVS-11 virus following neutralization with antibody 101-1 as described in Methods.
§ MR, Mortality ratio.
|| Proportion of mice from which variants were isolated.
¶ NT, Not tested.

passage level (4.0 to 5.0) and remained constant thereafter. This indicates that antigenic variants can accumulate within a virus population due to non-immunological mechanisms.

Selection of variants in vivo

Mice were injected i.p. with monoclonal antibody 101-1 ascitic fluid as described in Methods, and inoculated i.c. with CVS-11 virus after 24 h. At this time the titre of circulating neutralizing antibody in the infected mice was 1/16000. This level of antibody delayed but did not prevent infection, and mice became paralysed after 8 or 9 days. Variant viruses resisting neutralization by antibody 101-1 were isolated from the brains of two mice infected with 7.5 × 10⁵ p.f.u. of virus and from the brains of four mice infected with 1.5 × 10⁵ p.f.u. of virus (Table 4). When virus was mixed with neutralizing antibody prior to inoculation into mice, mortality was greatly reduced, and of three viruses isolated from mouse brain, only one variant virus was found. As expected, no variant viruses were isolated from control mice infected with non-neutralized virus.

DISCUSSION

We present evidence that three independent antigenic sites exist on the rabies virus (CVS-11) glycoprotein molecule, based on an antigenic analysis of glycoprotein variants of CVS-11 virus using 12 neutralizing monoclonal antibodies. A similar analysis of the ERA strain of rabies virus has revealed a fourth antigenic site, in addition to three others corresponding to those described
here for CVS-11 (M. Lafon et al., unpublished data). Recently, Flamand et al. (1980) reported nine ’epitope groups’ on the CVS-11 viral glycoprotein. However, our results indicate that these epitopes lie within the three larger antigenic sites described.

An analysis of the influenza A haemagglutinin has shown that antigenic sites defined by this method are located in discrete areas on the surface of the molecule (Wiley et al., 1981; Gerhard et al., 1981). Two lines of evidence indicate that this is also the case for the rabies glycoprotein. Firstly, maximum competition between two monoclonal antibodies for binding sites on the glycoprotein was observed if the two antibodies recognized the same antigenic site. By this criterion, site I is clearly independent, whereas sites II and III may be located in close proximity (Table 2). Secondly, we have previously shown that each of three large cyanogen bromide peptides isolated from the ERA virus glycoprotein induced neutralizing antibody when inoculated into mice (Dietzschold et al., 1982). Work is in progress to determine which of the cyanogen bromide peptides carry each particular antigenic site.

An important impetus for analysing antigenic variation in the CVS-11 virus was the provision of a model system that could be extended to street viruses. A large panel of street viruses has been assembled from different geographical areas (Wiktor & Koprowski, 1982; P. Sureau, P. Rollin & T. J. Wiktor, unpublished results) and preliminary results indicate that antigenic variants do exist among these viruses. Furthermore, these variants differ in the regions of the glycoprotein molecule represented by antigenic sites I, II and III in CVS-11 (M. Lafon et al., unpublished data; Wiktor & Koprowski, 1982). The effectiveness of an inactivated vaccine in protecting mice against these viruses correlated with the degree of antigenic disparity detected by analysis with monoclonal antibodies (Wiktor & Koprowski, 1980, 1982). We have previously shown that mice immunized with CVS-11 vaccine were protected against variants of CVS-11 exhibiting single differences in antigenic sites IIA or IIC. Interestingly, vaccines prepared from these same variants failed to protect against challenge with CVS-11 (Wiktor & Koprowski, 1980). In order to determine the threshold of common antigenicity required for protection, we are currently attempting to isolate viruses with variations in more than one antigenic site by sequential selection.

Our results demonstrating selection of antigenic variants by monoclonal antibody in vivo have a number of implications. Firstly, in view of the danger of an antigenic variant arising as a direct result of treatment, a single monoclonal antibody should not be used in place of immune gamma globulin in the post-exposure treatment of rabies. It may be possible, however, to use mixtures of monoclonal antibodies directed against several antigenic sites, thus reducing the probability of variant selection to an acceptable level. Secondly, it is tempting to extend this observation to the mechanism of antigenic variation in nature. It is assumed that immunity following vaccination or infection is directed against all antigenic sites. In this report we have shown that the frequency of variant selection is dramatically reduced when this is the case (Table 1). However, Natali et al. (1981) have reported that some influenza variants (differing in a single antigenic site) were not recognized by human antibody, especially that of children. They suggested that a restricted anti-influenza clonal repertoire may allow such variants to escape humoral immune mechanisms. Another possibility is that the selection pressure is not immunological, and that such non-immunological selection results in viruses showing a concomitant change in antigenic profile. This is supported by our observations that changes in antigenic composition can appear in vitro after several passages and cloning of virus in cell cultures (Table 3). The accumulation of particular variants in this case was presumably due to differences in replication efficiency in the tissue culture system used. However, which of these two models best describes the mechanism of antigenic variation in street rabies viruses remains to be seen.

We thank Dr W. Wunner for providing the 125I-labelled antibodies, and Dr A. Flamand for providing some of the CSV-11 variants. This work was supported in part by Public Health Science Research Grant AI-09706 from the National Institute of Allergy and Infectious Diseases.
Antigenic sites on rabies virus glycoprotein

REFERENCES


(Received 14 September 1982)