Use of Hybridoma Monoclonal Antibodies in the Detection of Antigenic Differences Between Rabies and Rabies-related Virus Proteins. I. The Nucleocapsid Protein

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SUMMARY

Twenty-one hybridoma cultures, obtained through the fusion of mouse myeloma cells with splenocytes of BALB/c mice immunized with either rabies virus or Mokola virus, secreted monoclonal antibodies specific for the nucleocapsid of the inducer virus. They displayed different specificities for the nucleocapsids of rabies and rabies-related viruses and could be classified into eight groups which are likely to correspond to different antigenic determinants on the nucleocapsid. Four strains of fixed rabies virus (CVS, ERA, Flury-LEP and Kelev) could not be differentiated by the nucleocapsid-specific hybridoma antibody. The Flury-HEP virus (derived from Flury-LEP) as well as the rabies-related viruses Mokola, Lagos bat and Duvenhage, showed marked differences in their reactivities with hybridoma antibodies to nucleocapsid. A selected panel of three of these hybridomas may be used for a rapid differential diagnosis among all members of the Lyssavirus group.

INTRODUCTION

The Lyssavirus serogroup of the rhabdovirus family consists of rabies and several related viruses, including Lagos bat, Mokola and Duvenhage. These viruses have been isolated in different parts of the world and from a wide variety of hosts (for review, see Shope, 1975). All except Lagos bat are known human pathogens.

We have recently produced and characterized a panel of hybridomas that secrete monoclonal antibodies against the glycoprotein or nucleocapsid protein of rabies virus. These hybridomas are being used to study the antigenic cross-reactivity of various strains of rabies and rabies-related viruses. This paper describes the results of an investigation of the nucleocapsid.

METHODS

Virus strains. Clone-purified fixed strains of rabies CVS, ERA, Kelev, Flury-LEP and Flury-HEP and rabies-related Duvenhage, Lagos bat and Mokola, were propagated in BHK-21 cell culture monolayers as described by Clark & Wiktor (1972, 1974), Wiktor (1973) and Tignor et al. (1977).

Production of hybridomas. Adult BALB/c mice were first immunized by intraperitoneal (i.p.) injection of concentrated, purified, $\beta$-propiolactone-inactivated vaccine (Wiktor et al. 1977).

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1969) prepared from the ERA, CVS, Kelev or Mokola strains. This was followed 6 to 8 weeks later by a booster inoculation of the same vaccine preparation diluted 1:5. Spleens were removed from mice 3 to 5 days after the booster inoculation, and splenocytes were processed and hybridized with P3 × 63 Ag8 mouse myeloma cells according to the method proposed by Koprowski et al. (1977) and Wiktor & Koprowski (1978). Hybrid colonies that produced antibodies were first tested by radioimmunoassay (RIA) for binding to virus-infected cells and then further screened by indirect immunofluorescence for the staining of intracytoplasmic nucleocapsid inclusions in acetone-fixed virus-infected cells (Wiktor & Koprowski, 1978). Hybridomas that did not stain membranes of rabies-infected live cells and did not neutralize homologous virus, were selected for this study and used either as a mass culture or after cloning according to Koprowski et al. (1977). With few exceptions, the clones had the same characteristics as the mass culture.

Preparation of ascitic fluid. BALB/c mice were primed by an i.p. injection of 0.5 ml pristane 2,6,10,14-tetramethylpentadecane, (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) and 2 weeks later were again inoculated i.p. with 1 × 10⁶ hybridoma cells. Tumours and ascites developed in approx. 2 weeks. The fluid was collected, separated from cells by centrifugation and used in immunoprecipitation experiments.

Isolation and purification of nucleocapsids. Nucleocapsids were isolated from infected cells as described by Sokol (1973). Briefly, infected cells were washed twice in phosphate-buffered saline (PBS), resuspended in water (1 × 10⁶ cells/ml) and disrupted with three strokes of a Dounce homogenizer. The cell suspension was centrifuged at 1200 rev/min for 30 min; MgSO₄ (final concentration 5 × 10⁻⁸ M) and 2 g CsCl were added to 4 ml of the supernatant, which was then centrifuged at 40000 rev/min for 24 h at 4 °C in a SW50.1 rotor. A nucleocapsid band clearly visible at a density of 1.32 (±0.1) g/ml was harvested by side-puncture of the tube, dialysed overnight against NT buffer (0.15 M-NaCl, 0.01 M-tris-HCl, pH 7.4) and further purified by a second centrifugation in CsCl. The nucleocapsid band was then harvested again, dialysed against NT buffer and kept at -70 °C until use. Protein concentrations of different nucleocapsid preparations, determined by the method of Bramhall et al. (1969), varied from 0.2 to 0.8 mg/ml. Purity of the antigen was controlled by 10% Laemmli's gel electrophoresis (Laemmli, 1970) and by negative staining and observation by electron microscopy (EM).

Virus purification. Virus was purified by polyethylene glycol precipitation followed by two cycles of centrifugation in a sucrose gradient, as described by Flamand & Bishop (1973).

Isolation of glycoprotein from purified virions. Purified virus (100 μg) was treated with 0.3% Triton in NTE buffer (0.15 M-NaCl, 0.05 M-tris-HCl, pH 7.4, 0.001 M-EDTA; 0.3 ml final vol.) for 20 min at room temperature with frequent mixing. The mixture was then loaded on to a 10 to 40% (w/v) sucrose gradient in NT buffer and spun in a SW50 Beckman rotor at 30000 rev/min for 1 h. The glycoprotein-containing top fraction was carefully removed, dialysed against PBS at 4 °C overnight and stored at -70 °C until used.

Immunoprecipitation of virus proteins from infected cells. For preparation of cell extracts, one roller bottle of BHK-21 cells (approx. 10⁸ cells) was either infected with ERA virus at a m.o.i. of 0.1 p.f.u. per cell or mock-infected. After 24 h of incubation at 33 °C in minimal essential medium (MEM) containing 0.1%, bovine serum albumin (MEM-BSA), cells were drained and covered with 40 ml MEM-BSA containing 1/5 the normal quantity of amino acids and 5 mM Ca²⁺-labelled leucine. After 18 h of incubation at 33 °C, cells were drained, washed twice with NT buffer and resuspended in 1.6 ml lysis buffer (0.5% NP40, 0.15 M-NaCl, 50 mM-tris-HCl, pH 7.4, 5 mM-EDTA and 2 mM-phenylmethyl-sulphonylfluoride; Sigma, St. Louis, Mo., U.S.A.). The cell suspension was sonicated for 15 s at the maximum power of the MSE sonicator and centrifuged in a SW50 Beckman rotor at 12000 g for 30 min. The top lipid layer and pellet were discarded and the supernatant saved and frozen.
Nucleocapsid of rabies and rabies-related viruses

in aliquots until use. Cell extracts from unlabelled uninfected cells were prepared as above. For reduction of non-specific binding in preparations for immunoprecipitation, cell extracts were pre-treated with a suspension of Staphylococcus aureus (1 vol. cell extract plus 1 vol. S. aureus at 4 °C overnight). They were centrifuged for 5 min in a bench Eppendorf centrifuge (8000 g), and 20 μl of the supernatant was then mixed with an equal volume of immune ascitic fluid diluted in PBS (1:10) and incubated at 4 °C overnight. Pre-treated anti-mouse IgG (10 μl anti-IgG plus 10 μl cold, uninfected cell extract kept at 4 °C overnight) was added to the mixture, which was then incubated at 4 °C for 4 h and centrifuged for 5 min in an Eppendorf centrifuge. The pellet was washed four times in wash buffer (0.5 M-NaCl, 15 mM-tris-HCl, pH 7.4, 5 mM-EDTA, 5% sucrose and 1% NP40), resuspended in 30 μl Laemmlli's protein resuspension buffer (62.5 mM-tris-HCl, pH 7.4, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue), boiled for 1 min and electrophoresed on a 10% discontinuous slab gel (Laemmlli, 1970). After electrophoresis for 4 h at 80 V, the gels were fixed in methanol/acetic acid/water (3:4:6), dehydrated in dimethylsulphoxide, infiltrated with 20% 2,5-diphenyloxazole in dimethylsulphoxide, dried and exposed to RP Royal X Omat film at −70 °C as described by Bonner & Laskey (1974).

RIA. All antigens were adjusted to a protein concentration of 2 to 8 μg/ml. Diluted antigen (25 μl) was placed at the bottom of plastic 96-well plates (disposable polyvinyl Flex U plates; Cooke Laboratory, Alexandria, Va., U.S.A.) and allowed to dry for 4 h at 37 °C. Plates were then washed with PBS containing 10% γ-globulin-free horse serum (HS-PBS; GIBCO). Hybridoma medium (25 μl) diluted 1:4 in 10% HS-PBS was added to each well. The plates were incubated at 37 °C for 1 h, washed three times in 1% HS-PBS and drained. Each well then received 25 μl125I-labelled rabbit anti-mouse F(ab')2 antibodies (kindly provided by W. Gerhard, of this Institute; 20000 ct/min, sp. act. 0.5 mCi/mg). The plates were again incubated at 37 °C for 1 h and washed three times with 1% HS-PBS. The bottoms of the wells were then cut with an incandescent wire and placed in a gamma-counter for determination of radioactivity.

RESULTS

Specificity of anti-nucleocapsid antibodies produced by hybridomas

Media from seven hybridomas derived from mice immunized with the ERA rabies strain, six hybridomas from mice immunized with the CVS strain, seven from mice immunized with the Kelev strain and one from mice immunized with Mokola virus were found to stain intracytoplasmic inclusions of acetone-fixed infected cells and not to stain membranes of live cells. These hybridomas were selected for this study. Further characterization was performed by selective immunoprecipitation of the N protein of ERA-infected cell extracts with ascitic fluid obtained from mice injected with hybridoma cells. Anti-nucleocapsid antibodies secreted by hybridomas 222, 239 and 237 precipitated a protein of mol. wt. 62000 (Fig. 1, tracks 1, 2 and 3), which is the same mol. wt. as that of the nucleocapsid protein of the virus (Fig. 1, track V). The same antibodies did not precipitate any protein of similar mol wt. from uninfected cell extracts. In addition, precipitation of a protein of mol. wt. around 70000 was observed with both infected and uninfected cell extracts and was considered non-specific. As a control, ERA-infected cell extracts were treated with antibody 101-1 directed against the glycoprotein of the virus. This antibody precipitated a protein (Fig. 1, track 4) of the same mol. wt. as that of the glycoprotein (Fig. 1, track V) as well as the 70000 mol. wt. contaminant and another protein, Gx, also present in the virion and likely to be a polymer of the glycoprotein (B. Dietzschold, personal communication). Immunoprecipitation of the nucleocapsid protein was also carried out with hybridoma supernatants, although at a lower rate of efficiency (data not shown).
Fig. 1. Immunoprecipitation of N or G virus proteins from ERA-infected cells by anti-nucleocapsid or anti-glycoprotein monoclonal antibodies. Autoradiography of a 10% discontinuous Laemmli gel treated for fluorography. V, 3H-leucine-labelled ERA virus dissociated in Laemmli's buffer. Tracks 1, 2 and 3, proteins precipitated from an ERA-infected cell extract by antibodies present in ascitic fluids 222, 239 and 237, respectively. Track 4, proteins precipitated from ERA-infected cell extract by antibodies present in ascitic fluid 101-1. C, proteins precipitated from an uninfected cell extract by antibodies present in ascitic fluid 222. Precipitation by the three other ascitic fluids gave similar results and are not shown in this figure.

Fig. 2. Purity of glycoprotein and nucleocapsid virus antigens. Autoradiography of a 10% discontinuous Laemmli gel treated for fluorography. The virus material (ERA virions, nucleocapsids or glycoproteins purified as described in Methods) was pelleted with alcohol, dried, dissolved into 30 μl of protein dissociation Laemmli's buffer, and boiled for 1 min before loading on to the gel. Track 1, 3H-labelled G protein. Track 2, 3H-labelled ERA virus. Track 3, 3H-labelled nucleocapsids.

When binding to purified antigens was tested, it was found that anti-nucleocapsid monoclonal antibodies reacted in RIA with purified nucleocapsids and purified virions but not with purified glycoproteins (see Table 1 for typical results). In contrast, anti-glycoprotein monoclonal antibodies reacted with purified virions and purified glycoproteins but not with the purified nucleocapsid. The purity of ERA nucleocapsids, glycoproteins and virion antigens used in RIA was controlled by gel electrophoresis and can be seen in Fig. 2. The five virus proteins, as well as Gx, are present in the virus preparation (track 2) with a small number of contaminants, probably of cellular origin. In addition to the major glycoprotein band, the glycoprotein suspension contains Gx and traces of M1 (track 1). The nucleocapsid preparation contains almost exclusively the nucleocapsid protein (track 3).
**Table 1. Immunoreactivity of hybridomas with ERA virus antigens: results of RIA**

<table>
<thead>
<tr>
<th>Hybridoma specificity</th>
<th>Antigen</th>
<th>Virus</th>
<th>Nucleocapsid</th>
<th>Glycoprotein</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-glycoprotein</td>
<td>5932*</td>
<td>2</td>
<td>810</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6107</td>
<td>10</td>
<td>800</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Anti-nucleocapsid</td>
<td>4155</td>
<td>1357</td>
<td>22</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3920</td>
<td>1247</td>
<td>22</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Other specificity</td>
<td>1737</td>
<td>72</td>
<td>7</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1682</td>
<td>75</td>
<td>57</td>
<td>25</td>
<td></td>
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</tbody>
</table>

* Counts of ^3^H-labelled anti-F(ab')\(_2^\) fixed. Each test was carried out in duplicate. A background of 100 ct/min was subtracted from each value.

**Fig. 3. Kinetics of the antibody-antigen reaction in RIA.** The antigen (purified CVS virus; 50 \(\mu\)g protein/ml) was diluted in PBS 1:25 (Δ——Δ), 1:50 (○——○) or 1:75 (●——●). The antibody (ascitic fluid obtained from hybridoma 222) was diluted in 10% HS-PBS.

**Kinetics of the antibody-antigen reaction**

Kinetics of the antibody-antigen reaction for three different concentrations of antigen are shown in Fig. 3. As long as antibody was in excess of antigens, the number of counts fixed in RIA did not decrease with the dilution of antibody. Counts at the plateau were roughly proportional to quantity of antigen.

When antibody was no longer in excess of antigen, the number of counts decreased exponentially. The three curves met the abscissa at the same point, which is considered the titre of the antibody suspension. For instance, ascitic fluid obtained from a mouse injected with hybridoma culture 222 had a titre of approx. 1/200,000, or 100 times higher than the supernatant of the corresponding hybridoma culture. Similar observations were made for several other hybridomas. The antibody titres of different hybridoma supernatants used in this study were between 1/40 and 1/2000.
Table 2. Immunoreactivity of hybridomas with nucleocapsid antigens in RIA

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>Origin</th>
<th>Number</th>
<th>CVS*</th>
<th>ERA*</th>
<th>Kelev†</th>
<th>Flury*</th>
<th>Duven- hage*</th>
<th>Mokola*</th>
<th>Lagos bat†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kelev</td>
<td>590-2</td>
<td>3428†</td>
<td>1633</td>
<td>4612</td>
<td>2260</td>
<td>1905</td>
<td>11</td>
<td>5</td>
<td>0</td>
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<tr>
<td>CVS</td>
<td>222-9</td>
<td>2921</td>
<td>2265</td>
<td>5709</td>
<td>1641</td>
<td>32</td>
<td>12</td>
<td>1398</td>
<td>2498</td>
</tr>
<tr>
<td>ERA</td>
<td>237-3</td>
<td>3947</td>
<td>2581</td>
<td>6375</td>
<td>1900</td>
<td>14</td>
<td>14</td>
<td>2465</td>
<td>8</td>
</tr>
<tr>
<td>Kelev</td>
<td>104-7</td>
<td>3342</td>
<td>2538</td>
<td>5516</td>
<td>2018</td>
<td>13</td>
<td>1188</td>
<td>1532</td>
<td>4696</td>
</tr>
<tr>
<td>Kelev</td>
<td>389</td>
<td>3173</td>
<td>1843</td>
<td>4571</td>
<td>1418</td>
<td>0</td>
<td>2260</td>
<td>65</td>
<td>2430</td>
</tr>
<tr>
<td>Kelev</td>
<td>502-2</td>
<td>6407</td>
<td>3791</td>
<td>6649</td>
<td>3859</td>
<td>3781</td>
<td>7444</td>
<td>4943</td>
<td>5369</td>
</tr>
<tr>
<td>Mokola</td>
<td>422-7</td>
<td>233</td>
<td>177</td>
<td>140</td>
<td>152</td>
<td>180</td>
<td>1773</td>
<td>3030</td>
<td>3462</td>
</tr>
</tbody>
</table>

* Purified nucleocapsids (50 to 100 ng).
† Purified virus (100 to 200 ng).
‡ Counts of 125I-labelled anti-F(ab')2 fixed. Average values for duplicate samples with < 20% deviation. Values obtained in the controls (antigens without antibodies or antibodies without antigens; 0 to 150 ct/min) were subtracted in each case.

Table 3. Classification of nucleocapsid hybridomas

<table>
<thead>
<tr>
<th>Virus-strain</th>
<th>Rabies-related</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybridoma</td>
<td>CVS</td>
</tr>
<tr>
<td>CVS</td>
<td>11</td>
</tr>
<tr>
<td>Kelev</td>
<td>1</td>
</tr>
<tr>
<td>Kelev</td>
<td>1</td>
</tr>
<tr>
<td>ERA</td>
<td>3</td>
</tr>
<tr>
<td>CVS</td>
<td>2</td>
</tr>
<tr>
<td>CVS</td>
<td>1</td>
</tr>
<tr>
<td>Kelev</td>
<td>1</td>
</tr>
<tr>
<td>Mokola</td>
<td>1</td>
</tr>
</tbody>
</table>

Classification of anti-nucleocapsid hybridomas

Purified nucleocapsids were isolated from cells infected with one of four fixed strains of rabies virus (CVS, ERA, LEP or HEP) or with one of the rabies-related viruses (Duvenhage or Mokola). Purity of the antigen was controlled by EM (data not shown) and/or gel electrophoresis (see Fig. 2 for example of ERA virus). Purified virions of the Kelev and Lagos bat viruses were also used in this study.

RIA was performed with 50 to 100 ng purified nucleocapsids or 100 to 200 ng purified virions and a 1:4 dilution of hybridoma supernatant. With few exceptions, antibody at this dilution was in excess of antigen.

Representative results are shown in Table 2. When less than 300 ct/min were fixed in RIA, the antibody was considered to have no affinity for the antigen. In all cases, results were either significantly above or below this limit and could therefore be easily interpreted as positive or negative. The 21 monoclonal antibodies displayed different specificities for the different viruses and were thereby categorized into eight groups (Table 3). All hybridomas
induced by the fixed strains of rabies (i.e., groups A to G in Table 3) secreted antibodies which reacted with the ERA, CVS, Flury-LEP and Kelev strains of rabies. The hybridoma induced by Mokola virus (group H) secreted antibodies which reacted with rabies-related viruses but not with fixed strains of rabies. Flury-HEP, a derivative of the Flury strain of rabies. was not recognized by antibodies of groups D to G. Duvenhage reacted with antibodies of groups B, C, D, G and H, Mokola with antibodies of groups C, D, E, F and H, and Lagos bat, with antibodies of groups C, D, E, G and H.

DISCUSSION

Twenty-one hybridomas were derived from mice immunized with rabies strain CVS, ERA or Kelev or with Mokola virus. These hybridomas secrete antibodies which react with the nucleocapsid of the inducer virus and show different specificities for the nucleocapsids of heterologous viruses. On the basis of their reactivities, they were classified into eight groups. However, it is obvious that the patterns of immunoreactivity of these hybridomas with other strains of virus could lead to the further subdivision of the groups. For instance, the reactivity of one hybridoma of group A toward several other strains of street rabies differs from reactivities of the other hybridomas of the same group (T. Wiktor, unpublished results). Hybridomas of different groups are likely to recognize different antigenic determinants on the nucleocapsid. Using protease digestion of the nucleocapsid, we are now investigating whether or not different antigenic determinants correspond to different parts of the nucleocapsid. Monoclonal antibodies secreted by hybridomas of groups A to G show similar specificities for the four fixed strains (ERA, CVS, Flury-LEP and Kelev). The surprising fact is that hybridomas representing groups D to G do not react with nucleocapsids of the Flury-HEP virus, despite the fact that the HEP virus is a derivative of Flury-LEP (Koprowski, 1954).

On the basis of a serological reaction with polyclonal antibodies produced in animals, four serotypes, i.e. rabies, Lagos bat, Mokola and Duvenhage, have been classified as constituting the Lyssavirus genus (World Health Organization, 1977). As shown in Table 3, the nucleocapsid antigens of the Mokola, Lagos bat and Duvenhage viruses show as good cross-reactivity with the four fixed strains of rabies virus as does the HEP strain, which is definitely classified as a rabies serotype. Thus, the three viruses of African origin (Duvenhage, Mokola and Lagos bat) may be variants of rabies virus with same relation to their ‘parental’ virus as Flury-HEP has to Flury-LEP.

Rabies virus has been classified as a member of the rhabdovirus group on the basis of its morphology and ultrastructural features (Melnick & McCombs, 1966). Modality of virus RNA synthesis (Ermine & Flamand, 1977; Kawai, 1977; Flamand et al. 1978) and similarities in the genetic maps of vesicular stomatitis virus and rabies virus genomes (Flamand & Delagneau, 1978) support the hypothesis that rhabdoviruses may have evolved from a common ancestral prototype (Sokol & Koprowski, 1975). The study of serological cross-reaction with animal sera between various rhabdoviruses has not supported this contention. However, if studies of the reactivity of monoclonal antibodies are extended to other members of the rhabdovirus group, it is possible that a cross-reaction may be discovered that might contribute to our understanding of the evolution of the group.

Finally, it should be mentioned that the diagnosis of rabies virus infection is presently based on the staining of intracytoplasmic rabies nucleocapsid in the neurons of the infected animal or human by anti-rabies animal serum conjugated with fluorescein isothiocyanate. Antibodies secreted by three of our hybridomas (510, 422-5 and 237-3) can differentiate HEP
from other fixed strains of rabies virus, and from the rabies-related Mokola, Lagos bat and Duvenhage viruses, and this test can be performed directly on brain impressions. The practical importance of this finding in routine diagnosis must be emphasized.

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