Human monoclonal antibodies specific for the rabies virus glycoprotein and N protein

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Introduction

The prevention of rabies by post-exposure immunization is a phenomenon virtually unique in the field of infectious diseases. The efficiency of this vaccination is probably due to the long incubation period of the disease. Serotherapy alone is not sufficient to confer protection, as shown in a simian model (Sikes et al., 1971). However, in cases of severe exposure, the combined administration of both antirabies serum and vaccine is strongly recommended (WHO Expert Committee on Rabies, 1984). The efficacy of combining rabies immune serum with vaccine was first shown by Balthazard & Bahmanyar (1955), who treated 32 patients severely wounded by a rabid wolf. Of the 17 patients administered serum plus vaccine, only one died and three of the five patients given vaccine alone died.

Passive immunization has been proposed either to neutralize the free rabies virus particles inoculated through the bite, or to destroy early infected cells by complement-mediated immune lysis or antibody-dependent cellular cytotoxicity mechanisms. The neutralizing activity of immune rabies serum is estimated either by the seroneutralization technique in cell culture (RFFIT) (Smith et al., 1973) or in vivo in mice (Atanasiu, 1978). The recommended doses are 20 international units (IU)/kg of body weight for human rabies immunoglobulins (HRIG) and 40 IU/kg for equine rabies immunoglobulins. HRIG are the product of choice for early passive protection, since they do not induce the adverse reactions that occur in patients treated with equine immunoglobulin (Wilde et al., 1987, 1989). However, HRIG treatment is expensive and, in countries where rabies is still a major public health problem, few patients can afford it (Wilde et al., 1987).

Human monoclonal antibodies (MAbs) of selected specificity could become an alternative to products prepared from human blood donations. We report here the continuous in vitro production of three human MAbs against rabies virus after fusion of a non-secretor heteromyeloma line (human–murine xenohybrid) with splenocytes from a rabies-immune human donor, and their use in the investigation of the antigenic structure of the rabies virus.
Methods

Cells and rabies viruses. The non-secretor human–murine xenohybrid SPM-40, a mouse P3-X63-Ag8 myeloma variant fused with a human spleen cell, was propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 1% sodium pyruvate, 1% glutamine, 1% kanamycin and 1% ampicillin (DMEM 10%). BSR cells (Sato et al., 1977), a variant line of BHK-21 and clone CI300 mouse neuroblastoma cells (Neuro-2a) (MacMorris & Ruddle, 1974), were propagated in Eagle's MEM supplemented with tryptose phosphate broth and 8% foetal calf serum (MEM 8%).

Rabies virus strains (lyssavirus serotype 1) Pitman-Moore (PM), CVS, ERA and Pasteur virus (PV4) (Clark & Wiktor, 1972), Mokola virus (lyssavirus serotype 3) MOK-5 (Foggin, 1982) and European bat (E. Bat 1) lyssavirus (DUV4, F. R. G. Stade isolate) (Schneider, 1982) were propagated on BSR cells. Neutralization-resistant viruses (RV) each altered at one of three conformation-dependent antigenic determinants (sites I, II and III) on the rabies virus glycoprotein (Lafon et al., 1983a, b) and one which lacked the conformation-independent site VI (Bunschoten et al., 1989) were obtained from the parental CVS strain by treatment with neutralizing antibodies (Wiktor & Koprowski, 1978; Libeau & Lafon, 1983; Bunschoten et al., 1989) as described previously (Wiktor & Koprowski, 1980). The rabies variant viruses RV-509-6 and RV-3-5B7 (site I), RV-231-22, RV-220-8, RV-101-1 and RV-PVE3 (site II), RV-507-1 and RV-248-8 (site III) and RV-2-25-5 (site VI) were used in this study.

Source of B cells. Peripheral blood lymphocytes (PBL) of a donor who received a pre-exposure immunization regimen with rabies vaccine of the PM strain grown on human diploid cells and of the PV strain grown on foetal bovine kidney cells were separated 10 days after boost immunization by centrifugation of the fixed blood in a Ficoll-Hypaque density gradient. The virus neutralizing antibody (VNA) titre of the donor serum was determined by the RFFIT method using, as standard, rabies virus strain CVS and HRIG (Centre de Transfusion Sanguine, Nancy, France) adjusted to contain 10 IU/ml. The VNA titre at the time of blood donation was 66 IU/ml against CVS, 10 AU/ml against E. Bat 1 and 0-15 AU/ml against Mokola (AU stands for arbitrary unit, as previously defined by Lafon et al., 1986).

Epstein-Barr virus (EBV) transformation and cell fusion. EBV transformation of PBL was obtained by exposing cells for 2 h at 37 °C to the supernatant of the EBV-producing cell line B95-8 (Miller et al., 1972), washed and resuspended in DMEM 10%. The EBV-transformed cells were fused with human–mouse xenohybrid cells, SPM4-O, (ratio PBL/xenohybrid = 1) in the presence of 45% polyethylene glycol (PEG 1000) as already described (Köhler & Milstein, 1975). Selection of hybrids was carried out by culture in DMEM 10% containing hypoxanthine, aminopterin and thymidine (DMEM HAT) for 8 days. Hybridomas were cloned by limiting dilution (three cells per well) at least twice to ensure that they were monoclonal.

Concentration and purification of the cell culture supernatants. Supernatants of hybridoma culture were concentrated (16-fold) by filtration through an XM-100 Amicon membrane, dialysed against 15 mM-phosphate buffer pH 7.3 and then purified on a DE52 cellulose column.

Enzyme immunoassay (EIA) analysis for immunoglobulin secretion. BSR cells were infected with PM rabies virus (m.o.i. 1 to 5), plated (3 x 10^5 cells per well; 150 µl) into a 96-well tissue culture plate (Falcon 3072) and incubated for 24 or 48 h at 37 °C. Infected cells were gently washed with phosphate-buffered saline containing Ca2+ and Mg2+ (PBS, Ca, Mg) and fixed with acetone (80:20). Non-specific binding was prevented by incubation with PBS Ca, Mg containing 10% (v/v) sheep serum for 30 min at 37 °C. Supernatants of hybridoma cultures were incubated with the fixed cells for 30 min at 37 °C and washed with PBS Ca, Mg. Bound antibodies were detected with anti-human IgG biotinylated sheep antibodies (Amersham, RPM. 1003) and followed by horseradish peroxidase–streptavidin (Amersham, RPM. 1231). Substrate solution (H2O2—o-phenylenediamine) was added and the reaction stopped with 3 M-H2SO4. The absorbance was read at 492 nm.

Purified rabies virus or virus fractions (glycoprotein and ribonucleoprotein) were also used as plate-coating antigens. EIAs were performed as described above either on ready-to-use purified glycoprotein-coated plates (Platelia-Rage, Pasteur Diagnostic, 72200) or on microtitre plates (Immunolon, Nunc) coated 18 h at 4 °C with antibodies. The coating antigens were u.v.-inactive and rabbit anti-human immunoglobulin antibody diluted in 0.05 M-carbonate–bicarbonate buffer pH 9.5. Rabbit anti-human immunoglobulin antibody was found to be free of glycoprotein contamination by enzyme immunoassay using mouse antibodies specific for the glycoprotein.

Determination of the IgG concentration and of the IgG subclasses. Serial dilutions (100 µl per well) of purified IgG preparation (2 mg/ml) and rabies-specific MAbs were incubated for 18 h at 4 °C with 50 µl of carbonate–bicarbonate buffer pH 9.5 on 96-well plates (Immunolon, Nunc). After washing with PBS Ca, Mg plates were blocked with PBS Ca, Mg containing 10% sheep serum. The presence of human IgG was detected with anti-human immunoglobulin antibody and the EIA reaction was performed as described. Murine MAbs specific for human IgG1, IgG2 or IgG3 (Seralab; MAS 265, MAS 266 and MAS 267) were used for IgG subclass determinations. Human MAbs were coated on plates in carbonate–bicarbonate buffer as above described and the binding of murine MAbs was detected with sheep anti-mouse immunoglobulin antibodies. Goat anti-human IgM biotinylated antibodies (Biosys, BA 3000) and sheep anti-murine IgG biotinylated antibodies (Amersham, RPM 1001) were used for human IgM and murine IgG detection, respectively.

Immunoblots. Concentrated and purified human MAbs were separated on Cellogel strips (Chemetron) in 25 mm-barbitral buffer pH 9-6 containing 25 mM-sodium acetate and 25 mM-calcium acetate as already described (Bouvet & Pillot, 1985). IgG was transferred onto nitrocellulose membranes (Schleicher & Schuell BA85, 401180) by contact for 3 h at room temperature in PBS. The nitrocellulose membranes were saturated with 5% (w/v) skimmed milk in PBS for 18 h at 4 °C and incubated with rabies virus (PV4 Vero, protein content 194 µg/ml) or native glycoprotein for 2 h at 37 °C. After extensive washing, the transfers were incubated for 2 h at 37 °C with an anti-rabies virus murine MAb conjugated with horseradish peroxidase, and then washed with PBS and incubated in substrate solution (10 ml of 4-chloro-l-naphthol in methanol, 40 ml 50 mM-Tris–HCl pH 6-8 and 50 µl H2O2). Colour development was stopped by dilution in distilled water. Immunoblots with MAbs, HRIG or human sera were also performed on SDS gel-separated rabies virus proteins transferred onto nitrocellulose membrane (Dietzschold et al., 1987).

Immunoprecipitation. BSR cells infected with ERA virus (m.o.i. 1) were labelled with [35S]methionine (Amersham SJ 204) (3 µCi/ml) for 2 days. Cell extracts containing 5 x 10^6 c.p.m., obtained by treatment of cells with lysis buffer (0-5 M-Tris–HCl pH 7-8, 1 M-NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and 100 units/ml zymofrin) were incubated with antibodies for 18 h at 4 °C, then with either anti-human antibodies or anti-murine antibodies for 2 h, and finally with formalin-fixed Staphylococcus aureus Protein A (Bethesda Research Laboratories) for 2 h. After repeated washings in lysis buffer, the pellets were resuspended in degradation buffer, heated and separated by SDS–PAGE. Gels were dried and autoradiographs were exposed for 3 to 7 days.

Immune lysis. Complement-mediated immune lysis was performed on infected Neuro-2a cells as previously described (Dietzschold et al., 1982). Cells were infected with PV4 rabies virus (m.o.i. 1 to 5) or mock-
were of the IgG2 subclass and HRIG of the IgG1 subclass. Human IgM and of murine IgG. The heteromyeloma line specific MAbs showed that MAbs HUM1 and HUM2 contained 5 μg/ml. Subclass analysis by EIA using a standard IgG curve. Culture supernatants were of the Glycoprotein, the nucleocapsid, the NT protein and the Virus.

### Results

#### Generation and screening of human hybridoma

One month after fusion, the surviving colonies in the selective medium (DMEM 10% HAT) were screened by EIA for rabies-specific human immunoglobulin secretion on acetone-fixed rabies infected or non-infected cells. Out of 29 surviving colonies, 10 were rabies-specific (34-4%) and three of these were stable. The positive colonies were cloned twice by limiting dilutions. The study focused on three subclones: HUM1, HUM2 and HUM3. Hybridomas were grown in culture flasks of 25 cm² or 150 cm² (3 x 10⁴ cells per ml) and in roller bottles (2.5 x 10⁵ cells per ml) in DMEM 5%. Supernatants harvested after 6 or 7 days were concentrated and purified as described in Methods.

#### IgG concentration and subclass determinations

IgG concentrations were determined by interpolation on a standard IgG curve. Culture supernatants usually contained 5 μg/ml. Subclass analysis by EIA using specific MAbs showed that MAbs HUM1 and HUM2 were of the IgG2 subclass and HRIG of the IgG1 subclass. MAb HUM3 was found to be composed of human IgM and of murine IgG. The heteromyeloma line was confirmed to be a non-secretor xenohybrid, since no human or murine immunoglobulins were detectable in the supernatants. Nevertheless, a murine light chain and a human μ chain were detected in the cytoplasm and in the supernatants of aged cultures. The appearance of the murine component in the rabies-specific immunoglobulin could be the result of a rearrangement between chains after fusion.

### Table 1. Specificity of human MAbs in EIA

<table>
<thead>
<tr>
<th>Antibody source*</th>
<th>Glycoprotein</th>
<th>Nucleocapsid</th>
<th>NT protein</th>
<th>Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRIG</td>
<td>1350†</td>
<td>760</td>
<td>NT§</td>
<td>1440</td>
</tr>
<tr>
<td>Donor serum</td>
<td>1450</td>
<td>650</td>
<td>NT</td>
<td>1350</td>
</tr>
<tr>
<td>HUM1</td>
<td>212</td>
<td>11</td>
<td>NT</td>
<td>426</td>
</tr>
<tr>
<td>HUM2</td>
<td>185</td>
<td>2</td>
<td>NT</td>
<td>316</td>
</tr>
<tr>
<td>HUM3</td>
<td>2</td>
<td>276</td>
<td>102</td>
<td>324</td>
</tr>
<tr>
<td>HM</td>
<td>11</td>
<td>2</td>
<td>30</td>
<td>55</td>
</tr>
<tr>
<td>PVE-3</td>
<td>2282</td>
<td>109</td>
<td>94</td>
<td>NT</td>
</tr>
<tr>
<td>805-3</td>
<td>NT</td>
<td>1855</td>
<td>1740</td>
<td>NT</td>
</tr>
<tr>
<td>802-2</td>
<td>NT</td>
<td>1135</td>
<td>111</td>
<td>NT</td>
</tr>
</tbody>
</table>

* HRIG (0.1 IU/ml), donor serum (0.33 IU/ml) and murine MAbs (ascites fluid diluted 10⁻³) of known specificity were used as controls. MAb PVE-3 is specific for glycoprotein, MAb 805-3 for N and trypsinized N (NT) and MAb 802-2 for the N protein, but not the NT protein.

† Antigens were purified glycoprotein (PV4 rabies virus) (Platelia rage, Pasteur Diagnostics), CsCl-gradient-purified N protein (ERA rabies virus), NT and sucrose gradient-purified and u.v.-inactivated ERA rabies virus.

§ NT, Not tested.

#### Specificity of human–murine IgM monoclonal antibody HUM3

Acetone-fixed infected cells used as immunosorbents for hybridoma screening expressed the glycoprotein, the nucleoprotein (N), the non-structural protein and the matrix protein. To define the specificity of the supernatants further, the reactivity of supernatants was tested on purified antigens by the EIA technique (Table 1). Human–murine monoclonal antibody HUM3 did not bind the purified rabies virus glycoprotein (Platelia rage, Pasteur Diagnostics), CsCl-gradient-purified N protein (ERA rabies virus), NT and sucrose gradient-purified and u.v.-inactivated ERA rabies virus. EIA. In order to determine the specificity of MAb HUM3, immunoprecipitation of [³⁵S]methionine-labelled ERA-infected cell lysates was performed. As shown in Fig. 1, MAb HUM3 (lane 3) immunoprecipitated a protein corresponding to the N protein of the rabies virus as effectively as a murine MAb (PVA-3) of already known N protein specificity (lane 2). MAb HUM3 did not bind to the N protein transferred onto a nitrocellulose membrane after separation from the other viral proteins by SDS–PAGE (data not shown). This indicated that the epitope of the N protein recognized by MAb HUM3 is sensitive to SDS denaturation. In order to locate this epitope, the reactivity of MAb HUM3 was tested in EIA on trypsinized ribonucleoprotein. It had been shown that trypsin hydrolysed the carboxy terminus of the N protein and that further action of the enzyme...
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4

Fig. 1. Immunoprecipitation of [35S]methionine-labelled ERA rabies virus-infected cell lysate (lane 1) with a murine MAb specific for the N protein (PVA-3) (lane 2) and with the human–murine MAb HUM3 (300 mM-PBS concentrated and purified fraction) (lane 3). The migration of [14C]leucine-labelled virus proteins is shown in lane 4.

was blocked (Sokol & Clark, 1973; Dietzschold et al., 1987). The resulting trypsin-resistant N protein, NT, was still recognized by murine MAb 805-3, but not by murine MAb 377-7 (Dietzschold et al., 1987). MAb HUM3 was found to be reactive with the N protein but not with the NT protein; this suggests that the epitope recognized by MAb HUM3 was located within the trypsin-sensitive portion of the N protein, or that the loss of the N protein carboxy terminus modified the epitope located on the remaining NT protein. Because of the sensitivity to denaturation of the epitope recognized by MAb HUM3, a more precise localization of this epitope could not be undertaken when studying the reactivity of the MAb with digested fragments of N protein or corresponding synthetic peptides.

Specificity of human MAbs HUM1 and HUM2

Human MAbs HUM1 and HUM2 recognized both purified virus and glycoprotein but not CsCl gradient-purified ribonucleoprotein in EIAs (see Table 1) indicating that HUM1 and HUM2 are specific for the glycoprotein either purified or present on the whole virus. The reactivity of MAb HUM1 with the glycoprotein of the whole virus was also shown in an immunoblotting assay in which human concentrated IgG was transferred onto the nitrocellulose membrane and incubated with whole rabies virus (Fig. 2). No reactivity was obtained with the glycoprotein after separation by SDS-
Table 2. Neutralizing activity of human MAbs against rabies virus

<table>
<thead>
<tr>
<th>Antibody source</th>
<th>Virus</th>
<th>PM</th>
<th>Mokola</th>
<th>E. Bat 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRIG (0.1 IU)</td>
<td>3.0*</td>
<td>0-4</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>(0.01 IU)</td>
<td>1.2</td>
<td>0-0</td>
<td>0-3</td>
<td></td>
</tr>
<tr>
<td>HUM3</td>
<td>0-0</td>
<td>0-0</td>
<td>0-0</td>
<td></td>
</tr>
<tr>
<td>HM</td>
<td>0-0</td>
<td>0-0</td>
<td>0-0</td>
<td></td>
</tr>
<tr>
<td>HUM1</td>
<td>1.2</td>
<td>1-2</td>
<td>0-3</td>
<td></td>
</tr>
<tr>
<td>HUM2</td>
<td>1.2</td>
<td>0-9</td>
<td>0-9</td>
<td></td>
</tr>
</tbody>
</table>

* Neutralization index.

PAGE from the other viral proteins transferred onto the nitrocellulose membrane. This indicated that the epitope recognized by MAb HUM1 is sensitive to denaturation.

Neutralizing activity of MAbs HUM1 and HUM2 against PM rabies virus and rabies-related viruses (Mokola and European bat virus)

The neutralizing activity of supernatants from hybridoma cultures was tested in vitro against the rabies virus strain PM and against two rabies-related viruses: Mokola and E. Bat 1 lyssavirus. MAbs HUM1 and HUM2 neutralized PM (neutralization index of 1-2) (Table 2); MAb HUM3 and the supernatant of the heteromyeloma culture had no neutralizing activity. The neutralizing activity of non-concentrated supernatants of hybridoma cultures was found to be equivalent against the PM rabies virus strain (in the VNA test) to that of 0.01 IU of HRIG. MAb HUM1 neutralized the Mokola virus but not E. Bat 1 lyssavirus; MAb HUM2 neutralized the two rabies-related strains. These differences in specificity indicated that the two human hybridomas obtained in the same fusion originated from different lymphocytic clones.

Neutralizing activity of MAbs HUM1 and HUM2 against neutralization-resistant rabies virus variants

Studies of neutralization-resistant variant viruses, selected in an excess of murine neutralizing MAbs, revealed the existence of at least four distinct antigenic determinants (sites I, II, III and VI) on the CVS glycoprotein. Sites V and VI were found only on the ERA strain glycoprotein (Lafor et al., 1983b). In order to locate the epitopes recognized by human MAbs on the glycoprotein, the capacity of MAbs HUM1 and HUM2 to neutralize resistant variant viruses representing each of the four antigenic determinants was tested. MAb HUM1 neutralized resistant viruses variant at sites I, II and III (see Table 3) but did not neutralize RV-2-22-5 altered at site VI. This virus was found to be resistant to only one MAb (2-22-5) out of the 39 tested (data not shown). Taken together, these findings suggest that mutations in the glycoprotein amino acid sequences of site VI virus variants modify the epitope recognized by human MAb HUM1. Consequently, the epitope belongs to antigenic site VI. It is noteworthy that the mutation modifying the epitope recognized by the murine MAb 509-6 in site I enhanced the neutralization activity of human MAb HUM1. The neutralizing activity of this MAb is higher against the resistant variant virus RV-509-6 (neutralization index >2) than against the parental virus (neutralization index 1-2) (Table 3) suggesting that topographic relationships could exist between the two sites. However, MAb HUM2 did not neutralize any resistant variant viruses affected at sites I, II, III or VI, suggesting that this MAb describes a new antigenic site on the CVS glycoprotein.

Lytic activity of MAbs HUM1 and HUM2 in the presence of complement

The lytic activity of supernatants of human hybridomas against PV4 rabies virus-infected neuroblastoma cells in the presence of guinea-pig complement was compared to the lytic activity of HRIG and of murine MAbs specific for the glycoprotein. As a result of the low sensitivity of the complement assay, the lytic activity of these supernatants was detectable only with concentrated supernatants (Table 4). The lytic activity of human MAbs was equivalent to the HRIG preparation adjusted to contain 0.1 IU/ml. No lytic activity was obtained with MAb HUM3 specific for the N protein or with the concentrated supernatant of the heteromyeloma culture. Immune lysis was also obtained with anti-glycoprotein murine neutralizing antibody (PVE-3) as well as non-neutralizing antibody (1118-2), and with HRIG.
Table 4. Immune lysis

<table>
<thead>
<tr>
<th>Source of antibodies</th>
<th>Specific chromium release (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRIG</td>
<td>84-3</td>
</tr>
<tr>
<td>HUM1</td>
<td>79-6</td>
</tr>
<tr>
<td>HUM2</td>
<td>86-3</td>
</tr>
<tr>
<td>HUM3</td>
<td>8-2</td>
</tr>
<tr>
<td>HM</td>
<td>8-1</td>
</tr>
<tr>
<td>PVE-3</td>
<td>87-2</td>
</tr>
<tr>
<td>1118-2</td>
<td>89-6</td>
</tr>
</tbody>
</table>

† Immune lysis of rabies (PV4 strain)-infected Neuro-2a cells in the presence of HRIG (diluted $2 \times 10^{-1}$), anti-glycoprotein neutralizing murine MAb PVE-3 or anti-glycoprotein non-neutralizing murine MAb 1118-2 (diluted $10^{-2}$) and concentrated and purified (15 mM-PBS fraction) human rabies virus-specific MAbs.

Discussion

We report here the first isolation of three human MAbs specific for the rabies virus. These antibodies were generated so as to produce an alternative to HRIG obtained from blood donations and also to permit identification of immunogenic domains on rabies virus proteins. The hybridomas were obtained by fusion between a non-secretor heteromyeloma and PBL of a rabies-vaccinated donor which had been briefly infected in vitro with EBV. The heteromyeloma was chosen as a fusion partner because it has better fusion and growth abilities than the usual human myeloma cell lines. Even when the heteromyeloma was a non-secretor line, possible rearrangement between murine IgG components present in the cytoplasm and human IgG of the PBL could not be ruled out. Indeed, of the three antibodies, one had a hybrid composition (human IgM and murine light chain), while the rest were composed solely of human IgG. Early screening can easily identify such hybrid antibodies which are not therapeutically suitable, since hybrid human–murine as well as murine antibodies may induce an immune reaction preventing efficient serotherapy or inducing adverse reactions in the patient.

PBL are the most readily available source of lymphocytes for human fusions, although they are less efficient fusion partners than lymphocytes obtained from tonsils or from spleen. However, they may be suitable fusion partners for a short time after the booster. The high proportion (34.4%) of antibody-producing colonies in the reported fusion experiment suggests that a high level of antibody-secreting cells was present in the donor's circulation at the time of bleeding. The blood donation obtained 10 days after the antigenic boost with rabies vaccine corresponded to the suggested period of time (2 weeks) when specific antibody-producing cells circulate after a tetanus toxoid boost (Kozlor & Roder, 1981). Immunological stimulation which increases the number of circulating antigen-specific B cells seems to be essential for obtaining hybridomas, since no colony was obtained with PBL harvested 1 year after the last rabies vaccine boost, or with no boost (the donor had received five vaccine injections in a post-exposure vaccination regimen). However, multiple boosters may have a negative effect, since no hybridoma was obtained with PBL from donors who received more than three boosters every 2 years.

Variant viruses resistant to neutralization were used to describe four functionally independent antigenic sites on the CVS glycoprotein, numbered I, II, III (Lafon et al., 1983a, b) and VI (Bunschoten et al., 1989). In order to identify the region of the glycoprotein involved in neutralization by the human MAbs, neutralization-resistant variants representative of the four CVS sites were tested against the human MAbs. Resistance to neutralization by MAb HUM1 of variants describing antigenic site VI suggested that the human MAb also binds to this site, recently delineated with a murine MAb corresponding to a sequential epitope (Bunschoten et al., 1989). The recognition by murine and human antibodies of the same antigenic site could be related to the nature of the immogen since both a human donor and BALB/c mice received inactivated PM rabies virus in their immunization schedule. BALB/c mice used to produce murine MAbs recognizing the other antigenic sites were immunized with strains other than PM (ERA, CVS, Kelev, European fox, North America bat, PV4) (Flamand et al., 1980; Libeau & Lafon, 1983). However, the epitope recognized by MAb HUM1 is present on ERA, CVS and PV4, since it neutralizes these strains as well as the homologous strain PM. The reactivity of the murine MAb directed against site VI with glycoprotein enzymic cleavage fragments and synthetic peptides indicates that at least one peptide corresponding to residues 253 to 275 of the glycoprotein amino sequence belongs to site VI (Bunschoten, 1989). It seems unlikely that this peptide contains the entire epitope recognized by the human MAb, since the human MAb, unlike the murine MAb, does not recognize the glycoprotein once the viral proteins have been subjected to SDS denaturation.

In the light of experiments showing that antigenic variants can be recovered in mice after treatment of infected animals with a single neutralizing MAb (Lafon et al., 1983a), HRIG should not be replaced by a single MAb but rather by a mixture of several. After treatment of a virus population with a neutralizing MAb the frequency of appearance of a virus resistant to neutralization was as high as $10^{-4}$ (Wiktor & Kuprowski, 1980; Lafon et al., 1983a). The probability of the appearance...
of mutants simultaneously escaping the neutralizing activity of three distinct antibodies is theoretically in the order of $10^{-12}$. Since it is unlikely that saliva of rabid animals contains such a large number of infectious particles, the combination of three antibodies directed against three distinct sites would be sufficient to avoid variant selection.

Neutralization assays using anti-glycoprotein MAbs with a variety of rabies virus strains revealed the existence of antigenic variations between some street rabies viruses and vaccine rabies virus strains (Flamand et al., 1980; Koprowski et al., 1985; Dietzschold et al., 1988). It is still unknown whether these antigenic variations can account for vaccination failures (Koprowski et al., 1985; Wiktor, 1985). However, it does seem necessary that immunoglobulins which are expected to neutralize the virus before the onset of an active immune response should be as specific as possible for local virus strains. If necessary, the composition of a mixture of immunoglobulins could be adjusted to the antigenic characteristics of rabies viruses according to their geographical or species origins. Mixtures of MAbs should therefore be composed of antibodies having either strong or complementary cross-specificity. Such antibodies can be obtained from lymphocytes of donors immunized with the common vaccine rabies virus strain. The presence of Mokola-specific MAbs was described in the serum of donors immunized with vaccine strains, once they had developed a high anti-rabies humoral response (Wiktor et al., 1984; Lafon et al., 1986; Celis et al., 1988).

Within the pool of circulating lymphocytes of the vaccinated donor, the fusion process led to the identification of at least one lymphocyte with rabies virus internal protein specificity. This isolation is consistent with the presence, in donor serum, of antibodies with nucleocapsid specificity, as detected in the IEA using CsCl-purified nucleocapsid (Table 1). The presence of anti-nucleocapsid antibodies in donor serum is not an individual response, since the HRIG preparation obtained from blood donations on a national scale also contains nucleocapsid antibodies (Table 1). IEAs performed on several rabies vaccines with N- and non-structural protein-specific murine MAbs demonstrated that rabies vaccine prepared in cell culture did not contain detectable amounts of free nucleocapsid (M. Lafon, unpublished). This indicates that vaccine internal proteins as well as surface antigen are presented to the immune system. The role of anti-nucleocapsid antibodies in protection has not yet been demonstrated. However, recent studies suggested that MAbs directed to the N protein could enhance the protective effect of N protein fragments in a pre-exposure immunization murine model (Dietzschold et al., 1990). In the light of these results, it may be necessary to revise the nature of the antibody mixture to be used in place of immune gammaglobulins for rabies serotherapy.

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