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Evidence from the Anti-Idiotypic Network that the Acetylcholine Receptor Is a Rabies Virus Receptor

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We have developed idiotype–anti-idiotype monoclonal antibodies that provide evidence for rabies virus binding to the acetylcholine receptor (AChR). Hybridoma cell lines 7.12 and 7.25 resulted after fusion of NS-1 myeloma cells with spleen cells from a BALB/c mouse immunized with rabies virus strain CVS. Antibody 7.12 reacted with viral glycoprotein and neutralized virus infectivity in vivo. It also neutralized infectivity in vitro when PC12 cells, which express neuronal AChR, but not CER cells or neuroblastoma cells (clone N18), which have no AChR, were used. Antibody 7.25 reacted with nucleocapsid protein. Anti-idiotypic monoclonal antibody B9 was produced from fusion of NS-1 cells with spleen cells from a mouse immunized with 7.12 Fab. In an enzyme-linked immunosorbent assay and immunoprecipitation, B9 reacted with 7.12, polyclonal rabies virus immune dog serum, and purified AChR. The binding of B9 to 7.12 and immune dog serum was inhibited by AChR. B9 also inhibited the binding of 7.12 to rabies virus both in vitro and in vivo. Indirect immunofluorescence revealed that B9 reacted at neuromuscular junctions of mouse tissue. B9 also reacted in indirect immunofluorescence with distinct neurons in mouse and monkey brain tissue as well as with PC12 cells. B9 staining of neuronal elements in brain tissue of rabies virus-infected mice was greatly reduced. Rabies virus inhibited the binding of B9 to PC12 cells. Mice immunized with B9 developed low-titer rabies virus-neutralizing antibody. These mice were protected from lethal intramuscular rabies virus challenge. In contrast, anti-idiotypic antibody raised against nucleocapsid antibody 7.25 did not react with AChR.

Areas of controversy exist regarding the pathogenesis of rabies virus in the early stages of infection (53). There is a general consensus that the virus enters peripheral nerves at nerve endings in muscle tissue (8, 17, 43, 64). However, there is little agreement beyond that essential point. Whether virus entry involves sensory or motor nerves is subject to differing opinions (5, 8). Whether the uptake of rabies virus is mediated by the acetylcholine receptor (AChR) is also subject to dissenting views (32, 46, 54).

Rabies virus has been found localized at neuromuscular junctions (NMJ) by immunofluorescence (IFA) and electron microscopy of both intact muscle and isolated NMJ (5). Virus binding to chick myotubes was inhibited by cholinergic antagonists, which bind to the AChR (32, 60). However, the fact that rabies virus binds to other types of cultured cells that have no AChR was recognized initially (32) and led some investigators to question whether the AChR is an important receptor for rabies virus (60).

In addition, rabies virus spreads through the central nervous system transsynaptically (19, 50). It has been suggested that spread through nerves involves endings other than a nicotinic AChR (29). While a nicotinic AChR has been isolated from rat brain (65), the nature of some nerve endings through which rabies virus spreads is unknown (29). Thus, there are questions as to whether there are multiple receptors for rabies virus: one or more for cells in culture and two or more in the nervous system, whether central or peripheral (31).

Biochemical methods have been used in an attempt to resolve the question of the relationship between the AChR and rabies virus. The amino acid sequence of the rabies virus glycoprotein was shown to possess homology with those of snake venom toxins that bind to the AChR (35). Furthermore, synthetic peptides based on these regions of homology bound to the AChR (34) and behaved as AChR antagonists (47).

While these biochemical results provided additional support for the hypothesis that rabies virus binding might involve the AChR, they were not biologically conclusive. Sequence homologies can be misleading. For example, there is a low-level amino acid sequence homology between rabies virus glycoprotein and the c-*myc* protein. However, rabies virus has no transforming function and localizes to the cell surface (6). In addition, these biochemical experiments did not address the complexity of the concept of multiple virus receptors raised by other studies (29, 32, 46, 54). Thus, additional efforts were required to establish whether the sequence homologies between toxins and the rabies virus glycoprotein are biologically significant.

One approach that addresses biologic significance is based on the idiotype–anti-idiotype network (22). The rationale for using this approach is based on the theory that antigen is recognized and bound by an antibody (Ab1) because the antigen binding site (paratope) on Ab1 is, to a greater or lesser degree, complementary to an epitope on the antigen. If anti-idiotypic antibodies (Ab2) are made against Ab1, a proportion will possess paratopes that are complementary to the Ab1 paratope. These Ab2 paratopes may mimic the original antigen and display properties of the antigen.

Work previously done in our laboratory (5) suggested that an anti-idiotypic response might be used to define a rabies virus receptor at NMJ, as has been done for receptors of other viruses (2, 28, 38, 44). We found that polyclonal antibodies against rabies virus-neutralizing antibodies bound to AChR (5). In his review, Spriggs emphasized the importance of repeating these polyclonal antibody anti-idiotypic experiments with hybridoma technology (53). Subsequently,

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others referred to the concept, suggesting that the underlying hypothesis was amenable to experimental verification (35). The purpose of the work described in this report was to test the hypothesis that the AChR is a rabies virus receptor by use of the anti-idiotypic network.

In a review article, UytdeHaag et al. (62) briefly mentioned an experiment in which two different rabbit anti-idiotypic antibody preparations against a rat monoclonal anti-AChR antibody reacted with rabies virus. No additional details were given. In the present study, we used a rat monoclonal anti-AChR antibody described by Lindstrom (36) in an effort to make anti-idiotypic antibody reactive with rabies virus. At the same time, we sought to isolate murine rabies virus anti-idiotypic monoclonal antibody reactive with the AChR. Thus, part of the project involved making and testing antibodies to rabies virus proteins. Our rationale was that a monoclonal antibody specific for an AChR binding rabies virus epitope would neutralize rabies virus in vivo but not in vitro, since rabies virus-permissive CER and neuroblastoma (clone N18) cells have no known AChR.

In this report, two aspects of our results will be presented: (i) the development and serology of monoclonal anti-AChR (anti-idiotypic) antibody derived by immunization with a monoclonal antibody specific for rabies virus glycoprotein and (ii) the morphologic appearance of IFA staining in the peripheral and central nervous systems by antireceptor antibody.

MATERIALS AND METHODS

Cell cultures, virus strains, polyclonal antibodies, and laboratory animals. Rat neuron-like PC12 cells expressing a functional neural nicotinic AChR (66) were received from Theresa Perney, Department of Pharmacology, Yale University School of Medicine. Clone T of PC12 cells was derived in our laboratory by fluorescence-activated cell sorting with anti-AChR antibody produced during this study. The passage histories of CER cells and rabies virus strains 1820B and CVS were those described by Watson et al. (64). Particle counts for rabies, eastern equine encephalitis, and Sindbis (EgAr339) viruses were obtained by methods reported before (51). Congo-Crimean hemorrhagic fever (CCHF) virus strain 10200 (59), a nairovirus, was used as a control to monitor specificity. Affinity-purified virus was prepared by use of polyclonal antibody coupled to cyanogen bromide-activated Sepharose 4B as previously described (5). Neutralization tests in vitro with fluorescent-focus units and CER cells were done as described elsewhere (59). Neutralization tests in vitro with PC12 cells were done as described for CER cells but were graded qualitatively as the presence or absence of IFA because a suitable nutrient overlay could not be developed. Neutralization tests in vivo were done by incubation of virus-serum mixtures at 37°C for 1 h before inoculation of the mixtures into the hindlimb of adult mice or into the cranium of infant mice. The test dose of virus was between 50 and 100 intramuscular (i.m.) 50% lethal doses (LD_{50}s) of rabies virus strain 1820B. Crl:CF1 BR mice (Charles River Breeding Laboratories, Kingston, N.Y.) were used at between 4 and 10 weeks of age. BALB/c mice were purchased from either Jackson Laboratories (Bar Harbor, Maine) or Charles River Breeding Laboratories. Necropsy procedures, methods for IFA staining of cells and tissue sections, and protocols for neutralization tests in vivo in infant mice were described by Tignor et al. and by Watson et al. (59, 64). In comparative IFA tests, each antibody was used at a dilution fourfold lower than the homologous endpoint.

Included in this study were pre- and post-convalescent-phase sera from an earlier study in which two dogs, dogs 3 and 4, had been inoculated i.m. with Lagos bat virus, which is related to rabies virus (58). Serum from dog 3 on day 42 contained in vivo rabies virus-neutralizing antibody. Serum from dog 4 did not contain significant rabies virus-neutralizing antibody (log neutralizing antibody titer, <1.7, or failure to neutralize 50 LD_{50}s of rabies virus). These antisera had been stored at −20°C for 18 years, so tests were done to confirm the presence of rabies virus antibody in the day-42 serum from dog 3 and the absence of significant antibody in the serum from dog 4.

Affinity purification of *Torpedo californica* AChR. Affinity purification was carried out by the original protocol of Froehner and Rafto (13) as modified by Mosckovitz and Gershoni (42). Frozen *T. californica* (100 g; Pacific Biomarine, Venice, Calif.) was cut in very thin slices, to which was added 100 ml of buffer A [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.5 mM NaN_3, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)]. The mixture was homogenized for 1 min in a Sorvall mixer. The homogenate was filtered through four layers of cheesecloth and subsequently centrifuged at 15,000 rpm for 45 min in a Sorvall SS-34 rotor. The supernatant fluid was discarded, and the pellet was extracted with buffer B (buffer A plus 1% [wt/vol] Triton X-100; 60 ml of buffer per 100 g of tissue) for 2 h at 4°C. The supernatant fluid was collected and incubated overnight at 4°C with 5 to 6 g of *Naja naja siemensis* alpha-toxin in an affinity column (Sigma, St. Louis, Mo.). The column was washed sequentially with 150 ml each of buffer B, buffer B with 1 M NaCl, and buffer B with 0.1% (wt/vol) Triton X-100. Bound AChR was eluted by rotating the beads for 2 to 3 h at 4°C with 5 ml of buffer A containing 0.1% (wt/vol) Triton X-100 and supplemented with 1 M carbamylcholine chloride. The eluate was dialyzed against buffer A containing 0.1% Triton X-100. After the addition of PMSF (1 mM), the purified receptor was divided into aliquots and stored at −20°C.

Enzyme-linked immunosorbent assay (ELISA) conjugates. Peroxidase-conjugated goat anti-mouse antibodies were used for the following purposes in our experiments: (i) anti-immunoglobulin (Ig) Fc for the detection of all Ig isotypes (Southern Biotechnology Associates, Inc., Birmingham, Ala.); (ii) anti-immunoglobulin M (IgM) Fc (μ chain specific) for the exclusive detection of IgM (Boehringer Mannheim Biochemicals, Indianapolis, Ind.); (iii) anti-IgG Fc (γ chain specific) for the exclusive detection of IgG (Organon Teknika Corp., West Chester, Pa.); and (iv) anti-IgG (γ and light chain specific) for the detection of IgG Fab (TAGO, Inc., Burlingame, Calif.).

Preparation of antibodies and antibody fragments for immunization and the ELISA. Antibodies, isolated by elution from protein A, were tested serologically for reactivity by procedures described previously (5). Mouse Fab fragments from Igs were made by standard methods (15). Fragments were tested for serologic activity and protein concentration. The purity of each Fab fragment was evaluated by polyacrylamide gel electrophoresis (PAGE) and ELISA.

The ELISA for evaluating the purity of the Fab fragments was done by coating either Immulon-2 (Dynatech) polystyrene or Falcon 3911 flexible assay (Becton Dickinson, Oxnard, Calif.) plates with dilutions of Fab fragments in binding buffer (0.015 M sodium carbonate, 0.035 M sodium bicar-

bonate, 0.003 M sodium azide [pH 7.6]) overnight at 4°C. Replicate wells were incubated with either goat anti-mouse IgG (γ and light chain specific) or goat anti-mouse IgG Fc (γ chain specific), and detection was done with substrate [2-2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS); Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.]. Optical densities (ODs) were read at a wavelength of 415 nm in a Titertek Multiskan (Flow Laboratories, McLean, Va.) photometer.

Typically, test and control Fab fragments were standardized to equal values of between 10 and 20 μg/ml and used at dilutions of 1:30 to 1:100. ELISA plates were coated with 0.1 ml of diluted Fab fragments overnight at 4°C. ELISA reactions against Fab fragments were positive with anti-mouse γ- and light-chain conjugate and were negative with anti-mouse γ-chain Fc conjugate (both at a 1:3,000 dilution).

For immunization, Fab (1 μg) was injected intrasplenically (52). In some experiments, Fab (2 mg/ml) was conjugated to keyhole limpet hemocyanin (10 mg/ml) for use as an immunogen (67). A rat monoclonal antibody (Mab35) described by Lindstrom was obtained from the American Type Culture Collection (Rockville, Md.) (36). This antibody marks the major immunologic region of the alpha subunit of the AChR.

Fusions after immunization with rabies virus. Suckling mouse vaccines are still widely used for human (and dog) vaccination (55). Therefore, we initially chose infected infant mouse brain tissue as the immunogen. The technique has been used successfully elsewhere to generate virus-specific monoclonal antibodies when a virus can be propagated in infant mouse brain tissue (18). BALB/c mice were immunized by the following protocol. A 10% suspension of rabies virus (CVS strain)-infected mouse brain tissue from BALB/c mice was mixed with an equal volume of formalin (0.05%) and allowed to stand for 1 week. Each mouse was inoculated intraperitoneally (i.p.) with 0.2 ml and then with a second injection 1 week later. Seven days after the second injection, 0.2 ml of a 10% suspension of non-formalin-treated rabies virus-infected brain tissue was given i.p., and two more injections were given at weekly intervals. Three days after the last immunization, spleen cells were fused with NS-1 myeloma cells by use of polyethylene glycol 1450 (Eastman Kodak Co., Rochester, N.Y.) (26), and then hybrid selection, screening, and expansion were carried out as modified by Walker (63) and Hadas and Theilen (16). Supernatant fluids from hybridoma cultures were screened first by ELISA against rabies virus-infected, formalin-fixed CER cells or CCHF virus-infected control cells and second by neutralization both in vivo and in vitro.

For the solid-phase ELISA, infected and control cells were dispensed into 96-well polystyrene plates when greater than 75% of the cells were IFA positive. After overnight incubation, the cells were covered with formalin (10%) and allowed to stand for 4 days. The plates were washed and placed in individually sealed airtight bags for storage at −20°C until needed for the ELISA. At the time of testing, the plates were blocked with 3% gelatin and washed before supernatant fluids from hybridoma cultures were added. Following incubation (3 h, 4°C), the plates were washed and incubated (1 h, 37°C) with peroxidase-conjugated anti-mouse Ig. Reactions were developed by adding substrate after three washes with phosphate-buffered saline (PBS). Two hybridomas, 7.12, reactive with rabies virus glycoprotein, and 7.25, reactive with rabies virus nucleocapsid protein, resulted from these fusions.

To ensure that we did not have a spurious reaction resulting from the use of virus derived from infected mouse brain tissue, we performed an additional fusion after immunization of mice as described above with CER cell-propagated virus partially purified from sucrose gradients. Several hybridomas, screened as described above and with neutralizing properties similar to those of 7.12, were obtained.

Fusions for control (CCHF) virus reagents. Hybridomas producing monoclonal CCHF virus-neutralizing antibody were prepared 3 days after intrasplenic inoculation of virus by the otherwise standard methods described above. The monoclonal antibodies were isotyped with a commercial kit (Zymed Laboratories, Inc., San Francisco, Calif.). Screening was done by IFA and immunoprecipitation. Anti-idiotypic antibodies to selected CCHF virus-neutralizing monoclonal antibodies were prepared as control reagents. Control antibodies were matched to test antibodies by isotype.

Fusions after immunization with rabies virus antibody. Rabies virus antibody (7.12 Fab or 7.25) was inoculated into BALB/c mice intrasplenically, and spleen cells were fused with NS-1 myeloma cells 3 days later. Supernatant fluids from hybridoma cells were screened by an ELISA with immunizing and control Fab antibodies and affinity-purified AChR as coating reagents. Hybridoma cells secreting antibodies of appropriate specificity were cloned. Ascites were produced by priming of BALB/c mice with pristane (0.5 ml i.p.) 10 to 13 days before injection of hybridoma cells (10^7 cells per mouse).

Fusions after immunization with anti-AChR (Mab35) antibody. Hybridoma cells secreting Mab35 antibody were obtained from the American Type Culture Collection and grown in culture. Fab was prepared from ascites of X-irradiated mice treated with antilymphocyte serum before inoculation with Mab35-secreting hybridoma cells. Fusions with NS-1 myeloma cells were done 3 days after immunization with Mab35 Fab coupled to keyhole limpet hemocyanin and 3 days after intrasplenic immunization with uncoupled Fab. Supernatant fluids from hybrid cells were screened on rabies virus-infected (and control) ELISA plates and on plates coated with Mab35 (and control) Fab. Anti-rat conjugates were purchased commercially (Southern Biotechnology Associates).

ELISA for detection of anti-idiotypic antibody. An ELISA was chosen because it previously had been shown in comparative assays to be useful for the detection of anti-idiotypic antibodies to the anti-AChR immune response (45). Two different ELISA systems were used to screen for anti-idiotypic antibody. In the first ELISA system, immunizing (7.12) and control (CCHF virus) Fab antibodies were used to coat polystyrene plates in dilutions containing similar amounts of protein, none of which could be detected by anti-Fc conjugate. Plates were blocked with 3% gelatin, and then supernatant fluids from hybridoma cultures were added. After incubation and three washes with PBS–0.05% Tween 20, goat anti-mouse Ig Fc conjugate (1:3,000) was added. Positive reactions (i.e., ODs higher than the OD of the CCHF virus control antibody + 3 standard errors) were found when substrate was added after five washes.

In the second ELISA system, affinity-purified AChR (approximately 0.3 to 0.5 mg/ml) was diluted 200- or 300-fold and dispensed into microtiter plates for overnight incubation at 4°C. After being coated, plates were washed three times with assay buffer (2% fetal calf serum in PBS); the third wash was used for blocking (1 h, 4°C). Plates were incubated with test and control primary antibodies and then with anti-mouse Ig Fc conjugate and substrate. The negative control was supernatant fluid containing CCHF virus monoclonal antibody. The positive control was Mab35 hybridoma culture

fluid. ELISA results were confirmed by IFA staining of frozen sections of normal mouse muscle tissue.

Hybridoma culture fluids that reacted positively in both systems were tested on ELISA plates that had been coated with 16-day-old chicken embryo fibroblast membrane vesicles, as a negative control, prepared as previously described (33) and adjusted to a protein concentration similar to that of the AChR.

Competitive ELISA. Anti-idiotypic antibody from ascites was used in a competitive ELISA to determine whether AChR binding could be inhibited by pretreatment of antibody with purified AChR or rabies virus antibody. Serial dilutions of anti-idiotypic antibody were mixed with a single dilution of AChR (either 0.03 or 0.15 mg), control membranes (chicken embryo membrane vesicles; 0.15 mg), or control CCHF virus antibody (1:50). After a preliminary overnight incubation at 4°C, pretreated antibody was tested for reactivity with AChR in the ELISA. Inhibition tests with antibody 7.12 were done in the same manner as that described above for anti-idiotypic antibody. The positive control for AChR competition was the Mab35-AChR reaction.

Immunoprecipitations and Western blotting (immunoblotting). Immunoprecipitations with monoclonal and polyclonal antiviral antibodies were done by a modification of the method of Mason (39). Pelleted (100,000 × *g* for 2 h) radiolabeled (^{35}S) rabies or CCHF virus from CER cell culture supernatant fluids was solubilized in RIPB buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% Na deoxycholate, 1% Triton X-100) containing protease inhibitors (PMSF, 1 mM; aprotinin, 10 μg/ml; leupeptin, 2 μg/ml; benzamidine, 2 μg/ml; and *Nα-p*-tosyl-L-lysine chloromethyl ketone [TLCK], 10 μg/ml). Solubilized viral proteins (50 μl) were incubated overnight at 4°C with 1 to 2 μl of clarified monoclonal or polyclonal ascites or with antibody 7.12 conjugated to Sepharose beads (Sigma). Material immunoprecipitated by antibody alone was sedimented with protein A-Sepharose beads that had been washed three times with RIPA buffer (RIPB buffer with 0.1% sodium dodecyl sulfate). After being extensively washed with RIPB buffer, proteins were released from the beads by incubation in sample buffer for 5 min at 100°C. The proteins were separated on a 10% acrylamide gel (30), which was processed for autoradiography with Kodak film. Western blotting was done as previously described (5). Radiolabeled (^{125}I) α-bungarotoxin for identification of the alpha subunit of the AChR was purchased from New England Nuclear (Boston, Mass.).

Preparation and use of affinity columns. Anti-idiotypic antibody was precipitated from serum-free hybridoma culture fluid by use of polyethylene glycol 6000 (23). Concentrated antibody was examined by PAGE and conjugated to cyanogen bromide-activated Sepharose 4B as described previously (5). Purified AChR was added to the anti-idiotypic affinity column. Binding proteins were eluted with pH 3.0 buffer (Bio-Rad Laboratories, Richmond, Calif.). After elution, proteins from the affinity column were concentrated by filtration through a low-molecular-mass protein binding membrane with a 30,000-Da cutoff (Millipore, Bedford, Mass.) and characterized by PAGE. Affinity columns for partial purification of virus were prepared and used as described previously (5).

Production of antibody to anti-idiotypic polyclonal and monoclonal antibodies. BALB/c mice were hyperimmunized by three intravenous injections of anti-idiotypic B9 ascites. Mice were bled at intervals and monitored for the production of rabies virus antibody by an ELISA against rabies virus-infected and control CER cells and by IFA on coded rabies virus-infected and control cells. After IFA-positive serum was confirmed by in vivo neutralization tests, a hyperimmune mouse was reinjected intrasplenically and spleen cells were fused with myeloma cells as described above.

RESULTS

Fusions after immunization with rabies virus and selection of rabies virus antibodies to be used as immunogens. Mice were immunized with rabies virus, and spleen cells were fused with myeloma cells; fluids from wells producing antibodies reactive with rabies virus were tested for in vivo and in vitro neutralization of rabies virus. Two wells (7CC8 and 7CE9) produced antibody that, at least partially, neutralized rabies virus in vivo but not in vitro when cells that do not express the AChR, such as CER and neuroblastoma (N18) cells, were used. Each neutralization test included both positive and negative controls.

Neither supernatant fluids nor ascites neutralized rabies virus in vitro when either CER or N18 cells were used, despite repeated attempts including the addition of fresh complement. In contrast, neutralization in vitro with PC12 cells, which express a neural AChR (66), was easily demonstrated in qualitative IFA tests. In qualitative virus dilution-constant serum neutralization tests, antibody 7.12 in ascites diluted 1:4 neutralized $10^{5.4}$ PFU of rabies virus (CVS strain). Similarly, neutralization in vivo with a clone derived from 7CC8 (7.12; IgG2A isotype) was consistent and of a high titer. Antibody in ascites neutralized rabies virus infectivity (adult mouse, i.m. administration of 50 to 100 LD_{50}s), with titers ranging from 1:512 to 1:1,024. Similar titers were obtained with PC12 cells in vitro. When given passively (0.5 ml i.p.) at the time of virus inoculation, antibody prevented mortality, even when diluted 1:16. Polyclonal rabies virus antibody (Fig. 1A, lane 1) immunoprecipitated multiple viral proteins. On the other hand, antibody 7.12 affinity columns immunoprecipitated only the two characteristic forms of the rabies virus glycoprotein (Fig. 1B, lane 1), as seen elsewhere with other rabies virus anti-glycoprotein monoclonal antibodies (3). These results were duplicated after immunoprecipitation without the antibody 7.12 affinity columns by the method of Mason (39). The starting virus, partially purified from sucrose gradients, is shown in Fig. 1C, lane 1; the product of the immunoprecipitation is shown in Fig. 1C, lane 2, in which, once again, only the viral glycoproteins were immunoprecipitated. Antibody 7.12-producing hybridoma cells were cloned three times by limiting dilution. Secreted antibody consisted of only one light chain, as determined by PAGE, and immunoprecipitated only the viral glycoproteins. Thus, 7.12 was a monoclonal antibody, as determined by conventional standards. Neither supernatant fluids nor ascites from antibody 7.12-producing cells reacted with rabies virus proteins in Western blotting, suggesting that the antibody recognizes a conformationally dependent glycoprotein epitope.

In contrast to the results obtained with 7CC8, a clone derived from 7CE9 (7.25; IgM isotype) produced antibody that did not neutralize rabies virus. Some mice infected with mixtures of virus and antibody 7.25 occasionally had a prolonged survival time, but neutralization could not be demonstrated (data not shown). These negative neutralization test results were associated with immunoprecipitation of a protein with the approximate mobility of the rabies virus nucleocapsid (Fig. 1A, lane 3). Antibody 7.25 did not react with rabies virus proteins in Western blotting.

Immunization with rabies virus antibodies. After fusion of

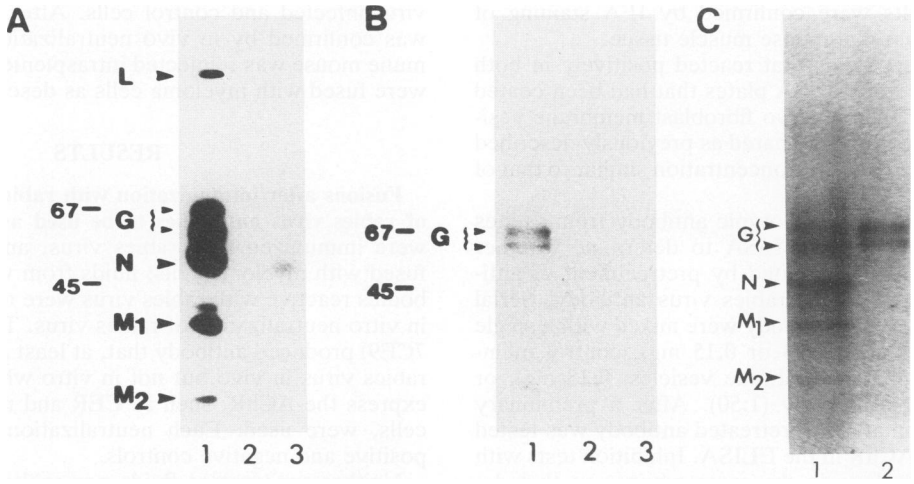

FIG. 1. Autoradiogram of ^{35}S-labeled rabies virus (CVS strain) proteins gel electrophoresed after immunoprecipitation by polyclonal and monoclonal antibodies. (A) Lanes: 1, immunoprecipitation with polyclonal rabies virus (CVS strain) antibody showing multiple viral proteins; 2, immunoprecipitation with polyclonal rabies virus antibody and control virus (CCHF virus); 3, immunoprecipitation with ascites from rabies virus hybridoma subclone 7.25 showing a reaction with nucleocapsid antigen. (B) Lanes: 1, immunoprecipitation with antibody 7.12-Sepharose beads showing only the ^{35}S-labeled rabies virus glycoproteins; 2, immunoprecipitation with control virus (CCHF virus) antibody-Sepharose beads; 3, control ^{35}S-labeled cells reacted with 7.12-Sepharose beads. (C) Lanes: 1, partially purified ^{35}S-labeled rabies virus (CVS strain) used in immunoprecipitations with antibody 7.12; 2, immunoprecipitation of viral proteins by antibody 7.12 with ascites and protein A-Sepharose beads.

spleen cells from mice intrasplenically immunized with 7.12 Fab, supernatant fluids from 96-well culture plates were screened for anti-AChR antibodies by an ELISA. Three wells were found positive, with ODs of 0.45 to 0.51. Mab35 supernatant fluids (positive control) had ODs of 0.54 to 0.59. Negative control ODs ranged from 0 to 0.03. Anti-idiotypic antibodies from these cultures reacted with 7.12 Fab and purified AChR in the ELISA and stained NMJ in IFA tests. One culture, designated B9, was cloned twice by limiting dilution and further characterized.

Other mice were immunized intrasplenically with 7.25 (IgM)-keyhole limpet hemocyanin prior to fusion. Anti-AChR antibody was not detected in the ELISA; negative results were confirmed by the absence of staining of muscle tissue in IFA tests. Mab35 was the positive control for both the ELISA and IFA tests.

Reactions of anti-idiotypic B9 antibody with monoclonal and polyclonal rabies virus antibodies. Supernatant fluids from replicate B9 cultures reacted in three different ELISAs with 7.12 Fab (1:30 dilution), with a mean titer of 1:512. B9 ascites reacted with 7.12 Fab (1:30 dilution), with a mean titer of 1:4,096. B9 ascites also reacted with dog serum (1:50 dilution) containing polyclonal rabies virus-neutralizing antibody. B9 titers in two different ELISAs with dog 3 (day-42) immune serum as the coating reagent were >1:6,400 whereas B9 antibody did not react (titer, <1:400) with nonneutralizing dog serum. The negative control sera included prebleed sera from dogs 3 and 4 and day-42 serum from dog 4, which did not make significant ($\log_{10}$ neutralizing index, <1.7) rabies virus-neutralizing antibody after infection with Lagos bat virus.

Reactions of anti-idiotypic B9 antibody with the AChR. One concern was that B9 antibody was reacting with a contaminant of the AChR. Seven different isolations of fish AChR were done with toxin affinity columns. All preparations contained the four AChR subunits; in addition, some preparations contained the 43-kDa polypeptide frequently associated with the four subunits of the AChR (12) (Fig. 2A, lane 2). B9 antibody reacted equally in ELISAs with the different AChR preparations, regardless of how much 43-kDa protein was present. The specificity of the AChR reaction was further established when B9 antibody did not react specifically with chicken embryo membranes (0.25 mg/ml diluted 1:300 in coating buffer).

After solubilized AChR was added to and eluted from a B9 antibody affinity column, the eluted proteins had the PAGE profile seen in Fig. 2B, lane 2. The four AChR subunits were

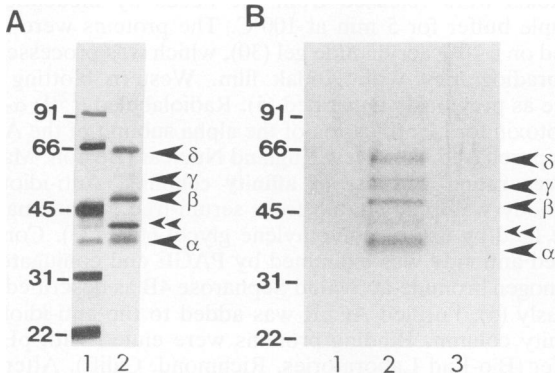

FIG. 2. Silver staining of AChR subunits electrophoresed on polyacrylamide gels. (A) Lanes: 1, molecular weight standards; 2, solubilized AChR isolated after elution from an *N. naja siemensis* alpha-toxin affinity column (the four subunits of the AChR can be seen; the gamma band is faint, whereas the contaminating 43-kDa band is more intense). (B) Lanes: 1, solubilized AChR eluted from a 7.12-Sepharose bead affinity column (detectable proteins were not eluted from these beads); 2, solubilized AChR eluted from a B9-Sepharose bead affinity column (the 43-kDa protein was scarcely present relative to the starting material; the gamma band had a slightly faster moving component); 3, solubilized 16-day-old chicken embryo fibroblast membranes reacted with B9-Sepharose beads (little or no material was eluted from these beads).

clearly visible, with the alpha subunit, as expected, being predominant. There was only trace evidence of the 43-kDa polypeptide relative to the starting material. The gamma subunit appeared as a doublet, as has been reported elsewhere for very pure AChR (37). The alpha subunit was identified by Western blotting with ^{125}I-labeled α-bungarotoxin and autoradiography (data not shown). B9 antibody did not react with the AChR in Western blotting.

Cumulatively, these data showed that B9 antibody did not react with a contaminant of the AChR. As is true of all monoclonal antibodies to the AChR, the subunit specificity could not be determined without competitive inhibition studies with a battery of AChR monoclonal antibodies (36). For this study, it was important to show that B9 antibody reacted with molecular structures on cells and tissues associated with rabies virus pathogenesis. Widespread rabies virus infection is limited almost exclusively to muscle cells and neuronal cells.

IFA staining of the AChR by anti-idiotypic B9 antibody. One of the standard ways of characterizing an AChR monoclonal antibody has been by its morphologic reaction with *Torpedo* muscle tissue. After cutting, mounting, and staining sections (4 to 6 μm) of fish muscle tissue with anti-idiotypic B9 antibody (either culture fluids or ascites), we observed membrane fluorescence similar to but distinguishable from that produced by Mab35 treatment. In both cases, fluorescence was limited to one surface of the muscle fiber. However, the fluorescence produced by B9 was more narrowly circumscribed than that produced by Mab35. In morphologic localization, the fluorescence produced by B9 antibody was essentially identical to that described in an earlier publication in which AChR antibodies were used (12).

IFA examination of alternate serial sections of mouse muscle tissue produced outcomes matching those described above for B9 (Fig. 3A) and Mab35 (Fig. 3B) antibodies. Morphologically similar conformations were found at virtually identical NMJ on adjacent sections, with the fluorescence again being more narrowly delimited on the section stained with B9 antibody.

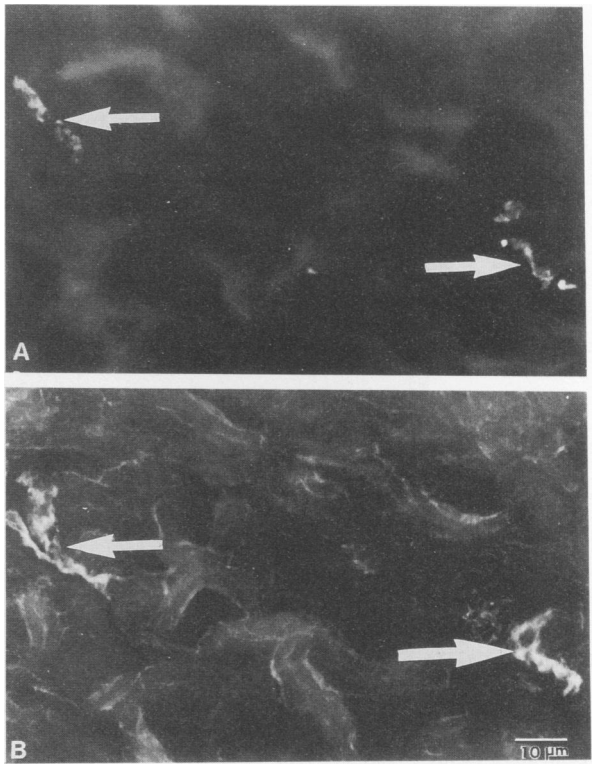

FIG. 3. IFA staining of mouse muscle tissue. Four-micrometer, acetone-fixed cryostat sections were stained with either B9 or Mab35 anti-AChR antibody at a concentration fourfold higher than the endpoint dilution for each. Bound antibody was detected with an anti-mouse fluorescein isothiocyanate conjugate. (A) Muscle tissue cut in cross section and stained with B9 antibody. Two configurations (arrows) typical in their morphology of NMJ are shown. (B) Adjacent section stained with Mab35 antibody. Two NMJ (arrows) are extensively stained.

B9 IFA in brain tissue. Although there are some exceptions, most anti-AChR monoclonal antibodies do not react with brain tissue; few react with both muscle and brain tissue (36). In IFA tests, B9 antibody labeled central nervous system cells similar in distribution to those frequently stained by virus antibody during rabies virus infection. Sagittal sections of mouse brain tissue have been presented, so that comparisons can be made with recent reports on rabies virus pathogenesis in mice (20, 21). For example, pyramidal cells in the hippocampus (Fig. 4a) and Purkinje cells in the cerebellum (Fig. 4b) were intensely reactive. In the hippocampus, the IFA was largely limited to discrete areas on the surface of the pyramidal cells; only an occasional cell in the molecular layer was stained, usually with punctate dots. In their morphology, these IFA-positive elements were suggestive of synaptic boutons (Fig. 4c). This staining pattern was typical of the reactions on neurons in the olfactory area, the thalamus, and the cerebral cortex. A different type of staining was seen in the cerebellum, in which the Purkinje cell bodies were labeled (Fig. 4b), sometimes with perinuclear fluorescence. There were numerous punctate dots of fluorescence on granule cells in the cerebellum. In other areas of both the brain and the spinal cord, a third type of staining was seen. Neuronal projections, probably representing nonmyelinated fibers, were stained, sometimes in configurations that resembled synaptic contacts (Fig. 4d). These general IFA results were consistent in both randomly bred and inbred mice. Variations among individual mice examined were not obvious. B9 antibody stained monkey brain tissue as follows. In monkeys, B9 stained mostly pyramidal cells, and the staining was similar in the frontal, motor, and somatosensory cortexes. The negative control IFA antigen was mouse liver tissue. B9 antibody did not react with paraformaldehyde- or formalin-fixed tissue (data not shown).

In striking contrast to the reaction with normal pyramidal cells in the murine hippocampus, B9 staining of rabies virus-infected hippocampal cells was greatly diminished, even with undiluted antibody. Serial sections from the brains of moribund mice, infected i.m. with strain 1820B, were treated with polyclonal antibody to strain 1820B (Fig. 5a) or with undiluted B9 ascites (Fig. 5b). In six moribund mice examined on two separate occasions, a reduction in B9 reactivity was more clearly defined in the hippocampus than in the cerebellum, in which staining appeared fragmented and disorganized compared with that in normal mice. In earlier stages of the disease course, a reduction in pyramidal cell staining by B9 antibody was less obvious. During these experiments, normal mouse brain tissue was treated with B9 antibody to ensure that the IFA system was working properly. The absence of an intense B9 antibody reaction on infected tissue from moribund mice suggested either that the virus infection destroyed the receptor or that there was

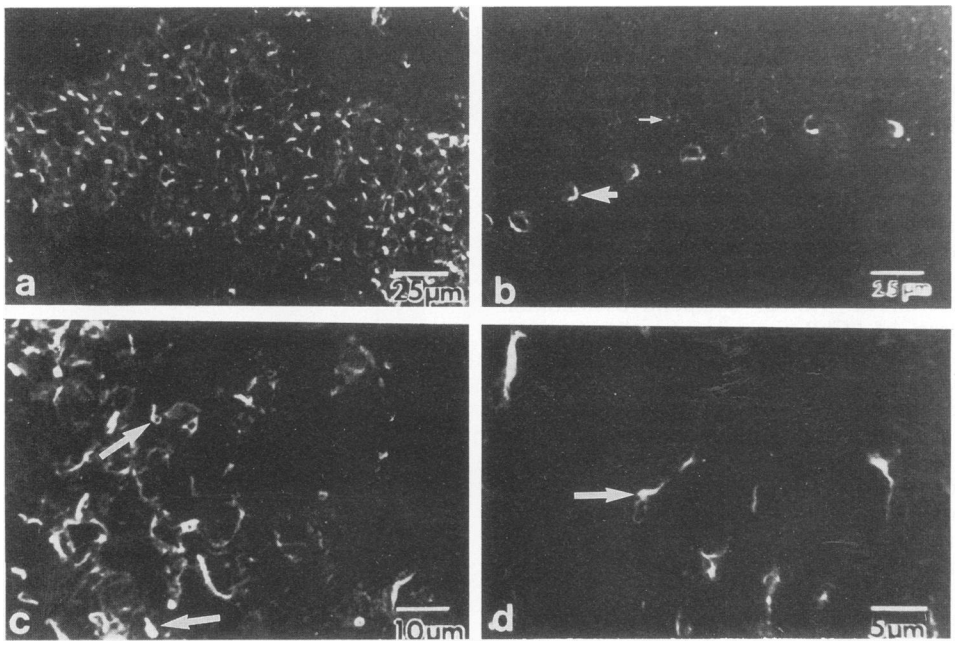

FIG. 4. B9 antibody IFA in the central nervous system of mice. Four-micrometer cryostat sections of acetone-fixed mouse brain tissue were stained with B9 antibody, which was detected with an anti-mouse fluorescein isothiocyanate conjugate. Bars, a and b, 25 μm; c and d, 10 and 5 μm, respectively. (a) B9 on pyramidal cells of the hippocampus. Staining is limited to a portion of the cell membrane. (b) B9 labeling (large arrow) of Purkinje cells in the cerebellum. Small punctate dots (small arrow) on individual granule cells can also be seen. (c) Higher magnification of the pyramidal cells of the hippocampus showing intense staining of portions of some membrane and synaptic bouton-like structures (arrows) on the surfaces of other cells. (d) Staining of neuronal projections in the corpus striatum. Some reactive areas were suggestive of nonmyelinated fibers making synaptic connections (arrow).

competition between rabies virus antigen and B9 antibody for binding sites. One important way to distinguish between these two possibilities was to do reciprocal inhibition tests.

Competitive inhibition (in vitro). Reciprocal competitive inhibition tests were done to show that B9 was an anti-idiotypic antibody to antibody 7.12. Preincubating B9 with antibody 7.12 significantly reduced the binding of B9 to AChR, as measured by ELISA OD readings (Fig. 6A). The B9-7.12 ODs were lower than those of the control (B9-CCHF virus) over the entire range tested ($P = 0.02$; paired t test). B9-AChR preincubation had a similar effect ($P = 0.04$; paired t test; Fig. 6A). In this experiment, the absorbing antibody was in the form of ascites for both 7.12 and CCHF virus. The total protein concentration of a 1:50 dilution of CCHF virus was slightly lower than that of B9, being 6 and 8 mg/ml, respectively. This difference was not considered significant in the outcome of the experiment because similar results were obtained when the experiment was repeated with CCHF virus and B9 culture supernatant fluids, with total protein concentrations of 5 and 4 mg/ml, respectively.

To address the question of specificity on a comparative basis with a known standard, we did competition experiments with both Mab35 and B9 antibodies. Attachment of Mab35 antibody to AChR was reduced ($P < 0.001$) by pretreatment with solubilized AChR compared with chicken embryo membrane vesicles (Fig. 6B). B9-AChR specificity was shown in a separate experiment by a lack of effect after pretreatment of B9 with chicken embryo membrane vesicles, whereas pretreatment with AChR, at a similar protein concentration, significantly reduced reactivity ($P < 0.001$; paired t test; Fig. 6C). There was no difference in the B9-AChR reaction whether preincubation was done with control (CCHF virus) antibody or chicken embryo membrane vesicles (Fig. 6A and C).

The binding of antibody 7.12 to rabies virus in the CER cell ELISA was inhibited by pretreatment of the antibody with B9 at 1:3 ($P < 0.0001$) and 1:10 ($P < 0.0002$) dilutions (Fig. 6D). Even in this crude system, in which the 7.12 epitope probably was not predominant, inhibition of binding was observed. When affinity-purified virus was used as an antigen in the ELISA, 7.12 binding to rabies virus antigen was even more clearly inhibited by B9 antibody. In this ELISA, the detecting antibody was a goat anti-mouse IgG Fc (γ-chain-specific) conjugate. Twofold serial dilutions (1:40 to 1:320) of antibody 7.12 (IgG2A isotype), when mixed with control antibody (IgM isotype; 1 mg/ml), reacted with rabies virus antigen (approximately 1 μg per well), yielding the following mean ODs: 1.77, 1.61, 1.40, and 1.25. After aliquots of the same dilutions of 7.12 were mixed with B9 antibody (1 mg/ml) partially purified from hybridoma culture fluid with a Sephadex G-200 column, the ODs were 0.38, 0.23, 0.17, and 0.15. B9 and control antibodies together resulted in ODs of 0.15, 0.23, 0.17, and 0.19. The standard errors of the mean in this test did not exceed 0.04.

Competition for binding sites between B9 antibody and rabies virus. The experiments described above were necessary for defining B9 as an anti-idiotypic antibody. However, the question of competition between rabies virus and B9 antibody for binding sites remained. We have not yet purified the B9 brain receptor, so this question was indirectly approached in two different ways. First, the effect of rabies virus on B9 binding to the AChR was examined. Affinity-purified rabies virus antigen was used in an ELISA to determine whether rabies virus blocked B9 binding to the

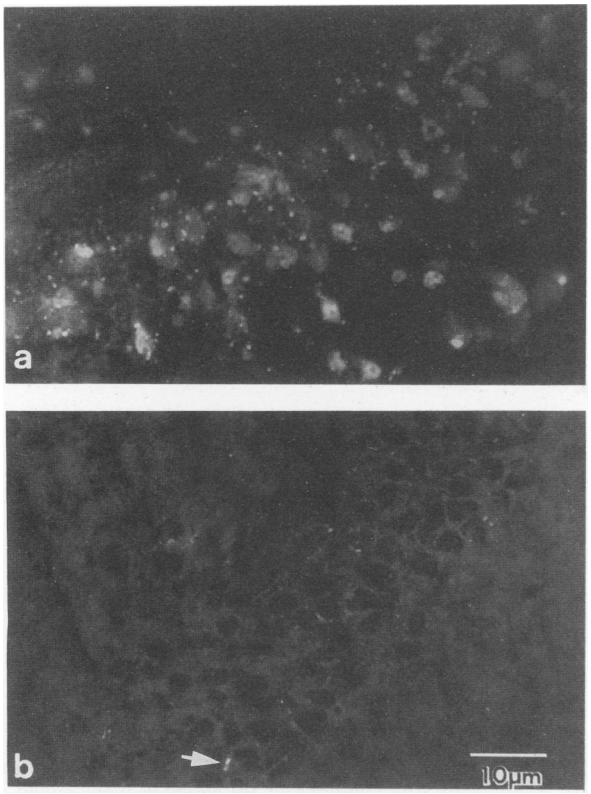

FIG. 5. Staining of serial sections of hippocampal cells from a moribund mouse infected i.m. with rabies virus strain 1820B. (a) Polyclonal rabies virus antibody reacted with antigen in pyramidal cells of the hippocampus. (b) Serial section of the same tissue showing that staining with undiluted B9 ascites was scarcely visible and much reduced (arrow) from that seen in panel a and Fig. 4c.

AChR. AChR (0.03 mg/ml) was dispensed into a 96-well plate in a 0.1-ml volume for overnight coating at 4°C. Dilutions of partially purified B9 antibody (1 mg/ml) were mixed with an equal volume of affinity-purified rabies virus antigen (approximately 5 μg/ml) or CCHF virus control antigen before incubation with AChR. In this ELISA, the detecting antibody was an anti-mouse IgM conjugate used on four replicates. At final dilutions of 1:500 and 1:1,000, the B9-control virus mixture reacted with AChR at mean ODs of 1.8 ± 0.07 and 1.5 ± 0.07, and the B9-rabies virus mixture reacted with AChR at mean ODs of 0.37 ± 0.01 and 0.24 ± 0.02, respectively. The control CCHF virus antibody-CCHF virus mixture reacted with AChR at mean ODs of 0.04 ± 0.02 and 0.01, respectively.

The previous experiments demonstrated competition between rabies virus and B9 antibody for binding to muscle-derived AChR. PC12 cells, which express the alpha subunit of the neural AChR (66), were also used to demonstrate competitive inhibition between B9 antibody and rabies virus. While B9 antibody did not stain CER or neuroblastoma cells in IFA tests, it did react with PC12 cells. The titer of B9 antibody (supernatant fluid) on acetone-fixed cells (clone T), in wells on printed slides, was ≥1:100 (Fig. 7a). After preincubation of PC12 cells (5×10^4 per well) with approximately 10^8 partially purified rabies virus particles, the titer of B9 antibody was ≤1:5 (Fig. 7b). Preincubation of PC12 cells with similar numbers of eastern equine encephalitis or Sindbis (strain EgAr 339) virus particles did not reduce the B9 antibody titer from ≥1:100 (Fig. 7c).

Competitive inhibition (in vivo). Myotube cultures, used in our earlier studies (32), and PC12 cells could not be used for in vitro competitive inhibition experiments because a suitable semisolid or solid nutrient overlay for quantitating the results does not exist, nor were we able to develop one. In addition, the interpretation of in vitro results would have been complicated by the fact that B9 binds to PC12 cells. However, the inhibition of 7.12 neutralization of rabies virus was demonstrated in vivo, but only when a relatively high concentration of B9 antibody was preincubated with 7.12 antibody. 7.12 antibody in ascites was diluted to 1:64 and mixed with an equal volume of partially purified B9 antibody (3 mg/ml), as in the previous experiment. The mixture was preincubated for 60 min at 37°C. Afterwards, it was mixed and similarly incubated with an equal volume of 1820B virus (100 i.m. adult mouse LD_{50}s). Groups of six adult mice were inoculated with 0.03 ml in the hindlimb. Antibody 7.12 mixed with control antibody (IgM isotype; 3 mg/ml) neutralized virus infectivity; six inoculated mice survived. In contrast, antibody 7.12 mixed with antibody B9 did not neutralize virus infectivity; six inoculated mice died. Lower concentrations (≤1 mg/ml) of B9 antibody (IgM isotype) did not inhibit neutralization, a result that may reflect the naturally low affinity of IgM antibody. A single i.m. injection (0.03 ml) of intact, partially purified B9 antibody had no obvious effect on survival.

Anti-B9 polyclonal and monoclonal antibodies. Syngeneic mice injected with ascites containing B9 antibody formed a rabies virus antibody (Ab3) 6 weeks after the first intravenous injection. 7.12 antibody (Ab1; Fig. 8a) stained rabies virus-infected CER cells similarly to the Ab3 antibody (Fig. 8b) but less extensively than the polyclonal antibody (Fig. 8c). Two months after the beginning of immunization, one of six immunized mice had a low titer (1:4) of in vivo neutralizing antibody. Spleen cells from that mouse were fused to myeloma cells, but without success in isolating a hybridoma clone producing Ab3. Other mice were treated by a different immunization schedule with B9 antibody (0.1 ml i.p., 1 mg/ml) partially purified on Sephadex G-200 as described above. Daily doses were given for 10 consecutive days. Seven days later, mice were bled and the serum was pooled. The in vivo neutralizing titer was 1:8. Six immunized mice resisted i.m. challenge (0.03 ml) with 1820B rabies virus (50 LD_{50}s) 2 weeks later.

Immunization with anti-AChR antibody Mab35. Despite extensive screening by both an ELISA and IFA tests, we were unable to isolate a rabies virus-specific anti-idiotypic monoclonal antibody following immunization with Mab35 antibody by two different protocols. The resulting monoclonal antibodies, which reacted with rabies virus-infected cells in the ELISA, also stained, in IFA tests, what appeared to be cytoskeletal elements of both normal and rabies virus-infected CER cells. Staining was indistinguishable from that which has been obtained with anti-idiotypic antibodies made from anti-AChR monoclonal antibodies (10).

DISCUSSION

The anti-idiotypic network has been used to show that a rabies virus epitope(s) utilizes the AChR during viral pathogenesis. We have developed an anti-AChR monoclonal antibody (B9) that is also anti-idiotypic to a monoclonal antibody (7.12) that neutralizes rabies virus in vivo and on cells that express the neuronal nicotinic AChR in vitro.

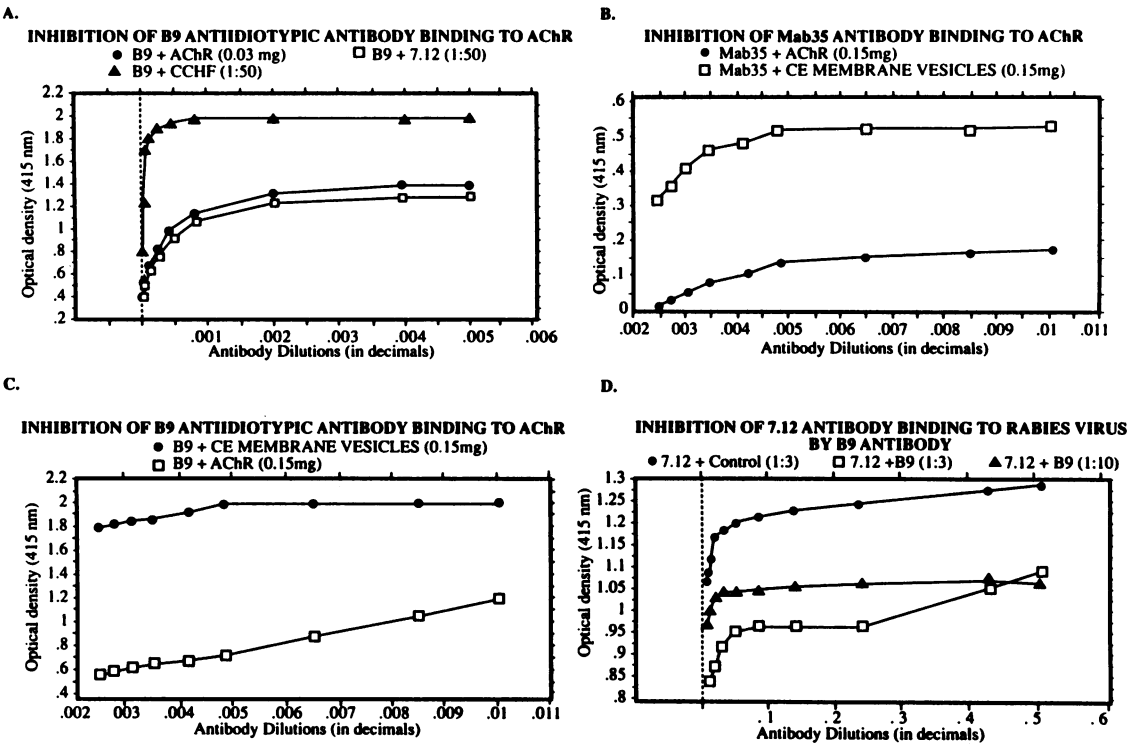

FIG. 6. Competitive inhibition with B9 antibody. The significance of differences in the binding curves was determined by a paired *t* test (one tail) that considers variations among individual samples in determining significance. (A) Dilutions of B9 antibody were mixed and incubated overnight at 4°C with AChR (0.03 mg) or one of the following antibodies: CCHF virus control (1:50 dilution of ascites; 6 mg of total protein per ml) or rabies virus 7.12 (1:50 dilution of ascites; 8 mg of total protein per ml). The pretreated B9 dilutions were tested in an ELISA on affinity-purified AChR (0.03 mg/ml). The binding of B9 to AChR was reduced after pretreatment with either AChR ($P < 0.04$) or 7.12 ($P < 0.02$), as determined by OD measurements. (B) In the control system, dilutions of Mab35 antibody were mixed and incubated with either AChR (0.15 mg/ml) or chicken embryo (CE) membrane vesicles (0.15 mg/ml) for 16 h at 4°C. Pretreated dilutions were tested in the ELISA on affinity-purified AChR (0.03 mg/ml). The binding of Mab35 to AChR was significantly reduced when Mab35 was pretreated with AChR compared with chicken embryo membrane vesicles ($P < 0.001$). (C) Dilutions of B9 antibody were mixed and incubated overnight at 4°C with either chicken embryo membrane vesicles (0.15 mg/ml) or AChR (0.15 mg/ml) before being plated on AChR (0.03 mg/ml) in the ELISA. B9 antibody binding to AChR was significantly reduced by pretreatment with AChR ($P < 0.001$). Pretreatment with control CCHF virus or control chicken embryo membrane vesicles did not yield significantly different results (compare panels A and C). (D) Dilutions of 7.12 antibody from hybridoma culture supernatant fluid were preincubated with control CCHF virus antibody (5 mg of total protein per ml) or with B9 antibody (4 mg of total protein per ml) at either a 1:3 or a 1:10 dilution. The pretreated antibody was titrated in the ELISA on formalin-treated rabies virus-infected or mock-infected CER cells. The background with mock-infected cells has been subtracted for each value shown. The binding of 7.12 to rabies virus was significantly inhibited ($P < 0.001$) after treatment with a 1:3 dilution of B9 antibody. The reduction in binding was lower after pretreatment with a 1:10 dilution of B9 antibody, but the difference was still significant ($P < 0.002$).

Antibody 7.12 does not neutralize rabies virus on cultured cells that are not known to have the AChR, at least insofar as we have searched. Guidelines, reviewed elsewhere (4), have been used to determine whether B9 is an internal image (Ab2β) of the virus antigen. B9 fullfils the following criteria, which suggest that it is an internal image. B9 reacts with the AChR, a host cell molecule for which there is significant evidence suggesting that it is a rabies virus receptor. B9 reacts not only with 7.12 but also with a heterologous polyclonal rabies virus antibody from a different species. There is reciprocal competitive inhibition, which is important in the evaluation of an anti-idiotypic antibody (1). The binding of B9 to affinity-purified AChR is inhibited by 7.12 but not by an irrelevant antibody. B9 binding to the AChR is also inhibited by rabies virus and AChR. Conversely, 7.12 binding to rabies virus is inhibited by preincubation with B9 antibody, both in vivo and in vitro. B9 antibody elicited an antibody response (Ab3) to itself in mice. The response was of a low titer; the low titer could have resulted from immunization with too little B9 or an otherwise inappropriate dose of B9 (11). Alternatively, the paratope of B9 may not have been perfectly complementary to the paratope of 7.12. However, despite the low neutralizing titers, mice treated with at least one immunization protocol were uniformly protected from i.m. challenge.

There are areas of debate regarding internal images (40). For example, cross-species reactivity between B9 and rabies virus antibodies could occur if a common V_h germ line gene coding for the idiotope were produced (25). However, our data suggesting a 2β internal image are supported by those of Rustici et al. (48). These workers raised monoclonal antibodies to the synthetic fragment of rabies virus glycoprotein (positions 190 to 203) and found, by using affinity chromatography, that the antibodies bound neurotoxins. They believed that their data indicated that the paratope of their monoclonal antibodies behaves as an internal image of the nicotinic cholinergic receptor binding site.

Our data unequivocally show that antibody against rabies

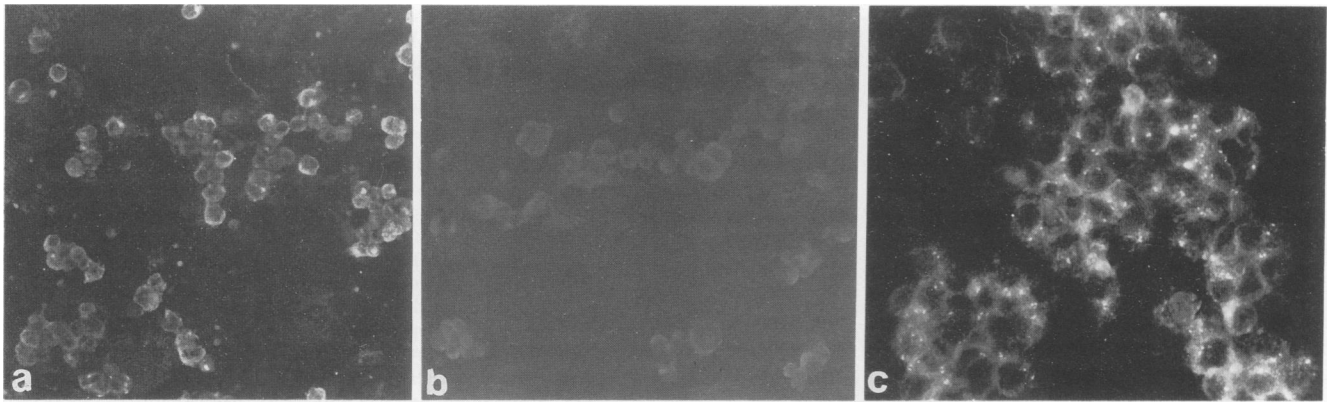

FIG. 7. IFA on acetone-fixed rat neuron-like PC12 cells distributed on printed slides. (a) B9 antibody staining (1:25 dilution of supernatant culture fluid) of uncloned PC12 cells detected by fluorescein isothiocyanate-conjugated anti-mouse IgM. The cells were synchronized by a cold block before distribution on printed slides. Between 30 and 50% of the uncloned cells expressed the AChR on the membrane, as detected by B9 antibody. Magnification, ca. ×106. PC12 cells (clone T) treated with B9 antibody (1:10 dilution of supernatant culture fluid) after being incubated for 30 min with approximately 10^8 particles of partially purified rabies virus (CVS strain). Little or no fluorescence was detected by the fluorescein isothiocyanate conjugate. Magnification, ca. ×211. (c) Cells similar to those in panel b, pretreated with approximately 10^8 particles of partially purified eastern equine encephalitis virus before being stained with B9 antibody (1:10 dilution of supernatant culture fluid). Most cells were labeled, some with very distinct granules. Magnification, ca. ×211.

virus glycoprotein specifically bears an anti-idiotypic relationship to the AChR. Whether an anti-idiotypic relationship can be demonstrated with other potential receptor molecules is unknown, because it was not the subject of the hypothesis tested in this report. While this issue has not been resolved, three other issues in rabies virus pathogenesis have been partially clarified in the course of our studies.

First, the epitope recognized by Mab35 is not the rabies virus binding site, as measured by the anti-idiotypic network. Our results with anti-idiotypic antibody to Mab35 parallel very closely those of others who found that anti-idiotypes frequently bind cytoskeletal proteins (10). The observation that anti-idiotypic antibody to 7.25 (antibody against the rabies virus nucleocapsid) also binds cytoskeletal proteins suggests some form of mimickry between the viral and cytoskeletal proteins. This explanation would parallel observations of mimickry in other viral systems but would be quite distinct from the anti-idiotypic network explanation (14). Whether the cellular cytoskeleton plays a role in rabies virus replication is presently unknown (reviewed in reference 7).

Second, our data may provide an additional explanation for the reported lack of correlation between monoclonal virus-neutralizing antibody titers determined in vitro and protection in vivo (49). 7.12 antibody does not neutralize rabies virus on cells that do not express the AChR, such as CER and neuroblastoma (N18) cells, but it does neutralize rabies virus and protect passively in vivo. Neutralizing antibodies measured in vitro may not be directed against AChR binding sites on rabies virus. Thus, monoclonal virus-neutralizing antibodies measured in vitro with continuous cell lines that do not bear the AChR may not be completely relevant to protection in vivo, in which the AChR may play a larger role in initiating infection.

Since the effect of the reagents developed in this study depends on whether the host cell expresses the AChR, the question of differential receptors can now be explored in vitro in the type of model system advocated by Koprowski (27). Rabies virus is certainly not unique in apparently using different receptors on different cultured cell types. In reovirus studies, virus binding to idiotype-negative cells was not inhibited by anti-idiotypic antibody, although these cells were susceptible to virus infection (24).

Finally, the question of multiple receptors in vivo is somewhat better resolved by the results of this study. The AChR epitope recognized by B9 at the peripheral NMJ is also present in the central nervous system. However, there may be multiple receptors in the nervous system. A variety of escape mutants of rabies virus have been isolated on the basis of their acquired resistance to neutralizing monoclonal antibodies directed to the viral glycoprotein. Lafay et al. (31) suggested that the pathogenic strain of rabies virus (CVS) should be able to bind several different kinds of receptors to penetrate neurons, while the nonpathogenic virus strain (AvO1) would be unable to recognize some of them. On the other hand, Dietzschold et al. (9) found that pathogenic viruses spread within the brain more rapidly than nonpathogenic viruses. Tuffereau et al. (61) reported that a positively charged amino acid, such as arginine or lysine, at position 333 is essential for the virus to be virulent for adult mice. However, the studies of Morimoto et al. (41) strongly indicate that the arginine 333-containing region is not a receptor binding site but is essential for glycoprotein-induced cell fusion at a neutral pH. These authors further postulate that a neurotoxin-like sequence located at positions 189 to 214 is a collaborating region in giant cell formation and possibly in viral invasion of neuronal cells. Our present efforts are directed toward the isolation and characterization of a 7.12 escape mutant whose pathogenesis and glycoprotein sequence can be compared with those of other existing mutants that have been previously studied (8, 20, 31) to clarify some of these areas of uncertainty.

For the present, at the very least, we have succeeded in defining one receptor and producing antibody to it. Our brief study of the distribution of the receptor in the central nervous system is consistent with the findings of those who have used other means to detect cholinergic nerve terminals in the central nervous system (56). Whether the differing B9 staining patterns in various regions of the brain have any significance is as yet unresolved. However, staining of neuronal processes has been observed before and has been postulated to result from axonal transport of the nicotinic

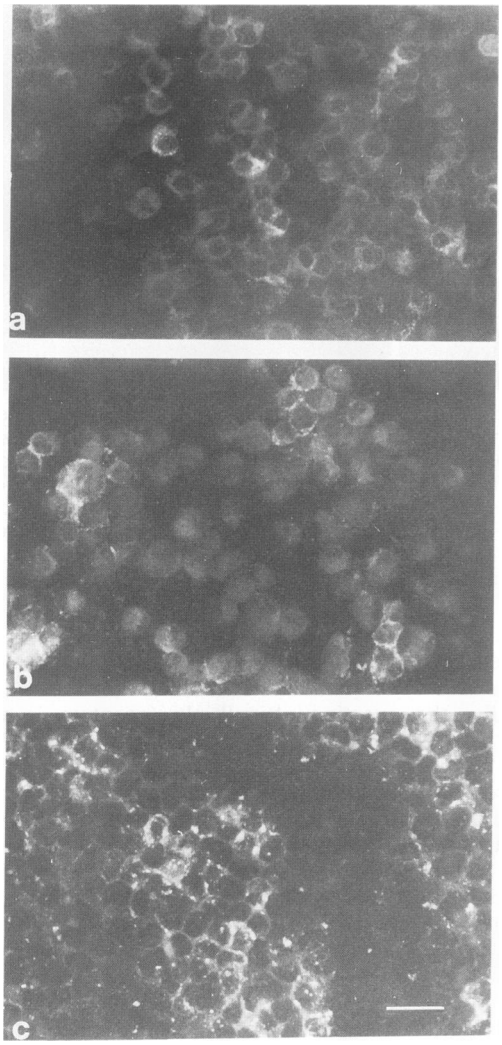

FIG. 8. IFA on acetone-fixed rabies virus-infected CER cells distributed on printed slides. Bar, 25 μm. (a) IFA on virus-infected cells after treatment with 7.12 monoclonal antibody to rabies virus (Ab1). There were discrete granules in cells, with some membranous association. (b) IFA on rabies virus-infected cells after staining with serum containing antibody (Ab3) to anti-idiotypic antibody B9 (Ab2). The approximate number of cells stained and the staining pattern were very similar to those in panel a, i.e., peripheral granular fluorescence. (c) IFA on rabies virus-infected cells after staining with polyclonal antibody to rabies virus (CVS strain). The pattern was similar to those in panels a and c, but the fluorescence was more intense and there were more cytoplasmic inclusions.

AChR receptor (56). We do not know, with certainty, from the present study whether virus infection destroys the B9 receptor in the brain. Whether B9 antibody targets a critical protein related to the spread of virus in cells of the central nervous system is another question that remains to be addressed before the importance of the B9 receptor in rabies virus pathogenesis can be properly assessed. These and other related subjects are treated in a separate report (57).

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We appreciate the technical help of Thomas Burrage and Elizabeth Gallagher in some portions of this research. Editorial assistance and advice were generously given by Francis Black, Fred Brown, Irene Greiser-Wilke, and Robert E. Shope.

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