Wurster-Hill and Maurer (*5*) reported cytogenetic studies of metastatic cells in the bone marrow of 26 patients with SCCL. No mention of chromosome 3 involvement is made in 18 patients with karyotypic abnormalities, but in two of five karyotypes shown in their report, the deletion of 3p is easily seen. Any discrepancies in their findings and ours need to be resolved by further studies of SCCL by several investigators. However, our findings of the 3p deletion in SCCL from 14 different patients, including tumor specimens freshly obtained after 2 days of culture, provide strong evidence for the association of the 3p(14–23) deletion with SCCL. In addition, one of our lines (NCI-N230) was derived from a Japanese SCCL tumor heterotransplanted into a nude mouse and then given to us by Shimosato (*6*). Thus, at least one example of the 3p(14–23) deletion has been found in an Asian SCCL. Chromosome studies of other neoplasms have not shown specific abnormalities of chromosome 3. The presence of this specific abnormality may prove to be a valuable aid in diagnosis, selection of therapy, and prognosis of lung cancer, with cytogenetic as well as light microscopic criteria used for the typing of lung cancer. When more is learned about the genes present in the 3p(14–23) region, their role in the genesis and maintenance of SCCL can also be determined.

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## Is the Acetylcholine Receptor a Rabies Virus Receptor?

Abstract. *Rabies virus was found on mouse diaphragms and on cultured chick myotubes in a distribution coinciding with that of the acetylcholine receptor. Treatment of the myotubes with* α*-bungarotoxin and* d*-tubocurarine before the addition of the virus reduced the number of myotubes that became infected with rabies virus. These findings together suggest that acetylcholine receptors may serve as receptors for rabies virus. The binding of virus to acetylcholine receptors, which are present in high density at the neuromuscular junction, would provide a mechanism whereby the virus could be locally concentrated at sites in proximity to peripheral nerves facilitating subsequent uptake and transfer to the central nervous system.*

Rabies virions have been detected within axons of animals infected with rabies virus, and their transport by axoplasmic flow has been described (*1–4*). Although this indicates a role for peripheral nerves in the pathogenesis of this disease, the receptor sites for virus binding in the peripheral nervous system have not been defined. We have recently shown that, 1 hour after intramuscular injection of mice with rabies virus (strain 1820B), antigen can be detected by immunofluorescence in leg sections at cholinesterase-positive sites which probably represent neuromuscular junctions (*5*). A similar distribution of radioactively labeled rabies virus (strain CVS) was seen at 6 hours after infection (*5*). These results suggested that rabies virus is present at the neuromuscular junction shortly after inoculation of virus. In this report we confirm that rabies virus binds initially at neuromuscular junctions and that the virus appears in nerves shortly thereafter. We also present data that suggest that virus-specific host-cell receptors for rabies virus are located at, or near, acetylcholine (ACh) receptor sites on cultured myotubes from chicken embryos.

Diaphragms and attached phrenic nerves were removed from both male and female random-bred mice (CF-1 strain, Charles River) and immersed in a 5-ml suspension of rabies virus strain 1820B containing $10^6$ $LD_{50}$ (the lethal dose for 50 percent of infant mice inoculated intracerebrally). The origin and passage history of this virus strain in our laboratory have been described (*5*). Specimens were held for either 30 minutes, 45 minutes, 60 minutes, 2 hours, or 4 hours at 23°C, after which each was washed extensively in phosphate-buffered saline (PBS), *p*H 7.2, and stained with fluorescein isothiocyanate (FITC)–conjugated antibody to rabies virus (Bio Quest, Cockeysville, Maryland; FITC-Rab, diluted 1:40 in PBS). Some stained preparations were counterstained for acetylcholinesterase (*6*), which is present at high concentrations at the neuromuscular junction. Other diaphragms, not exposed to virus, were stained with rhodamine-conjugated α-bungarotoxin (R-BTX) to determine the distribution of ACh receptors (*7*).

After 30 minutes of exposure to rabies virus, the mouse tissue showed the presence of virus antigen at sites that also stained for acetylcholinesterase. Virus antigen was not distributed over the entire neuromuscular junction at this time, nor always at later time periods. However, after 60 minutes of incubation in the virus suspension, the diaphragm often exhibited marked site-specific immunofluorescence. The FITC-Rab stained some of the neuromuscular junctions on the diaphragm so extensively that the characteristic morphology of these junctions was readily apparent by immunofluorescence (Fig. 1A). When the FITC-Rab–stained neuromuscular junctions were compared with such junctions on uninfected diaphragms stained only by R-BTX, the morphologic similarity was particularly obvious (Fig. 1B). After 4 hours of exposure to rabies virus, mouse diaphragms showed FITC-Rab–stained antigen in small peripheral nerves as they traversed the muscle fibers (Fig. 1C).

We also studied rabies virus binding


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and replication in chick myotubes cultured from 11-day-old embryos as described previously (*8*). In contrast to the diaphragm preparations, myotube cultures remain viable for as long as 2 weeks and possess surfaces that are readily accessible to both virus and drugs. Myotube cells contain ACh receptors within their membranes; these receptors are diffusely distributed in some areas and occur in high-density clusters or "hot spots" in others (*9, 10*). Myotubes cultured for 5 to 7 days were incubated in collagen-coated petri dishes or Lab-Tek slides (Miles Laboratories) with 0.2 ml of rabies virus, either strain 1820B or strain CVS ($10^7$ $LD_{50}$/ml). The passage history of the CVS strain has been described (*5*).

Two hours after exposure to strain 1820B at 23°C, myotubes stained with FITC-Rab showed small fluorescent patches. The pattern of fluorescence shown by the myotubes 3 days after exposure to strain 1820B at 37°C (Fig. 1, D and E) closely resembled the distribution of ACh receptors as revealed by the staining of uninfected myotubes with R-BTX (Fig. 1F). Six days after exposure to strain 1820B the myotubes showed widespread fluorescence, much of it apparently intracellular (Fig. 1G).

We then examined by electron microscopy myotubes that had been cultured for 5 days in Lab-Tek slides and incubated for either 2 hours at room temperature or 6 days at 37°C with rabies virus strain 1820B. After incubation with the virus the myotubes were washed four times with PBS and fixed with 1 percent glutaraldehyde and then postfixed with 1 percent osmium tetroxide, or fixed with a mixture of 2 percent glutaraldehyde and 2 percent paraformaldehyde and then postfixed in 1 percent osmium tetroxide. Myotubes exposed for 2 hours to strain 1820B revealed virus particles in close apposition to the plasma membrane of surface specializations or patches previously shown to be associated with the high-density clusters of ACh receptors (*8*). Bullet-shaped viral profiles were continuous with the sarcolemma at the margins of patches at both 2 hours (Fig. 2A) and 6 days after infection, suggesting that viral attachment and possibly exit occurred at these regions; virus particles were also closely associated with the extraneous coat of surface patches (Fig. 2B). The specialized patches on myotube surfaces stain with horseradish peroxidase–labeled α-BTX (*8*) (Fig. 2C).

The effect of α-BTX, an irreversible ligand of the ACh receptor, on myotubes infected with rabies virus was tested. α-Bungarotoxin has a high affinity for the cholinergic receptor in skeletal muscle and fish electric organ and is irreversibly bound at these sites (*11, 12*). The toxin interacts with the 40,000-dalton subunit of the ACh receptor; this subunit is also the ACh binding site (*13–15*). Prior incubation of myotubes for 2 hours with purified α-BTX (Miami Serpentarium Laboratories) at a concentration of $10^{-6}M$ followed by addition of the drug to the culture medium after virus sorption dramatically reduced infection by rabies virus strains 1820B and CVS, as determined by direct immunofluorescence. At all times up to day 6, the longest period of observation, the immunofluorescence in the toxin-treated cells was less than that in the controls (Fig. 1, H and I).

Chick embryo myotubes were treated with α-BTX ($10^{-5}M$ and $10^{-6}M$) or *d*-tubocurarine (*d*-TC) ($10^{-3}M$) prior to and after incubation with serial tenfold dilutions of strain 1820B at 37°C in Eagle's minimum essential medium. *d*-Tubocurarine is a cholinergic antagonist which, like α-BTX, interacts with the ACh binding site of the ACh receptor complex and competitively inhibits the binding of α-BTX (*15*). Since the binding of this antagonist is reversible, the myotubes were incubated with virus in the presence of *d*-TC. All cultures were examined by immunofluorescence after 3 days of incubation; cultures were regarded as positive at any single virus dilution irrespective of the number of infected (positive) myotubes. Titers were expressed as the $\log_{10}$

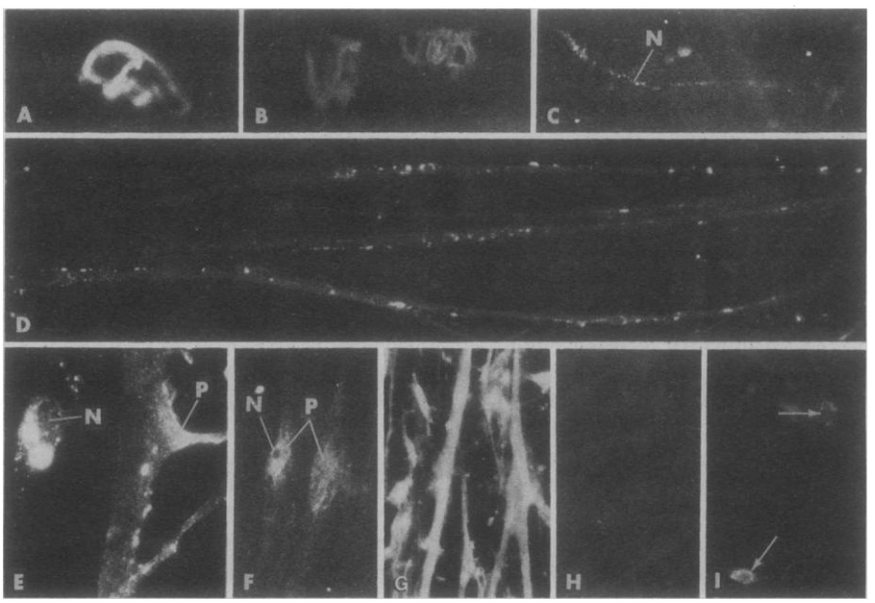


Fig. 1. Binding of rabies virus strain 1820B to neuromuscular junction of mouse diaphragm in vitro and to cultured mouse myotubes. (A) Localization of FITC-Rab–stained virus antigen at a neuromuscular junction of mouse diaphragm. The diaphragm was incubated in a suspension of strain 1820B for 1 hour and then stained with FITC-Rab and viewed with a fluorescence microscope (×960). (B) Neuromuscular junctions of mouse diaphragm stained with R-BTX which labels ACh receptors. The morphology of the junctions after staining for rabies antigen (A) and for ACh receptors (B) is the same (×560). (C) Diaphragm stained with FITC-Rab after 4 hours of incubation in a virus suspension. Fluorescent-stained antigen is present within a small nerve fiber (*N*) traversing the muscle fibers (×380). (D) Cultured chick myotubes incubated with rabies virus strain 1820B at room temperature for 2 hours and at 37°C for longer term exposures. Three days after infection the myotubes were fixed with acetone and stained with FITC-Rab. The elongated myotubes have a patchy, widespread distribution of high-intensity labeling and a faint, low-intensity labeling over the remainder of the surface (×100). (E) Higher magnification of myotubes stained with FITC-Rab 3 days after exposure to strain 1820B. Intense fluorescence occurs around nuclei (*N*), in broad patches (*P*), and in small focal areas. The remainder of the myotube surface exhibits weak fluorescence (×320). (F) Myotubes cultured for 8 days, fixed with methanol, and stained with R-BTX which labels regions of high-density ACh receptors (*7*). Staining occurs in large patches (*P*), often in the vicinity of nuclei (*N*). Weak fluorescence is present over other areas of the myotube surface (×230). (G) Myotubes stained with FITC-Rab 6 days after infection with rabies virus and showing widespread fluorescence (×220). (H) Myotubes were incubated in α-BTX ($10^{-6}M$) for 2 hours, exposed to the virus for 1 hour, and then cultured for 3 days in the presence of α-BTX. The myotubes were then stained with FITC-Rab. Virtually no rabies antigen can be detected in these myotubes by fluorescence microscopy, indicating that α-BTX prevented infection of myotubes by rabies virus (×160). (I) Myotube cultures treated as in (H) for a period of 6 days. α-BTX prevented the intense accumulation of virus antigen as seen in (G). Scattered small cells (arrows) contain perinuclear vacuoles staining for virus antigen. Examination of the same fields with phase microscopy revealed these cells to be fibroblasts (×520).

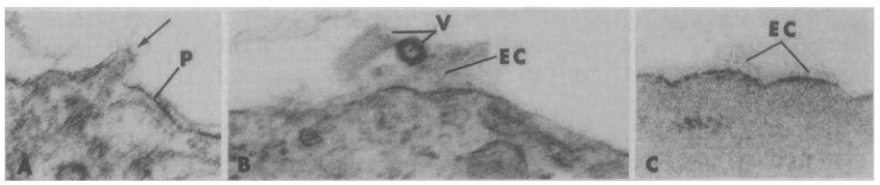


Fig. 2. (A) Electron microscopy of myotube surface 2 hours after exposure to rabies virus. A bullet-shaped profile (arrow) is continuous with the sarcolemma at the margin of a surface patch (*P*) characterized by ridges and extraneous coat. Stained en bloc with uranyl acetate and poststained with lead citrate (×135,000). (B) Myotube processed 6 days after exposure to rabies virus. Two virus particles (*V*), one sectioned longitudinally and the other transversely, attached to the extraneous coat (*EC*) of a patch characterized by surface ridges. The cell surface outside the patch region is unspecialized. Stained as in (A), (×127,000). (C) Myotube surface stained with horseradish peroxidase–labeled α-BTX as described previously (*8*). Dense reaction product demonstrates that ACh receptors are concentrated in the plasmalemma of the surface patch. The patch in this case consists of surface ridges and extraneous coating and closely resembles the patch illustrated in (B) (without heavy metal staining) (×106,000).

of the $TCID_{50}$ (median tissue-culture infective dose) per 0.5 ml. Treatment with α-BTX at $10^{-5}$ and $10^{-6}M$ reduced the virus titer by 3.5 $\log_{10}$ or greater (99.97 percent) and 1.5 $\log_{10}$ (97 percent), respectively, whereas treatment with *d*-TC at $10^{-3}M$ reduced the virus titer by 2.5 $\log_{10}$ (99.7 percent).

Cultures treated with α-BTX were susceptible to infection 3 days after the toxin was removed, during which time the myotubes may have regenerated new receptors, because the average lifetime of ACh receptors in chicken myotubes in primary culture is 16 to 28 hours (*16*). The α-BTX did not prevent rabies virus infection of scattered contaminating fibroblasts in the myotube cultures (Fig. 1I). These cells, unlike myotubes, do not have ACh receptors in high-density clusters. The inhibitory effect of α-BTX on virus binding was not attributable to obvious contaminants in the α-BTX preparation: polyacrylamide gel electrophoresis (*17*) of α-BTX resulted in a single band of approximately 8000 daltons. Further, the effect of the drug was exerted on the host cell and not on the virus itself, since infectivity with rabies CVS virus was unaltered compared to controls after the virus was mixed with α-BTX ($10^{-3}M$) and isolated on a sucrose density gradient. Addition of α-BTX to the myotubes after exposure to virus (1 hour) did not inhibit accumulation of antigen, suggesting that the drug-induced interference with virus growth was at an attachment step in the viral replicative cycle.

Binding assays with rabies virus strain CVS labeled with [$^3$H]uridine were conducted with myotubes at 4°C and analyzed as previously described (*18*). These experiments confirmed that α-BTX ($10^{-6}M$) reduced attachment of strain CVS to myotubes. For example, 29 ± 4 percent of the rabies virus, purified from CER cells (*5*), bound to untreated myotubes whereas 19 ± 3 percent bound to α-BTX–treated cells. Although virus binding was significantly reduced in the presence of α-BTX, this result was not as striking as one would expect from the results with immunofluorescence. One explanation for this difference is that the virus bound to other constituents in the cultures such as collagen or contaminating fibroblasts and that this binding was unaffected by α-BTX. To estimate the effect of possible contaminants on our results, we conducted binding assays with a nonneurotropic strain of Sindbis virus, Ar339 (*18*). This virus did not replicate extensively in the myotubes but readily infected the fibroblasts as determined by immunofluorescence. When the myotubes were exposed to Sindhis Ar339 in concentrations similar to those used for the rabies virus experiments, 6 percent of the Sindbis Ar339 became bound, largely to fibroblasts and possibly collagen.

Our data indicate that there are specific high-affinity host-cell receptor sites for rabies virus at the neuromuscular junction and suggest that the receptor may be the ACh receptor. Although other molecules associated with or located in the same region as ACh receptors (*19–22*) could act as the viral receptors, these molecules would have to be very closely associated with or a part of the ACh receptor complex to account for the blocking effect of α-BTX and *d*-TC.

Because of the high density of receptors at the tips of the junctional folds of the neuromuscular junction (about 30,000 α-BTX sites per square micrometer) (*23*), binding to the ACh receptor would effectively enhance the probability of virus entering motor nerves at this location for eventual transport to the central nervous system. These findings do not rule out the possibility that rabies virus may bind to other cell surface components on muscle fibers or other cell types, for example, fibroblasts in this study. However, binding to specific neurotransmitter receptors may represent the most significant mechanism in terms of the pathogenesis of rabies, because it would serve to localize and concentrate the virus on postsynaptic cells such as muscle or on neurons in close proximity to adjacent nerve terminals.

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