

Monoclonal antibody identification of a 100-kDa membrane protein in HeLa cells and human spinal cord involved in poliovirus attachment

(virus receptor/picornavirus/central nervous system)

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Communicated by Bernard N. Fields, June 21, 1988 (received for review April 11, 1988)

ABSTRACT Unique receptor sites for poliovirus are considered to be the primary determinant of the virus' cell and tissue-type specificity. To study the poliovirus-cell interaction, eight monoclonal antibodies that specifically block the cytopathic effects of poliovirus were generated by using HeLa cell preparations as immunogen and a newly developed colorimetric screening assay. Plaque-inhibition assays confirmed the viral specificity of the antibodies, and when one antibody, AF3, was used as a probe in immunoblots of cell membrane preparations, it detected a 100-kDa band in only those cell lines and tissues permissive for poliovirus infection. AF3 also specifically inhibited radiolabeled poliovirus binding to cells. In terms of tissue specificity, AF3 detected the 100-kDa band in membrane preparations from human spinal cord but not in organ homogenates of human kidney or in murine tissue, including the central nervous system. Furthermore, AF3 detected the band in a human-mouse hybrid cell line containing human chromosome 19, which confers permissivity for poliovirus infection, but the antibody did not detect the band in a human chromosome 19-deficient subclone. In an immunohistochemical study of the human brainstem, AF3 stained neurons in the reticular formation and clusters of brainstem neurons, consistent with the known pattern of damage caused by poliovirus infection in the brainstem. Furthermore, AF3 reacted with human peripheral mononuclear cells, consistent with the known replication of poliovirus in Peyer's patches and tonsils. These results strongly suggest that the 100-kDa band detected by antibody AF3 is, or is closely associated with, the poliovirus receptor site.

Poliovirus, a neurotropic picornavirus, is the causative agent of paralytic poliomyelitis. Most strains infect only primates and replicate primarily in the intestine, tonsils, deep cerebellar nuclei, brainstem, motor cortex, and anterior spinal gray matter (1). Studies utilizing minced organ tissue and organ homogenates suggest that the tissue tropism and species specificity of poliovirus is determined primarily by the presence or absence of unique cell-surface receptors (2). For instance, while intestine, brain, and spinal cord homogenates bind poliovirus, skeletal muscle and kidney organ homogenates do not. The hypothesis is further supported by experiments that show that bypassing the receptor binding step by introducing viral genetic material into the cell makes virus-resistant cells susceptible to poliovirus infection (3). Somatic cell hybrids between human and mouse cells have been used to show that the loss of both permissivity for poliovirus infection and binding correlates with the loss of human chromosome 19, indicating the loss or alteration of a cell-surface structure from these hybrids is responsible for

specific resistance to poliovirus infection (4, 5). Moreover, Mendelsohn *et al.* (6) have shown that transformation of mouse L cells with human DNA generates cells permissive for poliovirus infection.

A number of investigators have studied enterovirus-cell interactions by generating monoclonal antibodies against cell-surface structures to which the virus binds (7-10). Minor *et al.* (10) generated a monoclonal antibody that specifically blocked the cytopathic effect of all three serotypes of poliovirus while having no effect on the other viruses tested. Nobis *et al.* (8) generated a monoclonal antibody, D171, which blocked the binding of poliovirus and was itself blocked by poliovirus. Use of these blocking antibodies to identify the specific cell protein(s) involved in poliovirus attachment has not been reported. This may reflect the lability of the receptor; for example, poliovirus binding is lost after mild detergent treatment of receptor-bearing cells (reviewed in ref. 11). In contrast, Tomassini *et al.* (12) were able to use an anti-receptor monoclonal antibody to identify and isolate receptor protein involved in the attachment of the major group of the human rhinoviruses.

The present report describes (i) a newly developed colorimetric assay that was used to screen hybridoma supernatants for the ability to protect cells from poliovirus infection and (ii) the subsequent isolation of a monoclonal antibody, AF3, that identified a 100-kDa protein in membrane preparations of human tissues and cell lines susceptible to poliovirus infection.

MATERIALS AND METHODS

Viruses and Cells. Poliovirus serotype 1 Mahoney, attenuated serotype 2w2 (ATCC VR-301), serotype 2 Lansing, and serotype 3 Leon were obtained from the American Type Culture Collection. Coxsackieviruses B1 and B3 and echovirus 6 were provided by Richard Crowell, Hahnemann University School of Medicine, Philadelphia; coxsackievirus A18 and equine influenza virus (ATCC VR-317), by Richard Colonna, Merck Sharp & Dohme, West Point, PA; and adenovirus type 2, by Connie Cepko, Department of Genetics, Harvard Medical School, Boston. A pair of human-mouse hybrid cell lines that differ by the presence (KLEJ-42) or absence (KLEJ-42/P) of human chromosome 19 were provided by Howard Greene, Harvard Medical School (4, 5). Control viruses were grown in HeLa S3 cells, and lysates were used as stocks.

Abbreviations: PMNC, peripheral mononuclear cells; pfu, plaque-forming unit(s); INT, *p*-iodonitrotetrazolium violet; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAP, peroxidase-antiperoxidase.

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Coxsackievirus B3 was radiolabeled and purified as described (13). Poliovirus type 2w2 was radiolabeled and purified as described for radiolabeled rhinovirus 14 (14) except that cell monolayers were used and [³⁵S]methionine incorporation was allowed to proceed overnight. The specific activities of the poliovirus and coxsackievirus preparations were 2.2×10^{-4} cpm and 5.5×10^{-4} cpm per plaque-forming unit (pfu), respectively.

Production of Hybridomas and Screening by the Colorimetric "MTT/INT" Assay. Ten million live HeLa cells were recovered from T-150 flasks by scraping, were washed twice with cold phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS), and were resuspended in 0.5 ml of PBS before intraperitoneal immunization of 4- to 6-week-old BALB/c mice (Charles River Breeding Laboratories). One and 2 months after the initial immunization, each animal was administered a booster intraperitoneally with crude membrane preparations made by Dounce homogenization of 7×10^7 HeLa cells in 10% PBS at 4°C, followed by centrifugation at $100,000 \times g$ for 30 min at 4°C and resuspension in 0.5 ml of PBS. Three days after the second inoculation, spleen cells from two animals were fused with NS1 cells by standard procedure (15). Of 1600 wells, $\approx 97\%$ had viable cells, which corresponded to ≈ 5500 individual clones as calculated by Poisson distribution.

Supernatants of cultures were assayed for their ability to protect cells from poliovirus infection by using a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and *p*-iodonitrotetrazolium violet (INT). This assay is based on the ability of live cells to metabolize two tetrazolium dyes, which then form a deep-violet cytoplasmic precipitate. Replica 96-well plates of confluent HeLa cells were incubated with 70 μl of hybridoma supernatant for 2 hr at 37°C. Poliovirus type 2w2 was then added at the maximal dilution sufficient to achieve cell death of the entire monolayer as measured by the MTT/INT assay, determined to be 1 pfu per cell. The cells were incubated with virus and antibody for 30 min at 37°C and then were washed once with fresh medium and incubated at 37°C for 40–44 hr. Fifty microliters of dye solution [prepared fresh by heating 100 ml of PBS to 90°C, adding 50–200 μg of INT (Sigma I-8377) per ml and 30 μl of glacial acetic acid, cooling in the dark to room temperature, and adding 3 mg of MTT (Sigma M-2128) per ml] was added to each well, and the plates were incubated for 2 hr at 37°C. Medium was then removed, 200 μl of glacial acetic acid was added, and the plates were read on a multiscan ELISA plate reader (Flow Laboratories) at 415 nm. Positive wells were considered to be those that had the highest average optical densities in consecutive assays.

Hybridomas scoring positive were subcloned twice. Hybridoma supernatants were used for all of the described procedures. Supernatant from a hybridoma specific for an intracellular protein was used as a control supernatant. Antibody concentration of hybridoma supernatants was determined by radial immunodiffusion (Tago, Burlingame, CA; DIFFU-GEN 1345, procedure as described in the package instructions).

Plaque Reduction and Competitive Binding Assays. For plaque assays, hybridoma supernatants (8 μg of antibody in 400 μl) were preincubated with HeLa cell monolayers in six-well plates (Costar, Cambridge, MA) for 2 hr at 37°C, after which virus (30–100 pfu in 40 μl) was added, and the plates were incubated for an additional 30 min at room temperature. The assay was continued as described (14) with minor modifications in that plaques were allowed to develop for 40–44 hr in 1% agar with modified Eagle's medium (GIBCO 320-1935) containing 5% (vol/vol) fetal calf serum.

For binding assays, hybridoma supernatants (2 μg of antibody in 100 μl) were preincubated with HeLa cell monolayers in 24-well plates (Costar) for 2 hr at 37°C, after which radiolabeled virus (3000–6000 cpm in 20 μl) was added

for the appropriate time. Wells were then washed once with Hanks' balanced salt solution, containing 3% bovine serum albumin. The monolayers were dissolved overnight in 0.1 ml of 1 M NaOH and then assayed for radioactivity. Results are expressed as total cpm bound to the monolayers. Approximately 25–35% of radiolabeled virus bound to the monolayer when incubated at 37°C for 30–45 min in the absence of antibody.

Immunoblot Analysis. Membrane preparations of HeLa S3 cells were prepared as described for HeLa R-19 cells (9). L cells were harvested from suspension cultures and tissues were minced, after which they were washed twice and treated as described above. Human peripheral blood mononuclear cells (PMNC) were isolated from heparinized venous blood by means of centrifugation on a Ficoll/Hypaque density gradient (Pharmacia) and then treated as above.

Samples were electrophoresed on 10% polyacrylamide gels (16), electroblotted overnight onto nitrocellulose (17), and blocked with 20% fetal calf serum. Hybridoma supernatants were diluted 1:4 (final concentration, 5–15 $\mu\text{g}/\text{ml}$) and used as probe as described in the alkaline phosphatase-goat anti-mouse ProtoBlot immunoscreening system (Promega Biotec, Madison, WI).

Immunocytochemistry. The peroxidase-antiperoxidase method was used to localize antibody binding in the human brainstem. Specimens were obtained at postmortem intervals ranging from 4 to 12 hr from patients who had died from diseases not related to nervous system disorders. Blocks of brainstem tissue were cut and frozen immediately in embedding medium (Tissue-Tek 4583, Miles Laboratories, Ames Division, Elkhart, IN) on cryostat specimen discs and stored at -70°C . Sections (10 μm) were cut and dried on chromium potassium sulfate/gelatin-coated slides for 1 hr at room temperature. After rehydration in PBS for 5 min, the sections were fixed for 5 min at room temperature with 1% formalin in PBS and blocked for 1 hr with PBS containing 3% (wt/vol) bovine serum albumin and 0.5% sodium azide. Antibody supernatant was diluted 1:10 in the blocking solution with 0.1% Triton X-100 and incubated with the sections for 18 hr at 4°C. All subsequent steps were done at room temperature. The sections were washed three times in PBS/3% bovine serum albumin without sodium azide for 15 min and incubated for 2 hr with goat anti-mouse antiserum (Boehringer Mannheim) diluted 1:80 in PBS/3% albumin. Sections were washed three times as above and then incubated for 1 hr with peroxidase-antiperoxidase (PAP) monoclonal antibody-peroxidase complex (Clono-PAP, Sternberger-Meyer, Jarrettsville, MD) diluted 1:40. After three washes as above, the sections were incubated with diaminobenzidine and H_2O_2 as described (18).

RESULTS

Isolation and Preliminary Screening of Monoclonal Antibodies. Hybridoma supernatants were tested for their ability to protect HeLa cells from infection with poliovirus type 2w2 by using the colorimetric MTT/INT assay. Of 11 primary wells that met the criteria for a positive score—i.e., wells generating the highest optical densities in two consecutive assays—8 stable hybridoma clones were obtained. All 8 hybridoma supernatants had the following characteristics: (i) they blocked plaque formation by poliovirus type 2w2 (80–97% plaque reduction) but not echovirus type 6 or coxsackievirus B3; (ii) in ELISA the antibodies bound to HeLa cells while none bound to mouse L cells (data not shown), consistent with the known absence of poliovirus receptors on the latter (19). Upon immunoblot analysis, 1 antibody (AF3) reacted with both HeLa cell and human spinal cord membrane preparations (see below). Consequently, the present report deals primarily with antibody AF3.

Table 1. Monoclonal antibody AF3 inhibition of plaque formation

Virus	% inhibition*
Poliovirus	
2w2	74
2	66
1	44
3	5
Echovirus 6	0
Coxsackievirus	
B1	-5
B3	-7
A18	-7
Influenza	0
Adenovirus 2	0

*Percent inhibition is the number of plaques in control wells minus the number of plaques in antibody-treated wells multiplied by 100% and divided by the number of plaques in control wells.

Viral Specificity of Monoclonal Antibody AF3. The specificity of antibody AF3 was determined by its ability to inhibit plaque formation and to inhibit binding of radiolabeled virus to cells. Antibody AF3 inhibited plaque formation by poliovirus type 2, type 2w2, and type 1, while having little or no effect on type 3 (Table 1). Antibody AF3 did not inhibit plaque formation by the other viruses tested, which included an enveloped virus (influenza), a DNA virus (adenovirus), and other picornaviruses. In addition, AF3 inhibited the binding of radiolabeled poliovirus type 2w2 to HeLa cells (Fig. 1 *Upper*), whereas the antibody had no effect on the binding of radiolabeled coxsackievirus B3 to HeLa cells (Fig. 1 *Lower*).

Monoclonal Antibody AF3 Identifies a 100-kDa Band on Immunoblots of Cell Lines and Tissues Susceptible to Poliovirus Infection. Whereas HeLa cells and human spinal cord expressed the 100-kDa protein in immunoblot analysis, mouse spinal cord, mouse L cells, and human kidney organ homogenates did not (Fig. 2A). Note that the lane containing

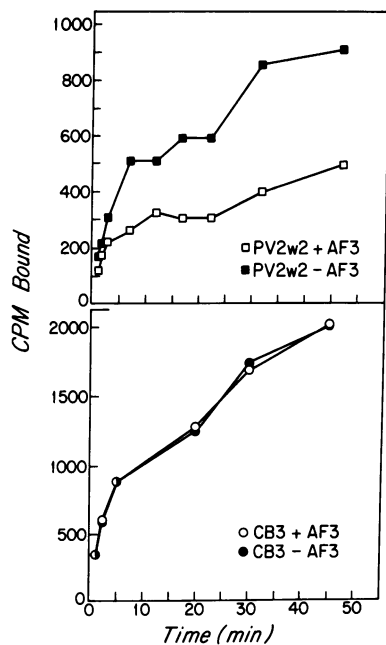


FIG. 1. Competition between radiolabeled viruses and antibody AF3 for binding to HeLa cells. (*Upper*) Binding kinetics of ³⁵S-labeled poliovirus type 2 (PV2w2) to cells in the presence (□) and absence (■) of AF3. (*Lower*) Binding kinetics of ³⁵S-labeled coxsackievirus B3 (CB3) to cells in the presence (○) and absence (●) of AF3.

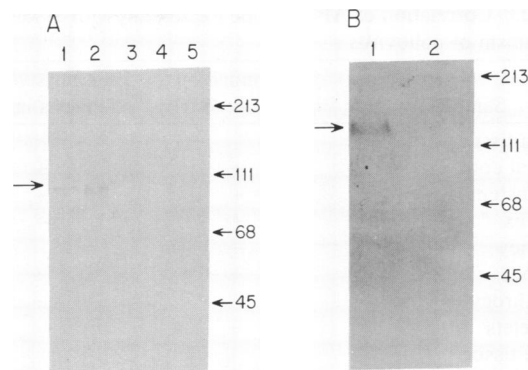


FIG. 2. Immunoblot detection of the AF3 epitope in membrane preparations from human and murine organ homogenates and cell lines. (A) Lanes 1-4 were loaded with 15 μ g, and lane 5 was loaded with 150 μ g of membrane protein. Lanes: 1, HeLa cells; 2, human brainstem obtained 5 hr postmortem; 3, mouse L cells; 4, murine brainstem; 5, human kidney obtained 4 hr postmortem. (B) Association of expression of the AF3 epitope with human chromosome 19. Lanes 1 and 2 were loaded with 14 and 20 μ g of membrane preparation, respectively. Lanes: 1, KLEJ-42 cells with human chromosome 19; 2, KLEJ-42/P cells lacking that chromosome. Sizes are shown in kDa.

the kidney membrane preparation had 10 times the protein as in the other lanes.

To further test the specificity of antibody AF3, a human-mouse hybrid cell line (KLEJ-42) that contains human chromosome 19, which confers permissivity for poliovirus infection, and a clonal subline of KLEJ-42 that lacks this chromosome (KLEJ-42/P) were used in immunoblot analysis. Antibody AF3 identified a faint band in a membrane preparation from the KLEJ-42 cells (Fig. 2B, lane 1) but not in that from the KLEJ-42/P cells (Fig. 2B, lane 2). The KLEJ-42 cell line expresses much less poliovirus binding activity than do HeLa cells (4, 5). The band migrated >100 kDa as the gel was electrophoresed under nonreducing conditions, which was necessary because 2-mercaptoethanol treatment of KLEJ-42 membrane preparations caused a decrease in antibody reactivity below the level of detection. HeLa cells expressed a band of similar molecular weight as KLEJ-42 cells when immunoblots were performed under nonreducing conditions, and the antigen derived from HeLa cells was similarly sensitive to 2-mercaptoethanol (data not shown). However, the large amount of antigen on HeLa cells allowed detection of the band under reducing conditions. Table 2 lists the tissues and cell lines that have been analyzed.

Immunocytochemistry. Antibody AF3 stained specific regions of the brainstem that previously have been shown to be damaged in poliomyelitis (1). Fig. 3A is a line drawing of the human brainstem at the level of the inferior olivary nucleus, which depicts the distribution of AF3 staining. The reticular formation and the hypoglossal nucleus were the most deeply stained, while sensory nuclei were less so, and the white matter was not stained at all. The reticular formation and the motor nuclei are the regions of the brainstem (observed at autopsy) to be most severely damaged by poliovirus infection of the brainstem (1). Little or no AF3 staining was observed in the inferior olivary complex, a region known to be spared in poliomyelitis of the brainstem (1).

DISCUSSION

A monoclonal antibody has been generated that identifies a 100-kDa protein in membrane preparations from HeLa cells and tissues known to be permissive for poliovirus infection. The 100-kDa protein identified by AF3 appears to fulfill the criteria for the biologic properties of the poliovirus receptor

Table 2. Correlation of AF3 epitope expression with tissue and cell tropism of poliovirus

Samples	Immunoblot immunoreactivity	Permissive for poliovirus infection
Human tissue		
Spinal cord	+	+
Brainstem	+	+
Cortex	+	+
Kidney	-	-
PMNC	+	+
Erythrocytes	-	-
Platelets	-	-
Mouse tissue		
Central nervous system	-	-*
Lymph node cells	-†	-
Cell lines		
HeLa S3	+	+
L cells	-	-
KLEJ-42	+	+
KLEJ-42/P	-	-

*Lansing type 2 poliovirus has been reported to infect mice (reviewed in ref. 24).

†AF3 epitope expression was determined by indirect immunofluorescence.

site or a component of it. Other investigators have obtained monoclonal antibodies against the poliovirus receptor site (8, 10). However, identification of a specific solubilized protein has not been reported. A new colorimetric cell protection assay was used here to identify monoclonal antibodies that protect cells from poliovirus infection. Vital dyes have been

used previously in the differentiation between live and dead cells and in proliferation assays (20, 21). Improvements were made on previous techniques to make this assay extremely sensitive. Changes in optical density occurred before virus-infected monolayers showed gross morphological changes; thus, the decrease in optical density was related to viral infection rather than to cellular degeneration.

The structure identified by AF3 appears to fulfill the criteria for the following biologic properties of the poliovirus receptor site. First, AF3 blocked plaque formation by poliovirus and did not block plaque formation by a number of other viruses tested, including other picornaviruses, a DNA virus (adenovirus), and an enveloped virus (influenza virus). Notably, the picornavirus coxsackievirus A18 shares its cell-surface receptor with the major group of human rhinoviruses, which comprises 89% of human rhinoviruses (9, 22). Antibody AF3 also specifically blocked the binding of radiolabeled poliovirus type 2w2 to cells. Second, expression of the AF3 epitope is consistent with the known species and tissue tropism of poliovirus, as the monoclonal antibody detected the 100-kDa protein in human spinal cord, HeLa cells, KLEJ-42 cells, and PMNC but not in murine spinal cord, L cells, KLEJ-42/P cells, or murine lymph node cells. In addition, the antibody did not react with human kidney organ homogenate, which is known to lack poliovirus binding activity (2), nor did it react with the inferior olivary complex of the brainstem, which was reported by Bodian to be characteristically spared in poliovirus infection of the brainstem (1). Finally, the antibody reacted with a human-mouse somatic cell hybrid containing human chromosome 19, which has been shown to be associated with expression of permis-

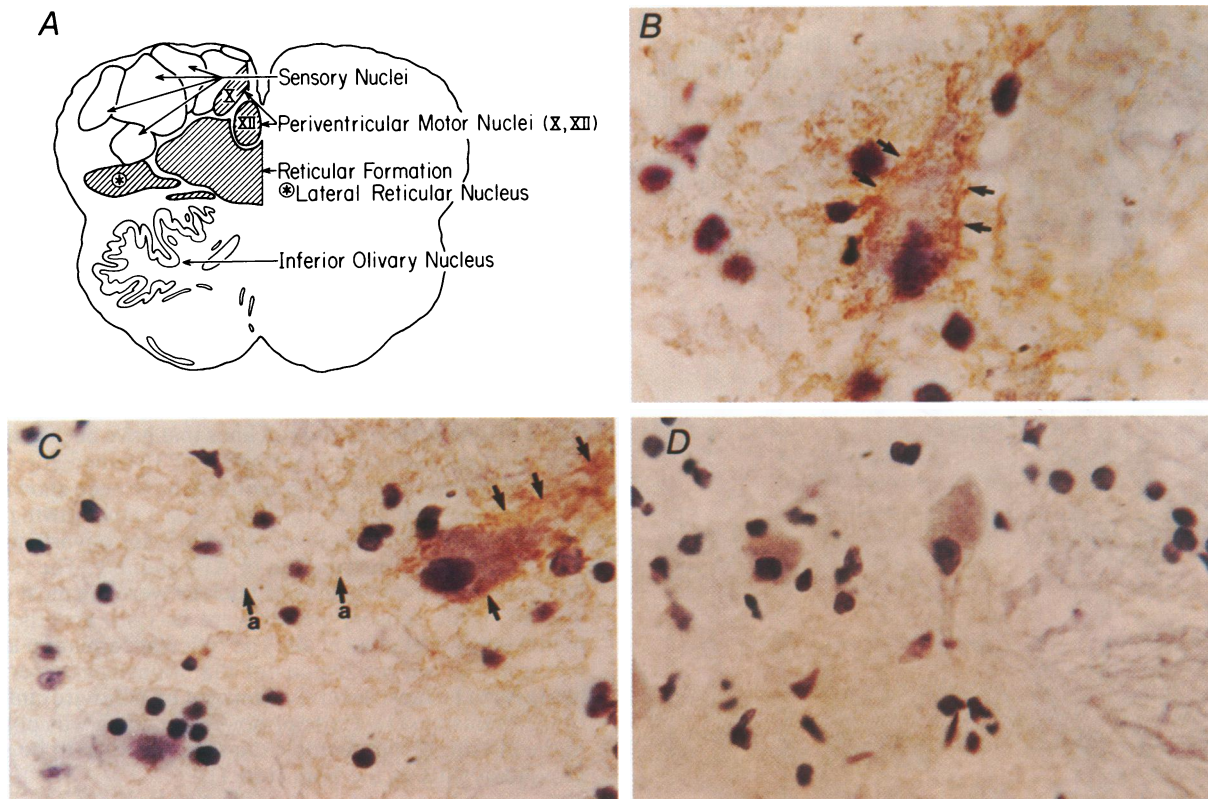


FIG. 3. PAP immunohistochemistry of the human brainstem at the level of the inferior olivary complex. Sections were counterstained with hematoxylin (blue). (A) Schematic summary of the regions stained by antibody AF3. Shaded areas represent the anatomical distribution of peroxidase reaction product (brown). Immunoreactivity was observed in the periventricular motor nuclei, including the hypoglossal nucleus (XII), the reticular formation, the lateral reticular nucleus, and the dorsal accessory olivary nucleus. (B and C) Photomicrographs of neurons from the reticular formation (B) and the lateral reticular nucleus (C) showing immunostaining of cell bodies (unlabeled arrows) and the surrounding neuropil. Note that the axon "a" in C is not stained by AF3. ($\times 75$.) (D) Photomicrograph of a neuron from the inferior olivary complex. Note the absence of the brown peroxidase reaction product. ($\times 75$.)

sivity for infection by poliovirus, whereas it did not react with the somatic hybrid lacking this chromosome.

The ability of human chromosome 19 to confer permissivity for infection by all three serotypes of poliovirus suggests that the three serotypes recognize a common gene product(s). Interestingly, AF3 blocked infection of HeLa cells by poliovirus type 2 best, type 1 intermediately, and type 3 to the least extent or not at all. Nobis *et al.* (8) generated an anti-poliovirus receptor monoclonal antibody, D171 by screening for the ability to protect cells from infection by poliovirus type 1 rather than type 2. This antibody preferentially blocked viral cytopathic effects of type 1 and type 3 best and to a lesser extent type 2. Thus, the serotype specificity of a protecting monoclonal antibody may be related to the poliovirus serotype used in screening for protection. Minor *et al.* (10) reported a poliovirus receptor site antibody that appeared to block serotypes 1, 2, and 3 equally. However, their assay for cytopathic effect may not have been sensitive enough to discern slight differences in the ability of their antibody to preferentially block a particular serotype of poliovirus.

The results from the cell protection experiments with antibody AF3 and those done by Nobis *et al.* with antibody D171 suggest that poliovirus serotypes 2 and 3 differ the most in their interactions with cellular receptors. Notably, previously published data from cross-competition experiments are least documented for competition between serotypes 2 and 3 (ref. 23; reviewed in refs. 24 and 25). La Monica *et al.* (26) have suggested that the ability of poliovirus type 2 Lansing and not other serotypes to replicate in mice is determined, at least in part, by viral capsid differences. Such variations in capsid structure might result in serotype-specific virus-cell interactions.

The results we have obtained suggest several hypotheses for the structural nature of the poliovirus binding site. First, individual serotypes may interact differentially with a single receptor molecule. In this hypothesis, poliovirus type 3 would have a much higher affinity for receptor than would antibody AF3. Second, the serotypes may interact differentially with a heterogeneous population of receptor molecules that are encoded by a single gene and are subsequently modified by alternate splicing and/or posttranslational modification. In this hypothesis, antibody AF3 and poliovirus type 3 would recognize alternate versions of the gene product. This hypothesis is consistent with the finding of Racaniello *et al.* that HeLa cells express several RNA messages that hybridize to cloned DNA probes derived from a secondary transformant permissive for poliovirus infection (ref. 6, V. Racaniello, personal communication). Third, the serotypes may interact differentially with components of a heterogeneous multimolecular receptor site. Distinction between these hypotheses must await isolation of the component(s) involved in poliovirus attachment.

With the generation of a monoclonal antibody that identifies a 100-kDa protein that appears to fit the criteria of the poliovirus receptor site, isolation of the protein may now be possible. It may also be possible to determine whether the poliovirus receptor site is a single molecule or is composed of a number of nonidentical structures. Finally, if the receptor site is composed of a single molecule or a number of homologous receptor molecules, then immunization of animals with protein isolated with antibody AF3 should result in an antiserum that blocks binding of all three serotypes of poliovirus to susceptible cells.

Of interest is the observation that antibody AF3 binds to PMNC. While poliovirus does not replicate efficiently in quiescent lymphocytes, phytohemagglutinin-activated lymphocytes do support poliovirus replication (27). Furthermore, poliovirus replicates in tonsils and Peyer's patches of the ileum in the early stage of infection. These tissues are rich

in lymphocytes and other mononuclear cells. Receptors for neurotropic viruses have been shown to exist on lymphoid tissue, and in the nervous system as exemplified by human immunodeficiency virus (28).

The poliovirus receptor site presumably serves a cellular function, and identification of a protein may prove useful in determining that function. Initial immunohistochemical studies suggest that the AF3 epitope is a synaptic antigen (data not shown). In addition, previous studies raise the possibility that poliovirus preferentially binds to synaptosomes (29). The functional role of the receptor site may provide insight into the neurotropism of poliovirus and unique properties of the neurons that are infected by poliovirus.

We gratefully acknowledge Linda Powers Simon, Gary Conboy, Dr. Nabila Haikal, and Dr. Marion Willinger for assistance with the immunochemical procedures; and Drs. Bernard Fields, David Johnson, Patrick Hogan, and Richard Colonno for invaluable criticism and advice. This work was supported by National Institutes of Health Grant NS169980. M.P.S. is a Medical Scientist Trainee supported by National Institutes of Health Grant 5T32GM07753-08. B.S. is a DuPont Fellow of the Life Sciences Research Foundation.

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