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Identification of a Putative Alphavirus Receptor on Mouse Neural Cells

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Alphaviruses replicate in a wide variety of cells in vitro. The prototype alphavirus, Sindbis virus, causes an age-dependent encephalitis in mice and serves as an important model system for the study of alphavirus neurovirulence. To begin to understand the role of cellular virus receptors in the pathogenesis of Sindbis virus infection, we developed an anti-idiotypic antibody made in rabbits against a neutralizing monoclonal antibody specific for the E2 surface glycoprotein. The anti-idiotypic antibody (anti-Id 209) bound to N18 mouse neuroblastoma cells and inhibited adsorption of ^{35}S-labeled virus by 50%. Binding of anti-Id 209 was inhibited by pretreatment of N18 cells with various proteases but not with neuraminidase or phospholipase, while virus binding was inhibited by pretreatment with phospholipase as well as protease. Anti-Id 209 precipitated proteins of 110 and 74 kDa from N18 cells intrinsically labeled with [^{35}S]methionine. N18 cells grow with two phenotypes in culture, and immunoprecipitation of ^{125}I-surface-labeled cells showed that the 74-kDa protein was present on loosely adherent cells growing in aggregates, while the 110-kDa protein was present in smaller amounts on firmly adherent cells growing as a monolayer. Analysis of brain cells from newborn mice by flow cytometry showed that all cells expressed the receptor protein at birth, but by 4 days after birth half of the cells had ceased receptor expression. A survey of other cell lines showed the protein to be present on murine fibroblastic and other rodent neuroblastoma cell lines but rarely on human neural or nonneural cell lines. These studies suggest that one of the receptors for Sindbis virus on mouse neural cells is a protein that is regulated during development of the nervous system. Developmental down-regulation of receptor protein expression may contribute to the age-dependent nature of susceptibility of mice to fatal alphavirus encephalitis.

The interactions between specific viral surface proteins (cell attachment proteins) and receptors on target cells may play an important role in determining viral tropism. Any constituent of the cell membrane, including carbohydrates, lipids, and proteins, may be a viral receptor. These cell membrane components serve normal cellular functions and are subserved by viruses for attachment and entry. A number of cellular molecules have been identified as viral receptors, e.g., the epidermal growth factor receptor for vaccinia virus (6), complement receptor type 2 for Epstein-Barr virus (7), CD4 for human immunodeficiency virus (5, 17, 28), and ICAM-1 for rhinoviruses (10, 40). Neurotropic viruses must attach to receptors that are expressed on neural cells. Two neural cell-specific molecules, the acetylcholine and β-adrenergic receptors, have been identified as attachment proteins for rabies virus and reovirus type 3 (4, 19, 20). Some molecules, like mannose-6-phosphate and heparan sulfate, which are used for initial binding by varicella-zoster and herpes simplex viruses (8, 43), are present on many types of cells. Tissue tropism of these viruses must not be controlled by these ubiquitous receptor molecules alone. The role of the fibroblast growth factor receptor as a more specific receptor for herpes simplex virus type 1 (15), with heparan sulfate serving as an accessory molecule (45), is controversial (38). Most neurotropic viruses replicate in extraneural as well as neural tissue and therefore must have receptors on multiple types of cells. The receptor for poliovirus, for instance, is a member of the immunoglobulin (Ig) superfamily present on cells in the gastrointestinal tract as well as on neurons (29).

The neurotropic alphaviruses are important causes of epidemic encephalitis and must replicate in cells of their insect vectors and their natural avian hosts as well as the mammals susceptible to encephalitis. Whether this broad host range is accomplished through use of an ubiquitous receptor or multiple receptors is not clear. In animals susceptible to encephalitis, neurons are primary target cells within the central nervous system (11, 13) and therefore must bear an alphavirus receptor. Sindbis virus (SV) infection of mice serves as a model system for the study of alphavirus-induced acute encephalitis. Initial replication occurs in peripheral tissue, primarily muscle, with subsequent spread to the nervous system. The severity of infection is age dependent, with more-widespread infection and larger amounts of viral replication in newborn mice than in mature mice (14). Binding of virus to susceptible cells is a property of the E1 and E2 glycoproteins, which form a heterodimer on the surface of the virion, and the efficiency of binding to neural cells is influenced by single-amino-acid changes in the E2 glycoprotein (41). In the present study, we used an anti-idiotypic antibody produced against an anti-E2 monoclonal antibody (MAb) that neutralizes SV to identify a potential receptor for SV on mouse neural cells.

MATERIALS AND METHODS

Viruses. Two strains of SV were used: AR339 (American Type Culture Collection [ATCC], Rockville, Md.) and TE, a recombinant virus constructed by using the full-length cDNA clone Toto1101 (34) as the background with insertion of fragments coding for the E1 glycoprotein of AR339 and the E2 glycoprotein of NSV, a neuroadapted strain of AR339 (24). This recombinant virus has previously been shown to

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be virulent for mice and to bind efficiently to mouse neural cells (41). Stock viruses were propagated and assayed in BHK-21 cells and had titers of approximately 10^9 PFU/ml.

Neural cell lines. N18 mouse neuroblastoma cells (an acetylcholine- and catechol-negative clone of C-1300 cells obtained from M. Nirenberg, National Institutes of Health) (2) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS), glutamine, and gentamicin. With repeated passage, N18 cells gradually changed from a nonadherent to an adherent phenotype. NB41A3, another subclone of C-1300 mouse neuroblastoma cells, was cultured in Ham's F10 medium with 15% horse serum and 2.5% FBS. IMR32, a human neuroblastoma cell line, was cultured in MEM with 10% FBS, nonessential amino acids, and gentamicin. SHEP-1, SHEP-13, and SY5Y, cloned cell lines from the human neuroblastoma line SK-N-SH, were cultured in RPMI 1640 with 10% FBS, glutamine, and gentamicin (35). SHEP-1 is epithelial in appearance and lacks tyrosine hydroxylase and dopamine β-hydroxylase, enzymes unique to catecholaminergic neurons. SHEP-13 is morphologically intermediate between neuronal and epithelial cells and expresses both enzymes. SY5Y is neuronal and also expresses both enzymes. PC-12, a rat adrenal pheochromocytoma cell line, was cultured in RPMI 1640 containing 8.5% horse serum and 10% FBS (9).

Nonneural cell lines. BHK-21 cells (ATCC) were cultured in DMEM supplemented with 10% FBS, glutamine, and gentamicin. Primary mouse embryo fibroblasts were prepared from 16- to 18-day-old mouse embryos and grown in MEM plus 5% FBS. Chicken embryo fibroblasts (SL-29 [ATCC]) and *Aedes albopictus* (mosquito) C6/36 cells (ATCC) were grown in MEM with nonessential amino acids, 10% FBS, glutamine, and 10% tryptose phosphate broth. Human skeletal muscle cells and human skin fibroblasts, kindly provided by Orest Hurko, Johns Hopkins Medical School, as primary cell cultures, were cultured in DMEM with 10% FBS.

Purification and radiolabeling of virus. ^{35}S-labeled SV (strain TE) was prepared as described previously (41). Briefly, BHK-21 cells were infected at a multiplicity of infection of 0.1 and incubated at 37°C in the presence of 20 μCi of [^{35}S]methionine per ml and 20 μCi of [^{35}S]cysteine per ml until >90% of cells showed cytopathic effects. The supernatant fluids were harvested and clarified, and virus was precipitated with polyethylene glycol 8000 (10% in 0.5 M NaCl, pH 7.4). The pelleted virus was banded on a continuous potassium tartrate gradient (15 to 40% in phosphate-buffered saline [PBS], pH 7.4) at 27,000 rpm in a Sorvall OTD 65B ultracentrifuge for 16 h at 4°C, dialyzed against 0.05 M Tris (pH 7.4), and stored in aliquots at −70°C in binding medium (RPMI 1640, 0.2% bovine serum albumin, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]).

Preparation of anti-idiotypic antibody. Three previously described (30) SV-neutralizing MAbs, 202 (IgG3, E2-ab), 106 (IgG2b, E1-c), and 209 (IgG3, E2-c), were purified from ascitic fluid by 33% $(NH_4)_2SO_4$ precipitation. F(ab')$_2$ fragments were prepared by pepsin digestion using immobilized pepsin (Pierce Chemical Company, Rockford, Ill.) and an 8-h incubation period. The supernatant fluid was harvested, neutralized, and applied to a staphylococcal protein A column to remove Fc fragments and undigested IgG.

New Zealand White rabbits (Bunnyville Farm, Littlestown, Pa.) were immunized by subcutaneous injection in multiple sites with 300 μg of IgG emulsified in complete Freund's adjuvant. The animals were boosted subcutaneously on day 7 with 100 μg of F(ab')$_2$ emulsified in incomplete Freund's adjuvant and on day 30 with 100 μg of F(ab')$_2$ in PBS. The rabbits were bled 10 days after the last injection.

Competitive ELISA. For a competitive enzyme-linked immunosorbent assay (ELISA), 96-well polystyrene plates (Immulon II; Dynatech, Chantilly, Va.) were coated with gradient-purified SV (AR339; 3 μg/ml) in coating buffer (1 M Na_2CO_3 and $NaHCO_3$, pH 9.6) overnight at 4°C. Dilutions of anti-idiotypic antibodies were preincubated with an equal volume of MAb at 37°C for 1.5 h. After incubation, 200 μl of the reaction mixture was added to each well in triplicate and incubated for 1.5 h at 37°C. The plates were washed three times with washing buffer (1% Tween 20 in PBS) before 200 μl of peroxidase-conjugated rabbit anti-mouse Ig (1:100; Dako, Santa Barbara, Calif.) was added. *o*-Phenylenediamine dihydrochloride (Sigma, St. Louis, Mo.) at a concentration of 0.25 mg per ml of citrate buffer (pH 5.5) was used as the substrate. The optical density (OD) was read at 450 nm. The degree of inhibition was expressed as a percentage calculated as [1 − (OD in the presence of anti-idiotypic antibody/OD of MAb alone)] × 100.

Virus-binding assay. N18 cells (10^6 cells per microcentrifuge tube [1.5 ml; Elkay Products, Inc., Shrewsbury, Mass.]) were incubated with 0.3 ml of diluted anti-idiotypic antibody or control rabbit serum for 1.5 h at 4°C. The unbound antibodies were removed by washing two times with binding medium. ^{35}S-labeled SV was added (4,000 cpm per tube) and incubated with constant gentle agitation at 4°C for 4 h. At the end of this incubation, the cells were washed three times with cold binding medium. The supernatant and wash fluids from each tube were pooled, and the radioactivity was measured to determine unbound virus. The final pelleted cells were resuspended in 1% sodium dodecyl sulfate (SDS; BDH Chemical Ltd., Poole, England), and the radioactivity was measured to determine bound virus. Each assay was performed in triplicate.

Flow cytometry. Adherent cells were removed nonenzymatically with PBS containing 0.5 mM EDTA and 0.02% sodium azide. For indirect staining, 10^6 cells were incubated with 0.3 ml of anti-idiotypic antibody or control rabbit serum diluted 1:100 in PBS with 1% nonfat dry milk at 4°C for 1.5 h. After being washed two times with binding medium, the cells were stained with 0.3 ml of fluorescein-conjugated goat anti-rabbit Ig (Vector Laboratories, Inc., Burlingame, Calif.; 1:100 dilution in 1% milk in PBS). For direct immunofluorescent staining, anti-idiotypic Ig and normal rabbit Ig were labeled with fluorescein (5,6-carboxyfluorescein-*N*-hydroxysuccinimide ester; Boehringer, Mannheim, Germany) and used at a 1:4 dilution in PBS with 1% milk. Cells were analyzed by flow cytometry (FACSTAR PLUS; Becton Dickinson, San Jose, Calif.). Positive cells were those with a level of fluorescence above which at least 90% of control antibody-stained cells were negative.

Competition for binding to cells was assessed by incubating anti-idiotypic antibody (1:1,000 dilution) with an equal volume of serial 10-fold dilutions of MAb (1:10 to 1:1,000) at 37°C for 1.5 h. N18 cells (10^6 per microcentrifuge tube) were stained with 0.3 ml of the mixture and analyzed as described above by the indirect method.

For analysis of suckling mouse brain cells, mice were sacrificed at various ages after birth. Whole brains were minced through a very fine sieve to yield a single brain cell suspension in RPMI 1640 supplemented with 10% FBS. The brain cell suspension (10^7 cells per ml) was overlaid on lympholyte-M (Accurate Chemical and Scientific Co., West-

bury, N.Y.) and centrifuged at 800 × g for 30 min at room temperature. The band of brain cells at the interface of the lympholyte-M layer was harvested and washed three times with RPMI 1640 without FBS. The cells (10^6 cells per microcentrifuge tube) were stained and analyzed as described above.

Enzymatic inactivation of receptor on viable cells. The following enzymes were used in receptor inactivation assays: phospholipase A_2 (PLA_2) (50 U/ml), PLC (4 U/ml for N18 cells and 2 U/ml for BHK-21 cells), neuraminidase (5 U/ml), bromelain (100 U/ml), subtilisin (100 U/ml), proteinase K (100 μg/ml), trypsin (100 μg/ml), pronase (100 μg/ml) (Sigma), and phosphoinositol (PI)-specific PLC (PI-PLC; American Radiolabeled Chemicals, Inc., St. Louis, Mo.). Suspensions of N18 and BHK-21 cells (10^6 cells per microcentrifuge tube) were incubated at 37°C for 30 min with enzymes at concentrations previously determined not to affect cell viability (determined by trypan blue exclusion). After enzyme treatment, the cells were washed three times with binding buffer, cooled to 4°C, and then exposed to ^{35}S-labeled SV (4,000 cpm, or about 8 to 10 PFU per cell) for 1.5 h at 4°C. N18 cells were also stained with the anti-idiotypic antibody by the indirect method and analyzed by flow cytometry. Results were expressed as percent reduction in SV attachment or, in cells positive by flow cytometry, relative to the undigested control cells.

Immunoprecipitation procedure. (i) Metabolic labeling of cellular proteins. N18 and BHK-21 cells were washed once with MEM without methionine (MEM Selectamine kit; GIBCO) and cultured overnight in medium containing 20 μCi of [^{35}S]methionine per ml and 10% FBS. Labeled cells were lysed with Nonidet P-40 medium (150 mM NaCl, 50 mM Tris [pH 8.0], 1% Nonidet P-40, 100 μg of phenylmethylsulfonyl fluoride per ml, 0.7 μg of pepstatin A per ml, 0.5 μg of leupeptin per ml) for 30 min at 4°C. This lysate was used for immunoprecipitation.

(ii) Surface protein labeling. N18 cells (10^7) were washed twice with PBS and incubated with ^{125}I (1.0 mCi; Du Pont) and lactoperoxidase (250 mg/ml in 50 μl of PBS) (26). The labeling reaction was continued for 8 min, with H_2O_2 (10 μl of a 1:1,000 dilution of 30% stock solution) added every 2 min. The labeled cells were washed with PBS containing 10 mM KI to remove free ^{125}I-Na. The cells were lysed as described above, and the lysate was used for immunoprecipitation.

(iii) Immunoprecipitation. Lysate (50 μl; 4 × 10^6 cpm by trichloroacetic acid precipitation) was incubated with 0.3 ml of anti-idiotypic (anti-Id 209) antibody or control (anti-202) antibody diluted 1:50 for 4 h at 4°C. Antigen-antibody complexes were precipitated by staphylococcal protein A-Sepharose beads (Pharmacia, Piscataway, N.J.) and washed twice with washing buffer (50 mM Tris, 2 M NaCl) and twice with 10 mM Tris, pH 8.0. The precipitated proteins were analyzed by SDS–7.5% polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Anti-idiotypic antibody as an antireceptor antibody. Rabbits were immunized with neutralizing MAbs recognizing two distinct epitopes on the E2 glycoprotein (MAb 202, which recognizes E2-ab, and MAb 209, which recognizes E2-c) and one on the E1 glycoprotein (MAb 106, which recognizes E1-c). Sera were tested for the presence of anti-idiotypic antibody by a competitive ELISA (Table 1). Only rabbits immunized with MAb 209 produced antibody which blocked MAb binding to virus. Since anti-Id 209 had properties of an anti-idiotypic antibody, it was investigated further to determine whether it serves as a mirror image for the portion of the viral E2 glycoprotein which binds to a cellular receptor.

TABLE 1. Inhibition of MAb binding to SV by rabbit antiserum against three neutralizing MAbs

MAb	Rabbit antibody	% Inhibition at indicated dilution				
		Undiluted	1:10	1:100	1:500	1:1,000
209	Anti-Id 209 (rabbit 1)	100	54.2	25.2	14.5	0
	Anti-Id 209 (rabbit 2)	92.4	46.6	22.1	2.3	0
	Anti-202	0	6.9	2.3	0	3.8
	NRS[a]	9.2	0	0	0	0
202	Anti-202 (rabbit 1)	28.2	14.5	0	0	0
	Anti-202 (rabbit 2)	4.2	0	0	0	0
	NRS	0	0	ND[b]	ND	ND
106	Anti-106 (rabbit 1)	0	0	0	0	0
	Anti-106 (rabbit 2)	0	0	0	0	0
	NRS	0	0	ND	ND	ND

[a] NRS, normal rabbit serum.
[b] ND, not determined.

Two different cell lines susceptible to SV infection were stained with the anti-Id 209 antiserum and analyzed by flow cytometry (Fig. 1). BHK-21 cells replicate virus efficiently and are used for routine virus propagation but were not stained by anti-Id 209. Cells of the N18 murine neuroblas-

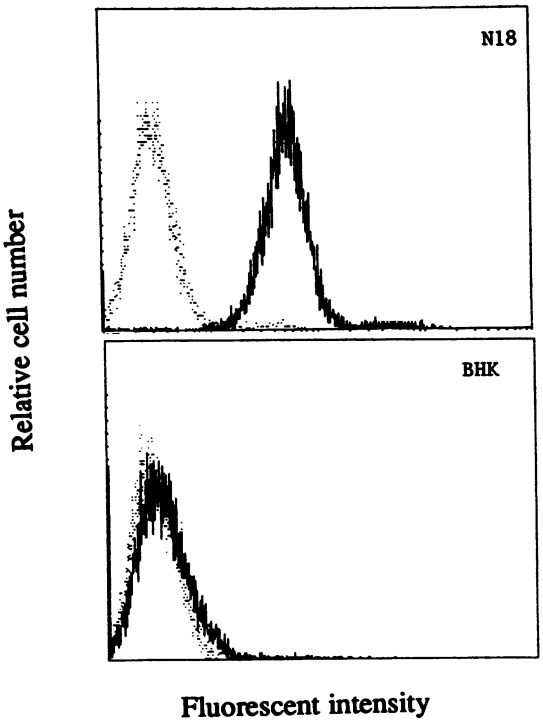

FIG. 1. Binding of anti-idiotypic antibody to surface molecules on N18 mouse neuroblastoma and BHK-21 cell lines. Solid lines represent binding of anti-Id 209; dotted lines represent binding of anti-202. Binding of preimmune anti-Id 209 and binding of normal rabbit serum were similar to that of anti-202.

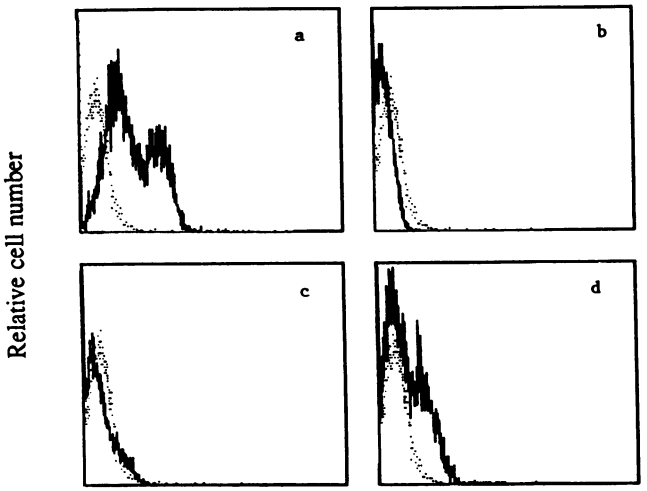

FIG. 2. Competitive inhibition of binding of anti-Id 209 to N18 cells by MAb 209. N18 cells (mixed phenotype) were stained with anti-Id 209 (1:1,000 dilution) (a), MAb 209 (1:10) alone (b), or anti-Id 209 (1:1,000) preincubated with MAb 209 diluted 1:10 (c) or 1:100 (d).

toma cell line, which binds and replicates virus in a neural cell-specific manner (41), were uniformly stained by anti-Id 209 but not by normal rabbit serum, preimmune anti-Id 209, or either of the anti-202 sera. Furthermore, MAb 209 competed with anti-Id 209 for binding to the N18 neuroblastoma cells (Fig. 2), further confirming the anti-idiotypic properties of the antibody.

To determine whether anti-Id 209 could block virus attachment to N18 cells, the binding of ^{35}S-labeled SV was assessed (Table 2). The anti-idiotypic antibody inhibited binding of ^{35}S-labeled SV to neuroblastoma cells by 50% at the lowest dilution tested (1:10), with significant inhibition still evident at a dilution of 1:100.

Enzymatic inactivation of receptor on viable cells. A panel of enzymes which cleave phospholipids, proteins, and carbohydrates was tested for cell toxicity (see Materials and Methods) and then for the effect of the maximal tolerated concentration on the binding of the anti-idiotypic antibody

TABLE 2. Ability of anti-Id 209 to inhibit binding of ^{35}S-labeled SV to N18 mouse neuroblastoma cells

Antibody	Dilution	% Bound virus (mean ± SEM)	% Inhibition
Anti-Id 209	1:10	12.7 ± 3.3	51.2
	1:50	18.0 ± 5.5	30.5
	1:100	18.9 ± 4.1	27.3
Anti-202	1:10	17.5 ± 3.6	32.7
	1:50	23.5 ± 4.6	9.3
	1:100	25.8 ± 0.5	0.5
NRS[a]	1:10	23.6 ± 4.6	9.1
	1:50	27.5 ± 5.8	0.0
None		26.0 ± 6.0	

[a] NRS, normal rabbit serum.

TABLE 3. Effect of enzymatic digestion of N18 mouse neuroblastoma and BHK-21 cells on binding of ^{35}S-labeled SV and anti-Id 209 antibody

Enzyme	% Reduction[a] in binding of:		
	^{35}S-labeled SV		Anti-Id 209 to N18
	N18	BHK-21	
Bromelain	71.9	67.8	20.1
Subtilisin	74.6	53.4	79.4
Proteinase K	74.6	49.5	63.1
Pronase	75.7	53.6	56.6
Trypsin	36.7	40.0	10.0
Neuraminidase	2.0	0	0
PLC	69.0	67.2	0
PLA_2	72.0	65.7	0

[a] Percent reduction was calculated by the formula [1 − (% binding by treated cells/% binding by untreated cells)] × 100.

209 (anti-Id 209) and ^{35}S-labeled virus to BHK-21 and N18 cells (Table 3). All of the proteolytic enzymes tested significantly decreased binding of SV and anti-Id 209, while neuraminidase had no effect on either. Treatment with phospholipases decreased virus binding to both BHK-21 and N18 cells but had no effect on anti-Id 209 binding.

To determine whether the phospholipase-sensitive molecule is PI linked, BHK-21 cells were treated with PI-PLC purified from *Bacillus thuringiensis*. Treatment with this enzyme did not affect binding of SV (Table 4). As a control for enzymatic activity, the effect of PI-PLC on the PI-linked, pronase-resistant protein Thy-1 (22) was assessed with the EL-4 thymoma cell line by flow cytometry.

These results suggested the possibility of two types of SV receptor on N18 cells. The combined effects of phospholipase digestion and anti-Id 209 on virus binding were therefore assessed (Table 5). The effects of the two reagents were additive, resulting in almost complete inhibition of SV binding to the neuroblastoma cells.

Immunoprecipitation of an N18 receptor for SV by anti-Id 209. The ^{35}S-labeled proteins from N18 and BHK-21 cells were immunoprecipitated by anti-Id 209 and by anti-202 as a control (Fig. 3). Anti-Id 209 precipitated two proteins from the N18 lysate, one of 74 and another of 110 kDa. These proteins were not precipitated from BHK-21 lysates by anti-Id 209 or from N18 lysates by control serum. Proteins of 35, 31, and 29 kDa were precipitated from both BHK-21 and N18 lysates and were considered nonspecific.

TABLE 4. Effect of treatment of BHK-21 cells with PI-PLC from *B. thuringiensis*

Exptl materials	PI-PLC (U/ml)	% Binding[a]	% Reduction
BHK-21 + ^{35}S-labeled SV		32.7	
BHK-21 + ^{35}S-labeled SV	1	34.2	0
BHK-21 + ^{35}S-labeled SV	2	33.8	0
EL-4 + anti-Thy-1		99.5	
EL-4 + pronase + anti-Thy-1		94.8	
EL-4 + anti-Thy-1	2	74.1	25.5
EL-4 + anti-Thy-1	4	60.8	38.8

[a] Percentage of cells positive by flow cytometry for EL-4 cells and percentage of ^{35}S-labeled SV bound to surface for BHK-21 cells.

TABLE 5. Inhibition of binding of ^{35}S-labeled SV to N18 cells by treatment with PLA_2 and anti-Id 209

Treatment	% Binding	% Inhibition
None	60.3	
Anti-Id 209	17.4	71.2
PLA_2	26.7	55.7
PLA_2 + anti-Id 209	6.8	88.8

The immunoprecipitation of ^{125}I-surface-labeled N18 proteins was performed to determine which of the proteins precipitated after intrinsic labeling is on the cell surface (Fig. 4). After passage in our laboratory, two phenotypes of N18 cells were distinguished. The original neuronal cells grew in loosely adherent aggregates (Fig. 4B) but gradually became more adherent (Fig. 4D) until all cells adhered and became more epithelial in appearance (Fig. 4F). N18 cells with the nonadherent growth pattern expressed only the 74-kDa protein on the cell surface (Fig. 4A). Both 74- and 110-kDa proteins were immunoprecipitated by anti-Id 209 from cells of mixed phenotype, while the adherent N18 cells expressed only the 110-kDa protein on the cell surface. None of the smaller proteins immunoprecipitated after intrinsic labeling were precipitated from ^{125}I-labeled cells. No surface proteins were immunoprecipitated by the control, anti-202. Both cell types are susceptible to SV infection (data not shown).

Immunoprecipitation (Fig. 4) suggested that smaller amounts of the 110-kDa protein than of the 74-kDa protein were generally expressed on the cell surface. The three types of cells were therefore analyzed by flow cytometry after being stained with anti-Id 209 to better quantitate the amount of surface protein (Fig. 5). Nonadherent N18 cells, from which the 74-kDa protein is precipitated, stained more intensely than adherent N18 cells, from which the 110-kDa protein is precipitated. Mixed-phenotype cells had a bimodal distribution. These data confirm that less of the 110-kDa protein than of the 74-kDa protein is present on the cell surface.

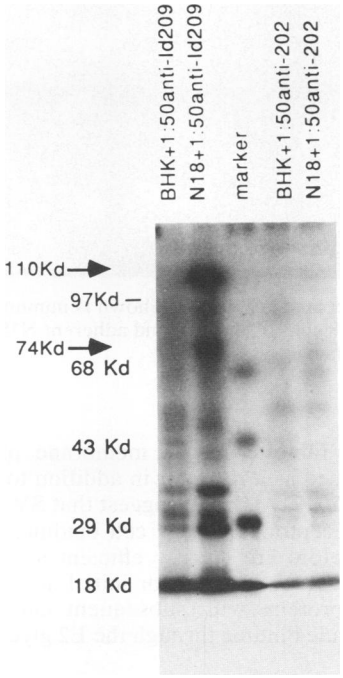

FIG. 3. Immunoprecipitation of ^{35}S-labeled protein from N18 mouse neuroblastoma and BHK-21 cells by anti-Id 209. Lane 1, BHK-21 lysate plus anti-Id 209 (1:50 dilution); lane 2, N18 lysate plus anti-Id 209 (1:50); lane 3, molecular weight markers; lanes 4 and 5, BHK-21 and N18 lysates, respectively, plus anti-202 (1:50).

Expression of the SV receptor recognized by anti-Id 209 on neural and nonneural cell lines. Seven neural cell lines and seven nonneural cell lines were investigated for the expression of the SV receptor identified by anti-Id 209 (Table 6). The receptor was highly expressed on most rat and mouse neural and nonneural cell lines but not on most human cell lines tested. The expression of the SV receptor on the N18 clone of C-1300 cells was significantly higher than on the NB41A3 clone, further suggesting variability of expression for different mouse neuroblastoma cell lines. Chicken and mosquito cell lines were intermediate in expression, and for chicken cells the distribution was bimodal, suggesting heterogeneity in the cell population.

Expression of the SV receptor recognized by anti-Id 209 on suckling mouse brain cells. The virulence of SV is age dependent, with younger mice being more susceptible than older mice (14). To determine whether receptor expression varies with age, brain cells obtained from mice from shortly after birth to 6 days of age were studied by flow cytometry. More than 90% of brain cells of newborn (<16-h-old) mice expressed this receptor (Fig. 6a). Shortly thereafter, expression was terminated in some populations of brain cells but retained in other populations, producing a bimodal distribution (Fig. 6b). During the first week of life, the proportion of cells expressing this receptor decreased from >90% to 40 to 50%.

DISCUSSION

An anti-idiotypic antibody made against a MAb which interacts with a virus at the site of virus binding to the host cell surface may provide an internal image of the cell attachment site and provide a reagent to identify the cellular receptor. Anti-idiotypic antibodies have proven useful in the identification of cellular receptors for polyomavirus, reovirus type 3, and leukemogenic retrovirus (3, 16, 27, 32). We have identified a candidate receptor for SV by using an anti-idiotypic antibody raised against neutralizing MAb 209, which maps by competitive binding to epitope E2-c. This antibody also protects against fatal encephalitis in adult mice and inhibits agglutination of goose erythrocytes by SV (30). Anti-Id 209 inhibited binding of SV to mouse neuroblastoma cells by 50% but did not block binding of SV to BHK cells.

There is increasing evidence that SV and perhaps other alphaviruses use multiple receptors for cell entry. In our studies, the receptor that must exist on BHK-21 cells is not identified by anti-Id 209 and anti-Id 209 does not completely block the binding of radiolabeled virus to N18 cells. In studies similar to ours, Wang et al. have identified a 63-kDa receptor on chicken cells by using an anti-idiotypic antibody prepared against a MAb that maps to a second neutralizing site (E2-ab) on the SV E2 glycoprotein (42). This anti-idiotypic antibody also only partially blocks virus binding to chicken cells and does not block binding to BHK cells. In addition, Maassen and Terhorst (25) have shown specific cross-linking of a 90-kDa protein from human lymphoblastoid cells to SV. None of these molecules has yet been identified.

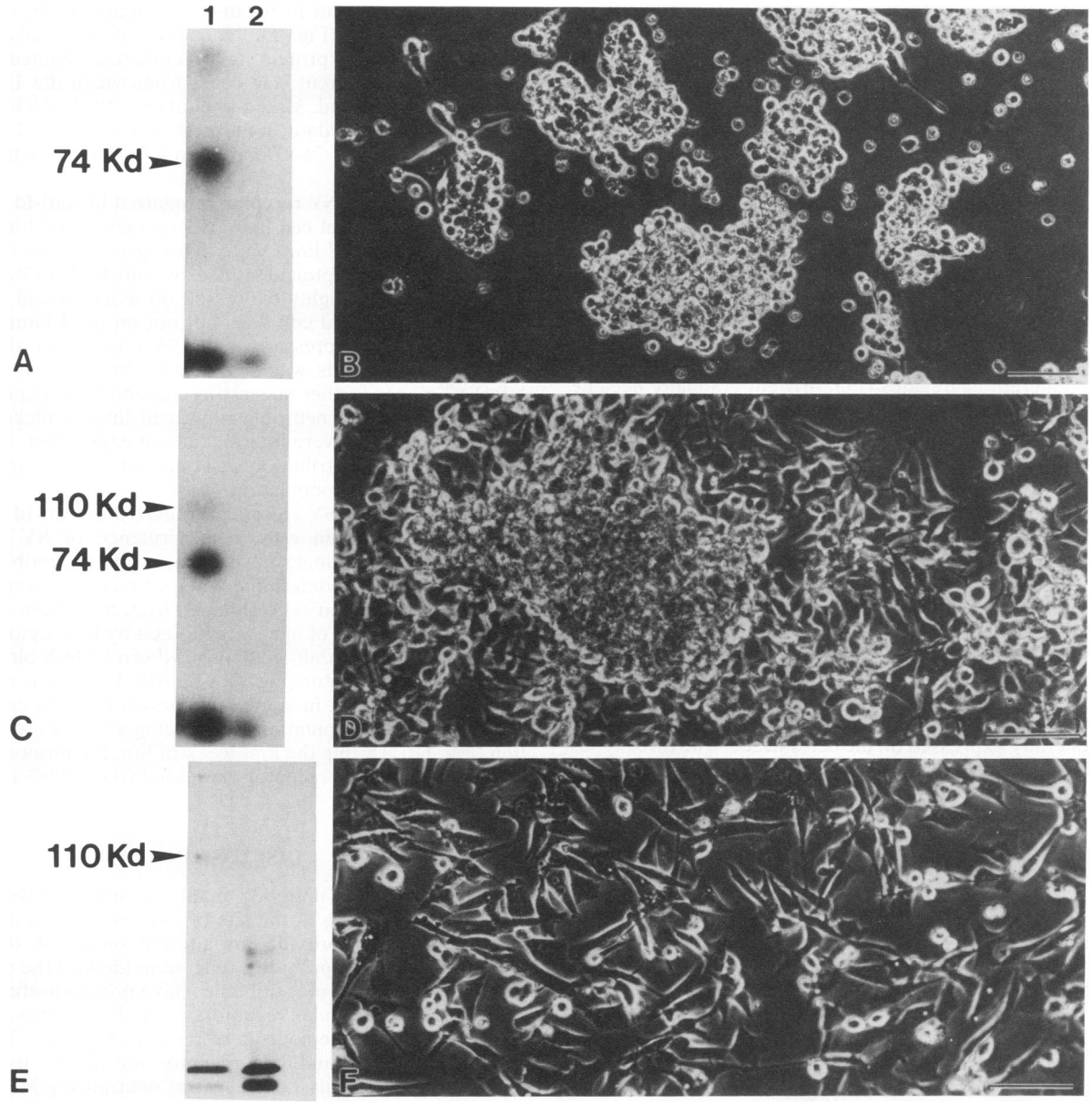

FIG. 4. Phenotypic change of mouse neuroblastoma cells (N18) and Sindbis virus receptor expression. Shown is immunoprecipitation of iodinated surface proteins of loosely adherent N18 cells (A and B), mixed-phenotype N18 cells (C and D), and adherent N18 cells (E and F). Lane 1, immunoprecipitation by anti-Id 209; lane 2, immunoprecipitation by anti-202. Bars = 50 μm.

Furthermore, the enzymatic data suggest that there is also a PL-sensitive receptor on BHK-21 and N18 cells in addition to the protease-sensitive receptor on N18 cells identified by anti-Id 209. It remains possible that a receptor on BHK-21 and N18 cells not identified by our anti-Id 209 is a phospholipid-linked protein, but we were unable to confirm this by using a PI-specific phospholipase. This is not conclusive evidence against a PI-linked protein, since different types of PI-linked proteins are sensitive to different sources of PI-PLC (23). It is possible that phospholipid is a structural part of a receptor or serves as an accessory molecule for receptor binding (1, 12, 36). Earlier studies suggested that phosphotidylethanolamine and cholesterol are important for SV hemagglutination through interaction with the E1 glycoprotein (18, 31). For rabies virus, a membrane phospholipid or glycolipid is used as a receptor in addition to the acetylcholine receptor (20, 21, 44). We suggest that SV uses more than one type of receptor for target cell binding. Whether these different receptors are equally efficient at mediating virus entry or whether one serves for initial attachment through the E1 glycoprotein, with subsequent entry mediated by another molecule binding through the E2 glycoprotein, is not clear.

SV can replicate in a variety of mammalian, avian, and insect cells in vivo and in vitro. Anti-Id 209 was used to survey a variety of neural and nonneural cell lines to determine the distribution of surface molecules reactive with this antibody. Essentially, all rodent neural and nonneural

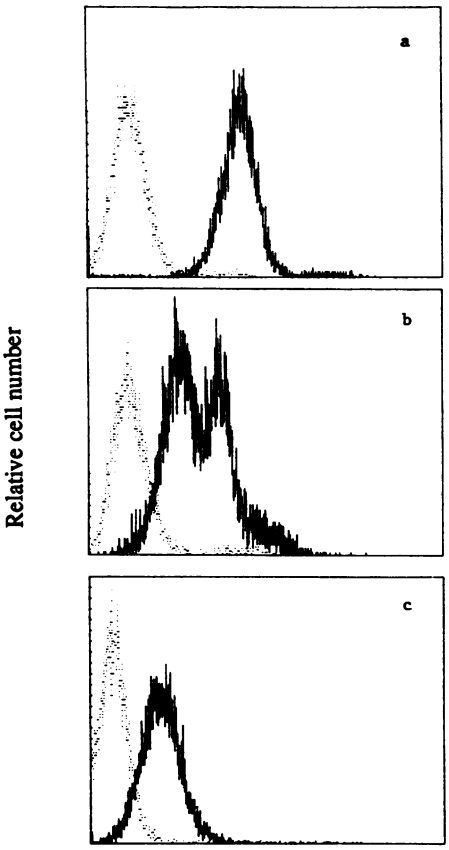

FIG. 5. Flow cytometry analysis of the amount of the receptor protein identified by anti-Id 209 on the surface of N18 cells differing in phenotype. (a) Nonadherent cells; (b) mixed adherent and nonadherent cells; (c) adherent cells. Solid lines represent cells stained with anti-Id 209; dotted lines represent cells stained with anti-202.

cell lines were positive, while most human neural and nonneural cell lines were negative. Chicken embryo fibroblasts and *A. albopictus* C6/36 cells were moderately positive, with about 50% scoring above background.

Although the receptor identified by our anti-Id 209 is not unique for neural cells, our data suggest that neural cells differ in their expression of this protein during development. Two clones, N18 and NB41A3, from the C-1300 mouse neuroblastoma cell line differed in expression, with N18 having greater expression. The N18 clone, identified by Smith and Tignor as having a large amount of a receptor for the AR86 neurovirulent strain of SV (39), does not synthesize acetylcholine or catechol (2) and binds anti-Id 209 well. NB41A3 has a more mature phenotype in culture, secretes acetylcholine, synthesizes choline acetylase and tyrosine hydroxylase, and binds anti-Id 209 poorly. Furthermore, surface expression of the candidate receptor differed in N18 cells of differing morphologic phenotypes. The 74-kDa protein was expressed in abundance on loosely adherent cells growing in aggregates, while the 110-kDa protein was expressed to a lesser extent on adherent cells growing as a monolayer. Whether these proteins represent two related molecules with differential splicing must await further inves-

TABLE 6. Binding of anti-Id 209 to neural and nonneural cell lines as assessed by flow cytometry

Cell line[a]	Origin	% of cells positive	
		Control antibody	Anti-Id 209
Neural			
IMR32	Human	0.9	15.3
SHEP-1	Human	0.2	3.8
SHEP-13	Human	4.4	64.2
SY5Y	Human	0.5	3.1
PC-12[b]	Rat	0.2	85.2
NB41A3	Mouse	9.9	22.6
N18	Mouse	4.8	97.5
Nonneural			
HSM[b]	Human	0.4	20.6
HSF[b]	Human	1.0	27.4
L cells	Mouse	4.0	84.0
MEF	Mouse	4.2	59.6
BHK-21	Hamster	0.4	1.6
CEF	Chicken	2.9	47.7
C6/36	Mosquito	8.2	50.4

[a] HSM, human skeletal muscle; HSF, human skin fibroblasts; MEF, mouse embryo fibroblasts; CEF, chicken embryo fibroblasts.

[b] Direct immunofluorescent staining using a fluorescein-conjugated normal rabbit Ig as a negative control antibody. All other cells were assessed by indirect immunofluorescence with anti-202 as the control.

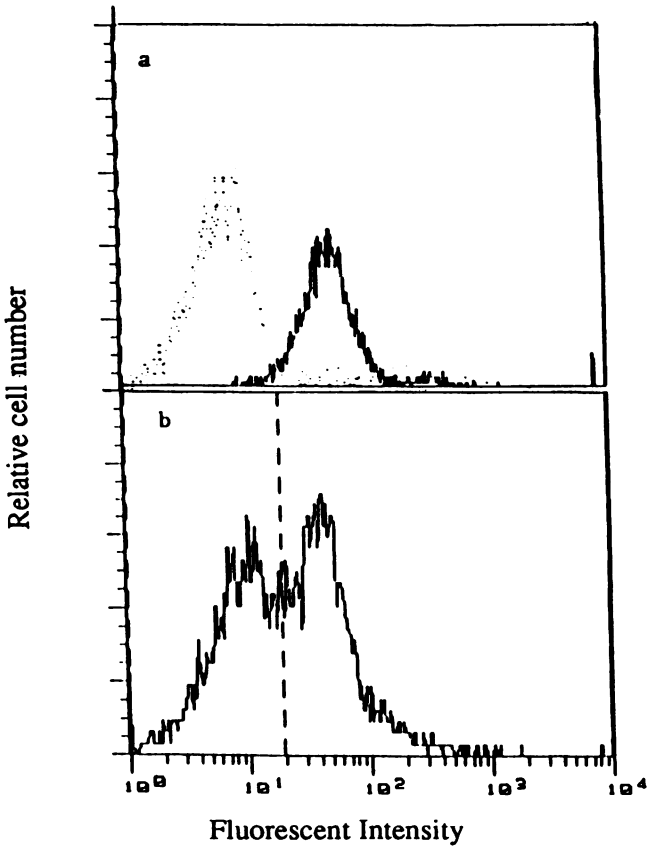

FIG. 6. Flow cytometry analysis of the expression of the receptor identified by anti-idiotypic antibody 209 on suckling mouse brain cells 16 h (a) and 96 h (b) after birth. Solid lines represent cells stained with anti-Id 209; dotted lines represent cells stained with anti-202.

tigation. Studies of newborn mouse brain also suggest that SV receptor protein expression is down-regulated with neural cell maturation. At birth almost all brain cells expressed this protein, but within a few days only half were positive.

The degree of neuronal maturity has been shown to be an important factor for neurotropism of a number of arthropod-borne viruses. SV replicates to a higher titer and causes more severe central nervous system infection in newborn mice than in more mature mice, and the virulence of SV for mice of different ages is altered by changes in the E2 glycoprotein (37, 41). Studies of Japanese encephalitis virus showed that after transplantation of embryonic neurons to adult rats, the virus infected the transplanted cells but not the mature neurons around the transplant (33). Since the expression of the SV receptor identified by anti-Id 209 decreased significantly within 48 h after birth, the amount of receptor available on the surface of neurons may be one of the factors which contribute to the increasing resistance to fatal SV encephalitis with age (14).

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