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Characterization of Saturable Binding Sites for Rabies Virus

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A specific, saturable receptor for rabies virus was analyzed on cultured cells of neural or non-neural origin. Viral attachment kinetics were enhanced by DEAE-dextran, an effect which in turn enhanced the apparent infectivity of the virus inoculum. Under optimized conditions, the attachment of metabolically labeled ERA strain rabies virus obeyed the laws of mass action, whereby the amount of virus bound to cells varied proportionally with the concentration of cells or virus. Attachment was sensitive to changes of temperature and pH, did not require divalent cations such as Mg^{2+} or Ca^{2+}, and occurred despite prior treatment of cells with proteolytic or sialic acid-specific enzymes. Saturation of the cell surface with rabies virus could be accomplished with 3×10^3 to 15×10^3 attached virions per cell. Competition for the rabies receptor occurred with rabies nonpathogenic variant virus, RV194-2, and vesicular stomatitis virus. Reovirus type 3, another neurotropic virus, failed to inhibit rabies virus binding, and West Nile virus only slightly inhibited rabies virus binding, suggesting independent cellular receptors were recognized by these viruses. Isolated rabies virus glycoprotein failed to compete in an equivalent manner. However, solubilization of BHK-21 cells with octylglucoside yielded a chloroform-methanol-soluble extract which blocked rabies virus attachment. The binding inhibition activity of this extract was resistant to proteases but could be destroyed by phospholipases and neuraminidase, suggesting a phospholipid or glycolipid component at the receptor site. These data provide evidence for a rhabdovirus-common mechanism for cellular attachment to cells in culture.

The attachment of viruses to specific receptors on the plasma membrane of host cells is an obligate step in productive infection and has been suggested to be a major determinant of virus-tissue tropism (7, 11, 18, 20, 36, 38). The existence of a limited number of specific cellular receptor sites (CRS) for a particular virus is suggested by the ability to demonstrate saturable binding (16 18, 22, 27, 32, 33, 35). The number of CRS has ranged between 5×10^2 and 5×10^5 in a variety of virus-cell systems (1, 17, 22).

Rabies virus is a strict neuropathogen in vivo (24), and yet it has a wide host range in vitro, infecting nearly all mammalian and avian cell types tested in primary cultures and continuous cell lines (4, 6, 39). Whether rabies virus uses a common receptor-mediated pathway for viral entry both in vivo and in vitro or instead recognizes more than one type of receptor on host cells is not known. A limited number of studies have suggested that the viral glycoprotein (G) mediates the attachment of rabies virus to cells (13, 26) and induces a pH-dependent fusion of membranes via adsorptive endocytosis (23, 28). A recent study has suggested that acetylcholine receptors may serve as CRS for rabies virus on differentiated myotubes in vitro and at neuromuscular junctions in vivo to facilitate uptake and transfer of virus to the central nervous system (15). However, not all cells susceptible to rabies virus infection in vitro express acetylcholine receptors for virus attachment (K. J. Reagan and W. H. Wunner, *in* E. Kuwert, ed., *Rabies in the Tropics*, in press), suggesting a difference in the nature of the receptor for rabies virus-host cell binding in vitro and in vivo. We describe studies here that establish the specificity of rabies virus receptor based on the saturability of the cellular receptors for rabies virus and on competitive binding. We also report experiments that suggest that the CRS for rabies virus in vitro has a lipid component.

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MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of hamster-derived BHK-21 clone 13 cells (34) and mouse neuroblastoma C1300 clone NA (NA) cells (5) were grown at 37°C in Eagle minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) as previously described (5). The ERA and CVS strains of fixed rabies virus (5, 6) and the rabies variant 194-2 (RV194-2) virus (10) have been described previously. Vesicular stomatitis virus (VSV), Indiana serotype, was provided by T. J. Wiktor (The Wistar Institute). Rabies viruses and VSV were propagated in BHK-21 cells infected at a multiplicity of infection of 0.1. Infected cultures were fed with MEM supplemented with 0.2% bovine serum albumin (MEM–0.2% BSA) as previously described (5). Rabies virus was labeled with [^{14}C]leucine (2 μCi/ml), [^{3}H]leucine (25 μCi/ml), or [^{35}S]methionine (20 μCi/ml) added to MEM–0.2% BSA in place of unlabeled leucine or methionine, respectively. Specific activities of 1.6×10^5 to 7.5×10^5 cpm per μg of virus protein were obtained. Rabies viruses and VSV were purified (40), collected after a single cycle of sedimentation through a sucrose gradient, and stored at −70°C or at 4°C after thawing once. Reovirus type 3, purified by isopycnic centrifugation in cesium chloride, was provided by J. Gentsch (University of Pennsylvania Medical School), and West Nile virus (WNV; the replication-efficient variant, was provided by M. A. Brinton (The Wistar Institute) and grown as described previously (3). In addition, replication-efficient WNV was purified in 20 to 70% (vol/vol) glycerol in 0.1 M NaCl–0.01 M EDTA–0.05 M Tris-hydrochloride (pH 7.5).

Purification and labeling of rabies virus G. Viral G, kindly provided by B. Dietzschold (The Wistar Institute), was extracted from ERA strain rabies virus and purified as

previously described (8). Soluble glycoprotein (G_s) was purified from virion-depleted, rabies virus-infected tissue culture fluid (9). Purified G and G_s were labeled with [^{125}I]iodine by the IODO-GEN method as described elsewhere (21).

Virus attachment assay. Standard attachment conditions utilized a suspension culture of BHK-21 cells (0.5×10^7 to 1×10^7 cells per ml) in siliconized vessels and attachment medium of MEM–0.2% BSA containing 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4, and DEAE-dextran (DEAE-D; 50 μg/ml). Volumes of the suspension cultures varied according to the type of assay as follows: 200 μl was used for direct binding and competition assays, whereas 1-ml batch cultures were used for viral attachment kinetic studies. In direct binding and competition assays, 10 μl of diluted radiolabeled virus probe was added directly to cells, bringing the final volume to 200 μl. Sufficient radiolabeled virus probe was added to the incubation medium to provide 5.5×10^3 cpm of ^{14}C label and 20×10^3 to 50×10^3 cpm of ^{3}H or ^{35}S label per assay sample. Batch cultures received 5 μl of undiluted radiolabeled virus probe, and at designated intervals, samples of 1×10^6 to 2×10^6 cells in 200 μl were assayed for bound radioactivity. Unattached virus was separated from cell-associated virus by a 10-s centrifugation in an Eppendorf microcentrifuge. Cell pellets were washed once in 200 μl of attachment medium and repelleted. Bound virus (cell associated), free virus (in original culture fluid plus wash), and cells resuspended in phosphate-buffered saline (PBS) were assayed by precipitation in 10% trichloroacetic acid with carrier BSA at 4°C. Precipitates were hydrolyzed in 0.15 M NaOH, and radioactivity was determined by liquid scintillation spectrometry (19). In saturation binding and competition studies, unlabeled virus, *n*-octylglucoside (OG) extract, or lipid fractions (see below) in attachment medium were added to cells ca. 10 min before the addition of radiolabeled rabies virus probe. A determination of virus attachment to cells in the presence of competing substances was made as described above. The amount of nonspecific attachment of radiolabeled viral probe to cells in these experiments was determined from the counts bound to cells in the presence of oversaturating amounts of unlabeled rabies virus relative to adsorption isotherm curves established for the system. Counts considered nonspecifically attached were subtracted from all calculations of viral counts bound.

Preparation of OG extract. Monolayer cultures of BHK-21 cells just before confluence were washed with PBS and removed from culture vessels by incubating the cells with 0.25% trypsin and 0.02% EDTA for 5 min at 37°C. Cells were washed three times at 4°C with cold PBS and treated for 2 min on ice with 50 mM octyl-β-D-glycopyranoside (Sigma Chemical Co.) in PBS at a ratio of 3.1 ml per g wet weight of cells as described by Schlegel et al. (31). The cellular extract was centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant, designated OG extract, was then dialyzed at 4°C with 1,000 equivalent volumes of PBS and stored at −70°C. OG extract was treated with chloroform-methanol (3:1) (30) to obtain a protein-free lipid fraction which was stored at −70°C under nitrogen.

Enzyme studies. BHK cells were treated with various enzymes as follows. Confluent monolayers of cells were dispersed by incubation at 37°C in 12 mM lidocaine in 10% FCS–MEM. Cells (2.5×10^6) were washed twice in PBS at 4°C and resuspended to a concentration of 5×10^5 cells per ml in the enzyme solution. Enzymes were each prepared in PBS at the appropriate pH (see below). A digestion of 30 min at 37°C was allowed, followed by quenching of the enzyme activity by the addition of 10% FCS–MEM. Cells were washed twice in 10% FCS–MEM and once in attachment medium. Viable cell counts were performed and appropriate numbers of cells were distributed among reaction vessels. Attachment of radiolabeled probe was performed as described above. Enzymes tested included trypsin [L-(tosylamido 2-phenyl)ethylchloromethyl ketone; 0.1%, pH 8.0; Worthington Diagnostics], chymotrypsin (0.1%, pH 8.0; Sigma), and neuraminidase (type V; 0.2 U/ml, pH 6.0; Sigma).

Dialyzed OG extract (capable of inhibiting rabies virus binding to BHK-21 cells by ca. 80%) was diluted in saline buffered at the pH values cited above for the various enzymes. In addition to the trypsin, chymotrypsin, and neuraminidase described above, phospholipases A_2, C, and D (5 U/ml each in PBS, pH 7.4; Sigma) were tested. OG extract was digested with enzymes for 1 h at 37°C. Enzyme activity was stopped by heating the digestion mixture to 100°C for 10 min. Samples were neutralized and added to cells. Attachment of radiolabeled virus was assayed as above.

RESULTS

Optimizing rabies virus binding to cells in culture. The interaction of virus particles with receptors on the cell surface is dependent upon a number of physical conditions including temperature, pH, virus concentration, and receptor density. To define specific CRS for rabies virus on BHK cells, a standard assay was developed. Attachment was first examined with suspensions of BHK-21 cells by adding radiolabeled virus probe in the presence of polyions (DEAE-D or dextran sulfate) (Fig. 1). DEAE-D had an enhancing effect on the initial interaction between virus and cell, in accordance with an earlier report by Kaplan et al. (14). A dose-response curve (not shown) demonstrated that 25 to 50 μg of DEAE-D per ml reproducibly enhanced the binding of radiolabeled virus to cells in 30 min at 37°C. Another polyion, dextran sulfate (50 μg/ml), was compared to DEAE-

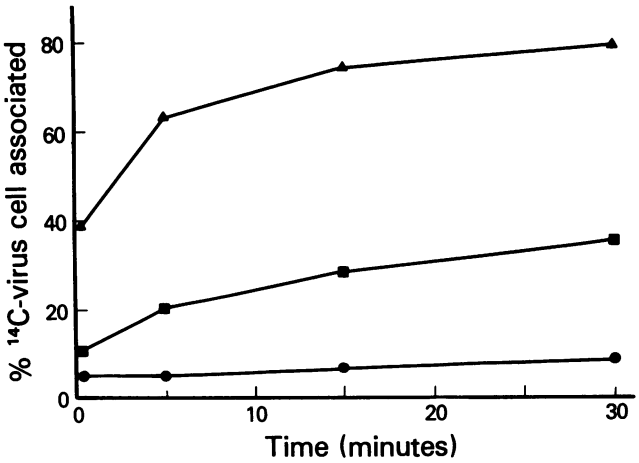

FIG. 1. Kinetics of ERA virus attachment to BHK-21 cells in the presence or absence of polyions. BHK-21 cells (10^7 cells per ml) were suspended in attachment medium alone (●) or with the addition of dextran sulfate (▲), or DEAE-D (■) at 50 μg/ml. [^{14}C]leucine-labeled ERA virus (5×10^4 cpm) was added to the cell culture and incubated at 37°C, and samples were taken at timed intervals and assayed for cell-associated virus as described in the text.

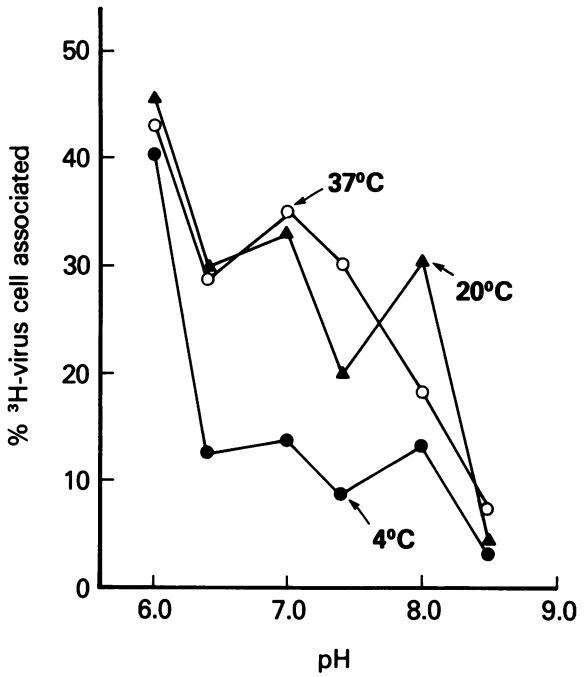

FIG. 2. Effect of temperature and pH on the attachment of ERA virus to BHK-21 cells. BHK-21 cells (5×10^6 cells per ml) were suspended in attachment medium buffered with 50 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.0 to 6.5), HEPES (pH 7.0 to 7.5), or Tricine (pH 8.0 to 8.5). [^{3}H]leucine-labeled ERA virus (2×10^4 cpm per sample) was added to the cells and incubated at the designated temperature for 30 min. The percentage of input virus which became cell associated was determined as described in the text.

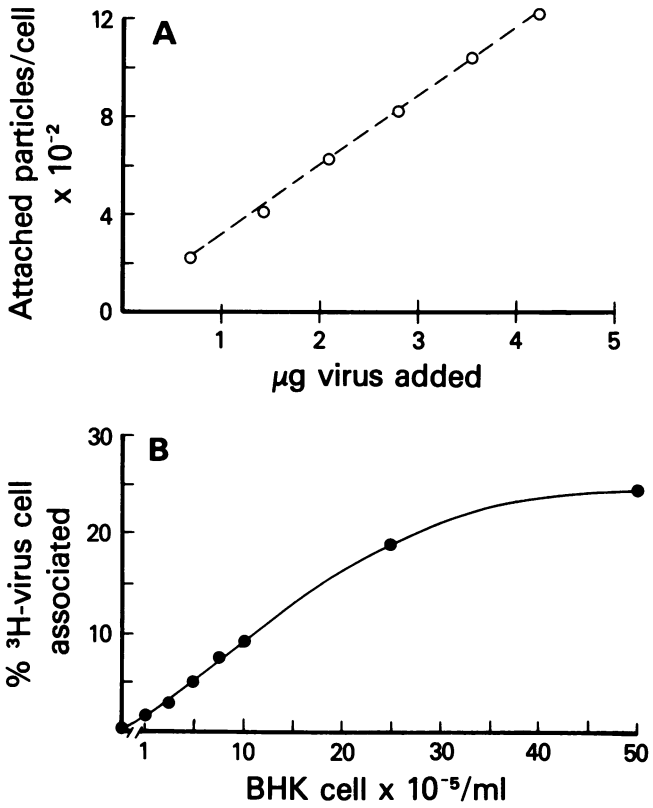

FIG. 3. Effect of alterations of input virus or cell concentrations on the attachment of ERA virus to BHK-21 cells. (A) BHK-21 cells at 10^7 cells per ml were suspended in attachment medium containing increasing amounts of [^{3}H]leucine-labeled ERA virus (1.6×10^5 cpm/μg of protein). After an incubation of 30 min at 37°C, cells were separated from the reaction mixture, and the virus particles attached per cell were determined (assuming 1.75×10^9 particles per μg of viral protein). (B) BHK-21 cells (0 to 5×10^6 cells per ml) were suspended in attachment medium containing 2×10^4 cpm of [^{3}H]leucine-labeled ERA virus. Attachment proceeded for 30 min at 37°C, and the percentage of input virus which became cell associated was determined.

D for its effect on the binding of rabies virus to the cell substrate. Unlike DEAE-D, the enhanced binding of virus probe (80% of input radiolabel) in the presence of dextran sulfate was attributed to a high degree of nonspecific binding, some of which represented binding to the plastic surface (40%). Indeed, only DEAE-D induced a significant biological enhancement of virus attachment to cells. This was indicated by a fivefold increase in viral antigen in ERA virus-infected cells as detected by immunofluorescence and a twofold increase in the number of plaques and the plaque size formed in strain CVS-infected cells. Moreover, the ability to demonstrate saturation of CRS when binding virus to cells in the presence of DEAE-D (as described below) attested to its biological enhancement of virus attachment to cells. In contrast, dextran sulfate-treated cells did not exhibit saturable binding of rabies virus and, moreover, inhibited antigen production and virus plaque titer.

Cells pretreated with DEAE-D before virus attachment exhibited a similar enhanced binding (data not shown), suggesting that the enhancement of virus attachment was due to an effect on the plasma membrane rather than on the virus. We did not determine whether the action of DEAE-D on the cell membrane is imparted solely by its ionic (positive) charge, whether it acts as a ligand bridging virus to cell surface receptors, or whether it induces a conformational change in the plasma membrane which in turn facilitates virus attachment. Electron microscopic examination of virus pretreated with DEAE-D (50 μg/ml) showed no aggregation of virus (data not shown).

The effects of temperature and pH on attachment of rabies virus to cells were measured at 4, 20, and 37°C (Fig. 2). The amount of attachment of [^{3}H]leucine-labeled virus to cells at 20 and 37°C was greater than that at 4°C. The effect of pH on binding is also illustrated in Fig. 2, which shows increased binding at pH lower than 7.4. The least binding was observed at pH 8.5. Since the enhanced binding at pH 6.5 did not correlate with an increase in infection of cells as measured by immunofluorescence, it was suggested that the enhanced virus binding at low pH was nonbiological. Divalent cations such as Ca^{2+} and Mg^{2+}, which may change the electrostatic interaction between ligand and receptor (7), did not affect rabies virus attachment at 1.8 and 0.8 mM, respectively, since virus attached to cells equally well when MEM was replaced with Ca^{2+}- and Mg^{2+}-free PBS in attachment medium (data not shown).

To demonstrate that attachment of rabies virus to susceptible cells under our binding conditions obeyed the laws of mass action, experiments were performed in which the concentrations of the individual interacting components were increased independently of each other, and the binding of virus particles to cells were measured. First, ^{3}H-labeled ERA strain rabies virus was mixed in increasing concentrations with a constant number of DEAE-D-pretreated BHK-21 cells (10^7 per ml) (Fig. 3A). The number of virus particles

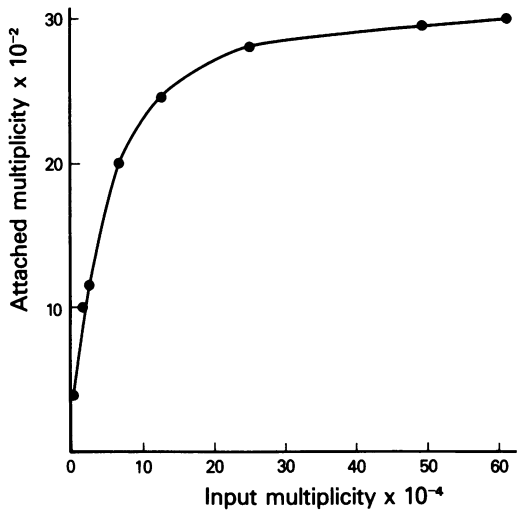

FIG. 4. Saturation of BHK-21 cells with ERA virus. Individual samples of BHK-21 cells (10^6 cells per 0.2 ml) were suspended in attachment medium containing rabies virus preparations of varied specific activity, which were created by the mixture of purified [^{3}H]leucine-labeled ERA virus with purified, unlabeled ERA virus. The amount of bound virus was determined after a 3-h incubation at 4°C.

(calculated from the specific activity of the ^{3}H-labeled virus probe) attached per cell in 30 min at 37°C increased linearly as the ratio of virus to cell concentration ([V]/[C]) increased. Inversely, by keeping the virus concentration constant and increasing the cell concentration from 1×10^5 to 5×10^6 cells per ml ([C]/[V]), the percentage of ^{3}H-labeled virus that became cell-associated after 30 min at 37°C increased to a maximum of 25% with 5×10^6 cells (the number of cells used in the standard assay) (Fig. 3B). No attempt was made in these experiments to distinguish specific from nonspecific viral attachment.

Demonstration of specificity in rabies virus-host cell interaction. Specific viral recognition sites on the cell surface could be defined for rabies virus in our cell culture system by demonstrating saturation and competition in the virus-receptor interaction. Saturation was demonstrated by binding ^{3}H-labeled ERA strain rabies virus probe to cells at 4°C (to prevent penetration) in the presence of increasing concentrations of unlabeled homologous virus particles. Samples were taken when apparent binding equilibrium was reached (3 h). By increasing the amounts of unlabeled virus (up to 357 μg) added to radiolabeled viral probe (the maximum ratio represented a 250-fold unlabeled virus excess), we observed a reduced percentage of cell-associated probe. The counts (190 cpm; 15% of the cell-associated radiolabel) that attached in the presence of a 500-fold excess (over probe) of unlabeled virus were considered nonspecifically attached and were subtracted from all values. From these data, the density of cell membrane receptors that specifically attached rabies virus was estimated from the adsorption isotherm shown in Fig. 4. By plotting attached multiplicity (virus particles per cell) versus input multiplicity, the level at which linear attachment reaches a plateau corresponds to the number of membrane sites which become saturated in the presence of excess amounts of virus (19, 20). In five separate experiments it was estimated that saturation of specific receptors for rabies virus was complete at ca. 3×10^3 to 15×10^3 attached virions per cell. Counts representing nonsaturable binding ranged between 15 and 19% of cell-associated counts. When ^{125}I-labeled purified viral G (viral attachment protein), or G_s (viral attachment protein minus its cytoplasmic, and most of the putative transmembrane, domains [9]) was used as probe, addition of unlabeled ERA strain virus had no effect on the percentage of cell-associated G or G_s probe, indicating a dissimilar cellular interaction of isolated G.

Another criterion for receptor specificity is based on competition for receptors by related or unrelated viruses. In these experiments, excess unlabeled ERA strain rabies virus (homologous competition), ERA strain nonpathogenic variant virus RV194-2, and VSV all competed with ^{3}H-labeled ERA strain virus probe for receptor sites on BHK-21 and mouse NA cells, causing 65 to 85% inhibition of probe with 100 μg of competing virus per ml (Table 1). In separate experiments, WNV, an enveloped neurotropic flavivirus, demonstrated only slight inhibition of viral probe attachment at the highest WNV concentration. These data suggest that WNV does not share receptors with rabies virus and VSV. Reovirus type 3, a nonenveloped neurotropic virus at 100 μg/ml did not inhibit attachment of viral probe, but rather enhanced the efficiency of rabies virus attachment.

Biochemical nature of the cellular binding site for rabies virus. To determine the type of virus-cell interaction that is responsible for the binding in vitro of rabies virus to specific cell surface receptors, the following modifiers of virus-cell interactions were examined. Cells were predigested for 30 min at 37°C with trypsin (0.1%), chymotrypsin (0.1%) or neuraminidase (1 U/ml) (Table 2). Rabies virus attachment to these cells was practically unaffected. Pursuant to the recent report by Schlegel et al. (31) of a soluble extract of cells with receptor activity for VSV, an OG extract was prepared from BHK-21 cells and tested for its ability to inhibit binding of radiolabeled rabies virus (Fig. 5). The addition of increasing

TABLE 1. Competitive inhibition by viruses of binding radiolabeled ERA virus to BHK and NA cells

Virus added (μg)	% Cell-associated rabies virus[a]		
	BHK		NA
	Expt 1	Expt 2	
Control	8.3 (0)	8.9 (0)	11.7 (0)
ERA			
10	5.0 (40.3)	4.9 (44.3)	7.4 (36.2)
100	2.9 (65.3)	2.4 (73.5)	2.3 (80.6)
RV194-2			
10	2.3 (72.8)		4.3 (63.4)
100	1.3 (85.0)		3.5 (69.8)
VSV			
10	5.4 (34.9)		7.4 (36.8)
100	1.9 (76.7)		2.1 (82.3)
WNV			
10		9.2 (0)	ND[b]
100		6.5 (27.1)	ND
Reovirus 3			
10	47.8 (0)		ND
100	48.8 (0)		33.8 (0)

[a] Cells (10^6 per 0.2 ml) were preincubated for 30 min at 37°C with 50 μg of DEAE-D per ml. These cells were then suspended in attachment medium containing the cited viruses. Cells were incubated with the viruses for 10 min at 4°C before the addition of ca. 2.5×10^4 cpm of [^{3}H]leucine-labeled ERA. Attachment was allowed to proceed for 2 h at 4°C, and the trichloracetic acid-precipitable counts that became cell associated were determined as described in the text. The numbers in parentheses are the percent inhibition in comparison to the control.

[b] ND, Not done.

TABLE 2. Effect of enzymatic digestion of host cells on the attachment of rabies virus[a]

Enzyme	Cell-associated rabies virus (cpm)[b]	% Of control
PBS (control)	7,227	100
Trypsin	5,628	77.9
Chymotrypsin	7,007	96.9
Neuraminidase	6,678	92.4

[a] BHK-21 cells (2.5×10^6 per 2.5 ml) were treated with various enzymes as described in the text. Washed, viable cells in attachment medium were assayed in duplicate for the ability to bind 2×10^4 cpm of [^{3}H]leucine-labeled ERA at 37°C for 0.5 h.

[b] The counts per minute binding in the presence of 200 μg of unlabeled ERA virus (385 cpm) was considered nonspecifically attached and was subtracted from all values.

amounts of the OG extract decreased the ability of virus to become cell associated. This effect occurred in the presence or absence of DEAE-D. To characterize the chemical nature of the attachment-inhibiting fraction, the OG extract was digested with a variety of enzymes (Table 3). Proteolytic enzymes (at an enzyme-to-substrate ratio of 1:50) had no effect on the inhibitory activity of OG extract as was indicated with whole cells. Denaturing conditions such as repeated freezing and thawing also had no effect. Unlike the results obtained with whole cells, predigestion of OG extract with neuraminidase totally eliminated its binding inhibitor activity. Phospholipase C, and to a lesser extent phospholipases A_2 and D, partially reduced OG attachment inhibitor activity. The chloroform-methanol lipid fraction from OG extract contained some of the inhibitory activity. It was concluded on the basis of resistance to proteases, sensitivity to phospholipases, and solubility in chloroform-methanol that the binding inhibitor in the OG extract was due to a phospholipid as shown for VSV (31). It is not known whether this phospholipid fraction constitutes the specific CRS.

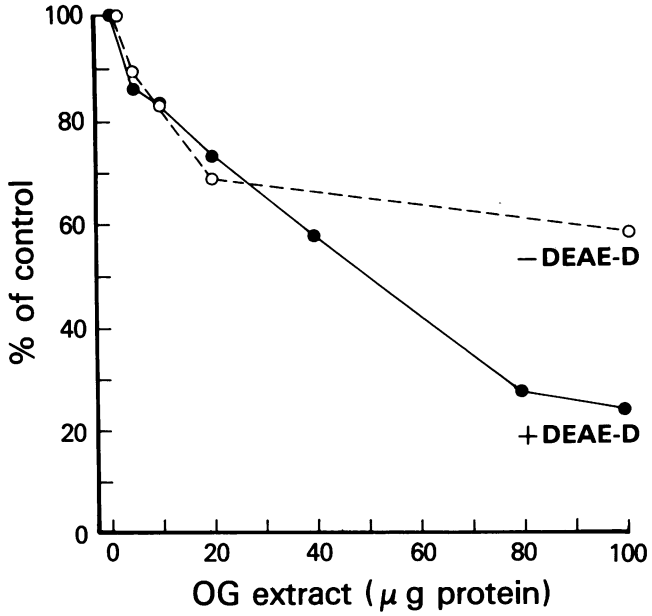

FIG. 5. Inhibition of ERA virus binding by an OG extract from BHK-21 cells. BHK-21 cells (5×10^6 cells per ml) were suspended in attachment medium with or without 50 μg of DEAE-D per ml. Increasing amounts of dialyzed OG extract from BHK-21 cells were added to the cell suspension before the addition of [^{35}S]methionine-labeled ERA virus (3.5×10^4 cpm per sample). The amount of virus bound after a 3-h incubation at 4°C was determined and expressed as a percentage of a control culture which received no OG extract.

TABLE 3. Effect of enzymatic pretreatment or lipid extraction on the ability of OG extract to inhibit rabies virus binding to BHK-21 cells[a]

Treatment	Cell-associated cpm[b]	% Inhibition
^{35}S-labeled ERA virus	10,194	0
+ OG extract	1,997	80.4
+ Chloroform-methanol extract (20 μl) of OG extract	6,161	39.6
+ OG extract, freeze-thawed	1,293	87.3
+ OG extract + neuraminidase	13,811	0
+ OG extract + trypsin	−200[c]	100
+ OG extract + chymotrypsin	840	91.8
+ OG extract + phospholipase A_2	3,380	47.2
+ OG extract + phospholipase C	8,502	16.6
+ OG extract + phospholipase D	5,762	43.5

[a] OG extract (99.3 μg of protein) was treated with various enzymes as described in the text. After a 100°C incubation to inactivate the enzymes, the OG extract was added to 10^6 DEAE-D-pretreated BHK-21 cells per 0.2 ml final volume and incubated for 10 min at 4°C before the addition of ca. 5×10^4 cpm of [^{35}S]methionine-labeled ERA virus. Cell-associated counts after 2 h at 4°C were determined.

[b] The counts per minute binding in the presence of 200 μg of unlabeled ERA virus (5,937 cpm) was considered nonspecifically attached and was subtracted from all values.

[c] Cell-associated counts accounted for less than was considered nonspecifically attached (see footnote *b*).

DISCUSSION

We conclude from these experiments that rabies virus binds to specific receptors on the plasma membrane of cells in vitro. Two major criteria for receptor specificity (saturability and competition) were satisfied in these studies to verify the existence of a finite number of CRS available for rabies virus in a cell culture system. Viral saturation of distinct binding sites on the cell surface is one of two independent modes of attachment in virus-cell interactions. The other is a nonsaturable (generally a low-affinity) binding of virus to cell surfaces, which may be variable and can represent a significant amount of virus adsorption, especially at high virus input multiplicities (26, 32, 36). One presumes that the specific, higher-affinity receptor is utilized preferentially under natural conditions of low virus concentration, although we cannot rule out the possibility that virus bound to nonsaturable sites might nonetheless become internalized and productively uncoated.

An alteration of the electrostatic environment can dramatically alter the amount of virus which binds to cell surfaces (Fig. 1). It is clear, however, that electrostatic attraction alone does not result in productive infection, since both dextran sulfate and DEAE-D increased cell-associated virus, whereas only DEAE-D increased infectivity. Thus, although DEAE-D certainly binds to a variety of negatively charged cell surface components (2a), it cannot be concluded from the present experiments that the enhanced binding is nonspecific.

Scatchard analysis for information such as affinity constants (K_a) can only be valid if all criteria on which it is based have been verified for any given virus-cell system (12). Of critical concern is the multivalency of the viral attachment protein per virus particle in the binding to CRS. The equation upon which the Scatchard plots are based assumes only a single link between ligand and receptor. Only in this case can K_a be determined. Thus, in virus attachment studies where the effect of multivalency in the binding reaction is not considered, theoretical interpretation of data derived from Scatchard analysis is not clear. Instead, we have estimated the number of CRS that specifically attach rabies virus from the relationship between input multiplicity and attachment multiplicity (19). Saturation was complete with ERA strain rabies virus at ca. 3×10^3 to 15×10^3 attached virions per BHK-21 cell.

Our findings also suggest that rabies virus binds to receptors which may serve to attach rhabdoviruses in general. The similarities between rabies virus (prototype of *Lyssavirus* genus) and VSV (prototype of *Vesiculovirus* genus) in the number of saturable binding sites on the cell surface, the chemical nature of the receptor, and the factors that influence the rate of attachment to cell surface receptors imply that a receptor exists for a family of related viruses. Moreover, we have demonstrated that VSV blocks rabies virus attachment to saturable cell surface receptors to the same extent as homologous virus inhibits attachment to receptors (Table 1). There are ca. 4×10^3 high-affinity, saturable binding sites for VSV on the surface of Vero cells (32), which are apparently involved in VSV entry into cells (25). Pretreatment of cells with trypsin (31, 37) and omission of Mg^{2+} or Ca^{2+} from the incubation medium (37) had little or no effect on VSV or VSV G binding. Cells pretreated with phospholipase A, on the other hand, produced a reduction in VSV binding to the cells (37), and phosphatidylserine extracted from cell membranes specifically inhibited VSV binding and infectivity (31). All of our experiments which characterized rabies virus binding and demonstrated virus-binding inhibition, including the use of an OG extract, produced results similar to those observed with VSV, suggesting that the two viruses interact with a similar component in the cellular binding site. Our results did not allow us to identify a phospholipid, such as the one detected in VSV binding studies, as the specific cellular receptor component for rabies virus. The identification of a specific lipid component(s) and its possible role in the neurotropism of rabies virus in vivo is under investigation.

In searching for a possible explanation for the strict neurotropism that rabies virus exhibits in vivo, it is worth noting that attenuated rabies virus, represented by the ERA variant virus selected by monoclonal antibody 194-2 (10), recognizes and competitively binds to the same "receptor" as does the wild-type virulent virus in cell culture. This suggests that the receptor site utilized in culture does not differentiate between viruses of pathogenic and nonpathogenic phenotype and that the pathogenic discrimination in vivo may be at a level subsequent to initial viral attachment. Similarly, rabies virus does not appear to discriminate between neural (mouse neuroblastoma) and non-neural (hamster kidney) cells in culture, suggesting that CRS of similar composition exist on the surface of these cell types. The relationship of these findings to the suggestion that nicotinic acetylcholine receptors may serve as the rabies virus CRS (15) is unclear.

The specific binding of rabies virus is probably mediated by rabies virus G (13, 26), yet the binding characteristics of purified G appear to be quite different from those of intact virions. Our results of blocking experiments with unlabeled virus and radiolabeled purified rabies virus G or G_s probe are in agreement with results described for VSV (37) and recently for rabies virus (26), which showed that isolated G failed to inhibit specific binding of virus. We also studied the inhibitory effect of G in virosomes reconstituted from viral lipids, and whereas attachment inhibition of radiolabeled viral probe was observed, the specificity of inhibition by G may not be valid since the radiolabeled virus probe may have interacted with a lipid component in the virosome rather than with the cellular receptor. However, one might speculate that the virion, with its lipid-containing envelope, holds the glycoprotein in a conformation suitable for attachment. This conformation may be lost upon glycoprotein isolation.

The sequence homology between the predicted amino acid sequences of rabies virus G (2) and VSV G (30) has been examined to find ancestral relationships between these two family-related viruses (29). Certain regions of the proteins show greater than 50% homology, although the overall sequence homology is 20% between these two glycoproteins. The identical positioning of one of the putative glycosylation sites and the similar positioning of several cysteine residues in both proteins show that portions of these molecules are highly conserved. It should be possible to determine whether conserved domains in each virus G are responsible for the viral attachment to common CRS.

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LITERATURE CITED

1. **Altrock, B. W., L. O. Arthur, R. J. Massey, and G. Schochetman.** 1981. Common surface receptors on both mouse and cells distinguish different classes of mouse mammary tumor viruses. Virology **109**:257–266.
2. **Anilionis, A., W. H. Wunner, and P. J. Curtis.** 1981. Structure of the glycoprotein gene in rabies virus. Nature (London) **294**:275–278.

2a. **Bailey, C. A., D. K. Miller, and J. Lenard.** 1984. Effects of DEAE-dextran on infection and hemolysis by VSV. Evidence that nonspecific electrostatic interactions mediate effective binding of VSV to cells. Virology **113**:111–118.

3. **Brinton, M. A., and A. V. Fernandez.** 1983. A replication-efficient mutant of West Nile virus is insensitive to DI particle interference. Virology **129**:107–115.
4. **Clark, H. F.** 1979. Systems for assay and growth of rhabdoviruses, p. 23–41. *In* D. H. L. Bishop (ed.), Rhabdovirus, vol. 1. CRC Press, Boca Raton, Florida.
5. **Clark, H. F., N. F. Parks, and W. H. Wunner.** 1981. Defective interfering particles of fixed rabies viruses: lack of correlation with attenuation or auto-interference in mice. J. Gen. Virol. **52**:245–258.
6. **Clark, H. F., and T. J. Wiktor.** 1972. Rabies virus, p. 177–182. *In* S. A. Plotkin (ed.), Strains of human viruses. S. Karger, Basel.
7. **Crowell, R. L., and B. J. Landau.** 1979. Receptors as determinants of cellular tropism in picornavirus infections, p. 1–33. *In* A. G. Bearn and P. W. Choppin (ed.), Receptors and human diseases. Josiah Macy, Jr., Foundation, New York.
8. **Dietzschold, B., J. H. Cox, L. G. Schneider, T. J. Wiktor, and H. Koprowski.** 1978. Isolation and purification of a polymeric form of the glycoprotein of rabies virus. J. Gen. Virol. **40**:131–139.
9. **Dietzschold, B., T. J. Wiktor, W. H. Wunner, and A. Varrichio.** 1983. Chemical and immunological analysis of the rabies soluble glycoprotein. Virology **124**:330–337.

10. **Dietzschold, B., W. H. Wunner, T. J. Wiktor, A. D. Lopes, M. Lafon, C. L. Smith, and H. Koprowski.** 1983. Characterization of an antigenic determinant of the glycoprotein which correlates with pathogenicity of rabies virus. Proc. Natl. Acad. Sci. U.S.A. **80**:70–74.
11. **Holland, J. J.** 1961. Receptor affinities as major determinants of enterovirus tissue tropisms in humans. Virology **15**:312–326.
12. **Incardona, N. L.** 1981. The chemical nature of virus-receptor interactions, p. 155–168. *In* K. Lonberg-Holm and L. Philipson (ed.), Virus receptors, part 2, animal virus. Receptor and recognition series B, vol. 8. Chapman and Hall, London.
13. **Iwasaki, Y., T. J. Wiktor, and H. Koprowski.** 1973. Early events of rabies virus replication in tissue cultures. An electron microscopic study. Lab. Invest. **28**:142–148.
14. **Kaplan, M. M., T. J. Wiktor, R. F. Maes, J. B. Campbell, and H. Koprowski.** 1967. Effect of polyions on the infectivity of rabies virus in tissue culture: construction of a single-step growth curve. J. Virol. **1**:145–151.
15. **Lentz, T. L., T. G. Burrage, A. L. Smith, J. Crick, and G. H. Tignor.** 1982. Is the acetylcholine receptor a rabies virus receptor? Science **215**:182–184.
16. **Linser, P., H. Bruning, and R. W. Armentrout.** 1977. Specific binding sites for a parvovirus, minute virus of mice, on cultured mouse cells. J. Virol. **24**:211–221.
17. **Lonberg-Holm, K.** 1981. Attachment of animal viruses to cells. An introduction, p. 1–20. *In* K. Lonberg-Holm and L. Philipson (ed.), Virus receptors, part 2, animal viruses. Receptors and recognition series B, vol. 8. Chapman and Hall, London.
18. **Lonberg-Holm, K., R. L. Crowell, and L. Philipson.** 1976. Unrelated animal viruses share receptors. Nature (London) **259**:679–681.
19. **Lonberg-Holm, K., and B. D. Korant.** 1972. Early interaction of rhinovirus with host cells. J. Virol. **9**:29–40.
20. **Lonberg-Holm, K., and L. Philipson.** 1974. Early interaction between animal viruses and cells. Monogr. Virol. **9**:1–148.
21. **Markwell, M. A. K., and C. F. Fox.** 1978. Surface-specific iodination of membrane proteins of viruses and eucaryotic cells using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril. Biochemistry **17**:4807–4817.
22. **McClintock, P. R., L. C. Billups, and A. L. Notkins.** 1980. Receptors for encephalomyocarditis virus on murine and human cells. Virology **106**:261–272.
23. **Mifune, K., M. Ohuchi, and K. Mannin.** 1982. Hemolysis and cell fusion by rhabdoviruses. FEBS Lett. **137**:293–297.
24. **Murphy, F. A.** 1977. Rabies pathogenesis. Arch. Virol. **54**:279–297.
25. **Pastan, I. H., and M. C. Willingham.** 1981. Journey to the center of the cell: role of the receptosome. Science **214**:504–509.
26. **Perrin, P., D. Portnoi, and P. Sureau.** 1982. Etude de L' adsorption et de la pénétration du virus rabique: interactions avec les cellules BHK21 et des membranes artificielles. Ann. Virol. (Inst. Pasteur) **133E**:403–422.
27. **Philipson, L., K. Lonberg-Holm, and U. Petterson.** 1968. Virus-receptor interactions in an adenovirus system. J. Virol. **2**:1064–1075.
28. **Reagan, K. J., and W. H. Wunner.** 1984. Early interactions of rabies virus with cell surface receptors, p. 387–392. *In* D. H. L. Bishop and R. W. Compans (ed.), Nonsegmented negative strain viruses. Academic Press, Inc., New York.
29. **Rose, J. K., R. F. Doolittle, A. Anilionis, P. J. Curtis, and W. H. Wunner.** 1982. Homology between the glycoprotein of vesicular stomatitis virus and rabies virus. J. Virol. **43**:361–364.
30. **Rose, J. K., and C. J. Gallione.** 1981. Nucleotide sequences of the mRNA's encoding for vesicular stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. J. Virol. **39**:519–528.
31. **Schlegel, R., T.S. Tralka, M. C. Willingham, and I. Pastan.** 1983. Inhibition of VSV binding and infectivity by phosphotidylserine: is phosphotidylserine a VSV binding site? Cell **32**:639–646.
32. **Schlegel, R., M. C. Willingham, and I. Pastan.** 1982. Saturable binding sites for vesicular stomatitis virus on the surface of Vero cells. J. Virol. **43**:871–875.
33. **Smith, A. L., and G. H. Tignor.** 1980. Host cell receptors for two strains of Sindbis virus. Arch. Virol. **66**:11–26.
34. **Stoker, M., and I. MacPherson.** 1964. Syrian hamster fibroblast cell line BHK-21 and its derivatives. Nature (London) **203**:1355–1357.
35. **Svensson, U., R. Persson, and E. Everitt.** 1981. Virus-receptor interaction in the adenovirus system. I. Identification of virion attachment proteins of the HeLa cell plasma membrane. J. Virol. **38**:70–81.
36. **Tardieu, M., R. L. Epstein, and H. L. Weiner.** 1982. Interaction of viruses with cell surface receptors. Int. Rev. Cytol. **80**:27–61.
37. **Thimmig, R. L., J. V. Hughes, R. J. Kinders, A. G. Milenkovic, and T. C. Johnson.** 1980. Isolation of the glycoprotein of vesicular stomatitis virus and its binding to cell surfaces. J. Gen. Virol. **50**:279–291.
38. **Weiss, R. A.** 1981. Retrovirus receptors and their genetics, p. 195–202. *In* K. Longberg-Holm and L. Philipson (ed.), Virus receptors, part 2, animal viruses. Receptors and recognition series B, vol. 8. Chapman and Hall, London.
39. **Wiktor, T. J., and H. F. Clark.** 1975. Growth of rabies virus in cell culture, p. 155–179. *In* G. M. Baer (ed.), The natural history of rabies, vol. 1. Academic Press, Inc., New York.
40. **Wiktor, T. J., B. Dietzschold, R. N. Leamnson, and H. Koprowski.** 1977. Induction and biological properties of defective interfering particles of rabies virus. J. Virol. **21**:626–635.