Specific Plasma Membrane Receptors for Reovirus on Rat Pituitary Cells in Culture

ELEFTHERIA MARATOS-FLIER and C. RONALD KAHN, *Research Division, Joslin Diabetes Center and Department of Medicine of Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115*

DALE R. SPRIGGS and BERNARD N. FIELDS, *Department of Microbiology and Molecular Genetics, Harvard Medical School and Department of Medicine of Brigham and Women's Hospital, Boston, Massachusetts 02115*

ABSTRACT Specific cellular and host tropism is a characteristic property of many viruses mediated by the interaction of viral attachment proteins with components of the plasma membrane of the cell. We have studied the binding of virus to cells quantitatively by using type 3 reovirus labeled with ^{125}I and GH_4C_1 pituitary cells in culture. Binding was rapid at both 4° and 15°C and was stable over a 9-h period. Unlabeled virus inhibited binding of the labeled virus in a dose-dependent manner. Scatchard analysis revealed 4,200 viral binding sites/cell with an apparent affinity of 1.2×10^{-11} M. Also, binding of type 3 reovirus was inhibited by antibodies directed against the viral hemagglutinin and partially inhibited by type 2 reovirus, but was unaffected by type 1 reovirus or a variety of other ligands that bind to receptors on GH_4C_1 cells. These data indicate that reovirus binds to a high affinity, specific receptor on target cells, which may control its tropism and ultimate disease expression.

INTRODUCTION

Viruses are well known to exhibit specific tissue tropism and have defined host ranges. This specificity is in part attributable to discrete protein moieties on the viral surface, termed viral attachment proteins, which mediate adsorption of virus to the host cells (1). For many viral systems, these proteins are well characterized. Adsorption of virus is also dependent on properties of the plasma membrane of the host cell, and there is some evidence that viruses interact with specific proteins or receptors on the cell plasma membrane to initiate their infection (2). However, little information is available about the nature of these viral receptors; their specificity and even their existence is debated (3).

Most data regarding the possible existence of viral receptors have been derived from relatively indirect or qualitative studies, such as measurements of viral adsorption to host cells, immunofluorescence of viruses on the cell surface, or electron microscopy. By contrast, membrane receptors for numerous other ligands that affect cellular function, such as peptide hormones and neurotransmitters, have been quantitatively characterized (4). The success of these studies has depended on the availability of a purified ligand that could be labeled to a high specific activity, and could be used to define specific binding sites (5). In the present study, we have applied these techniques to demonstrate and quantitatively characterize the interaction of type 3 reovirus with pituitary cells in culture.

Reovirus is a double-stranded RNA virus. The tropism of reovirus is known to be determined by a surface protein of the virus, the hemagglutinin, and the role of this protein has been assessed using recombinant viruses derived from the wild types (6, 7). Recently, binding of fluorescein-labeled reovirus to ependymal cells has been demonstrated (8).

There are three naturally occurring serotypes of reovirus, designated 1, 2, and 3. In mice, type 1 reovirus is known to produce an ependymitis and to infect cells of the endocrine pancreas and anterior pituitary,

---

Portions of this work were presented at the annual meetings of the American Society for Clinical Investigation, Washington, D.C., May 9, 1982.

Received for publication 10 January 1983 and in revised form 3 March 1983.

which leads to the development of autoimmune syndrome associated with diabetes, runting, and the development of antibodies to insulin and growth hormone (9). In vivo type 3 reovirus preferentially infects neurons, but can also infect endocrine cells (10). GH_4C_1 are a clonal line of rat pituitary cells (11–13). We have shown that type 3 reovirus infects cultured GH_4C_1 cells, and that this infection alters the membrane of these cells, particularly the receptor for epidermal growth factor (14; Maratos-Flier, E., A. H. Tashjian, B. N. Fields, and D. H. Rubin, submitted for publication). We now show that this infection is mediated by specific, high-affinity viral receptors on the surface of these cells.

METHODS

Viral growth and iodination. Reovirus type 3 was grown and purified from mouse L cells. Stability of the viral genome was assessed by polyacrylimide gel electrophoresis. 10 μg of type 3 reovirus (7.7×10^{-14} mol) was iodinated to a specific activity of 5 μCi/μg (range 2–8 μCi/μg) using lactoperoxidase beads (Bio-Rad Laboratories, Richmond, CA) and ^{125}I (New England Nuclear, Boston, MA). Iodinated virus was separated from free iodine by gel filtration over a Sephadex G-25 column (Pharmacia Inc., Piscataway, NJ); the virus eluted in the void volume. Radioactivity in the void volume was >85% precipitable by 10% trichloroacetic acid. The labeled virus was used only when initial binding studies demonstrated specific binding of at least 65%. Analysis of the labeled virus using sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated that >99% of the iodine was bound to three surface-associated proteins of the virus. Utilizing densitometry measurements of the gel autoradiogram, 73% of ^{125}I was found to be bound to σ3, which comprises 66% of the surface protein, and 24% was bound to μ1C, which comprises 30% of the surface protein; the additional 3% of viral-associated ^{125}I was bound to λ2, which is a core spike protein that is partially exposed to the surface. No measurable radioactivity was associated with the hemagglutinin that comprises <1% of the surface protein. 75–85% of the iodinated virus was precipitable by anti-reovirus antibodies. (Fields, B. N. Unpublished data.)

Time course of binding of reovirus to GH_4C_1 cells. GH_4C_1 cells were grown to confluency on 100-mm dishes in Ham's F10 media supplemented with 15% horse serum and 2.5% fetal calf serum. Cells were removed from plates by gently scraping with a rubber policeman, and washed three times in binding buffer containing 115 mM NaCl, 5.3 mM KCl, 1.4 mM CaCl, 0.8 mM $MgSO_4$, 0.9 mM NaH_2PO_4, 5.5 mM dextrose, 50 mM Hepes, 1% bovine serum albumin (BSA, fraction V; Sigma Chemical Co., St. Louis, MO), pH 7.5. Cells were then aliquoted into polyethylene 12 × 75-mm tissue culture tubes. Either buffer or excess unlabeled virus (final concentration, 200 μg/ml) was added. Cells were incubated for 10 min at room temperature, and then placed on ice or in a 15°C waterbath. Next, 20,000 cpm of iodinated virus (2.0 ng) was added. The incubation tubes were agitated at 10–15-min intervals. Samples were taken at the designated time and washed in a large excess of 4°C phosphate-buffered saline (PBS), 1% BSA, pH 7.5. Cell-associated radioactivity was determined using a gamma counter (model 1290, Tracor Analytic Inc., Elk Grove Village, IL).

Competition of ^{125}I-virus binding by unlabeled virus, anti-hemagglutinin, antibodies, and other ligands. 7.5×10^4 GH_4C_1 cells were aliquoted into 12 × 75-mm plastic culture tubes. Amounts of unlabeled type 3 reovirus ranging from 5 ng/ml to 500 μg/ml were added to the cells and cells were incubated for 10 min at 22°C. Control cells were incubated with buffer only. Cells were then placed in a 15°C waterbath and 5,000 cpm of tracer (0.5 ng) was added to each sample. After a 1-h incubation, cells were washed with 2 ml of 4°C PBS containing 1% BSA and centrifuged at 262 *g* in a GLC-4 centrifuge using an H-1000 rotor (E. I. DuPont de Nemours & Co., Inc., Sorvall Instruments Div., Newtown, CT). Experiments to determine the ability of other ligands to block binding of ^{125}I-type 3 reovirus were performed in the same manner. Concanavalin A, epidermal growth factor (EGF),[1] and insulin were preincubated with GH_4C_1 cells at a concentration of 1 μg/ml. Vesicular stomatitis virus (VSV) was added at a concentration of 200 μg/ml. Types 1 and 2 reovirus were tested at a concentration of 300 μg/ml.

In order to determine the ability of monoclonal anti-hemagglutinin antibodies to block viral binding, 5,000 cpm of ^{125}I-virus in 10 μl was incubated with 15 μl of diluted antibody or buffer for 20 min at 22°C in 12 × 75-mm tubes. Tubes were then placed in a 15°C waterbath; 75,000 GH_4C_1 cells suspended in 75 μl of viral binding buffer were added and tubes were incubated for an additional 90 min. Cells were washed and counted as described above.

RESULTS

When tracer quantities of virus were added to aliquots of GH_4C_1 cells, binding of ^{125}I-reovirus was rapid at both 4° and 15°C (Fig. 1). Equilibrium was reached at 60 min and was stable over the 9-h period of incubation. Nonspecific binding was determined by performing incubations in the presence of a 1,000-fold excess of unlabeled virus and it was 30% of the total tracer binding. Total and nonspecific binding was similar at both temperatures. In separate experiments, time points from 5 to 60 min were sampled; one-half maximal binding was noted at 20 min at 15°C (data not shown).

The ability of unlabeled virus to compete with labeled virus that binds to GH_4C_1 cells was studied at 15°C using variable amounts of unlabeled virus. Complete inhibition of specific binding occurred at a concentration of 100–200 μg/ml of unlabeled virus.

To determine the "apparent" binding affinity and number of receptors per cell and to allow comparison of the viral receptor to receptors for other ligands, Scatchard analysis (15) was performed (Fig. 2, inset). The Scatchard plot was somewhat curvilinear; for the purpose of this analysis, however, only a single class of high affinity sites was analyzed. From the slope of the line, the apparent affinity (K_D) of the virus for the cells was 1.2×10^{-11} M and there were 4,200 high

[1] *Abbreviations used in this paper:* EGF, epidermal growth factor; VSV, vesicular stomatitis virus.

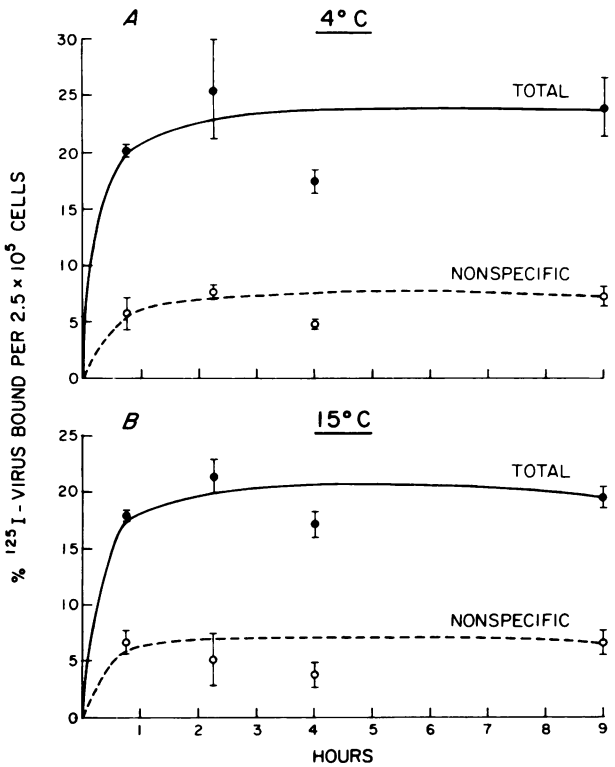

FIGURE 1 Time course of ^{125}I-reovirus type 3 binding to GH_4C_1 cells. Cells in suspension were incubated with (nonspecific) or without (total) unlabeled type 3 reovirus at a concentration of 200 μg/ml, for 10 min at 22°C, in 12 × 75-mm culture tubes. Tubes were then placed in either a 4° or 15°C water bath and ^{125}I-reovirus type 3 (2.0×10^5 cpm/ml) was added to each tube. At designated intervals, 100-μl aliquots containing 2.5×10^5 GH_4C_1 cells were sampled and washed as described in Methods. The results shown here are from one experiment; each point represents the mean of duplicate samples and bars show range. Similar results were obtained in three separate experiments.

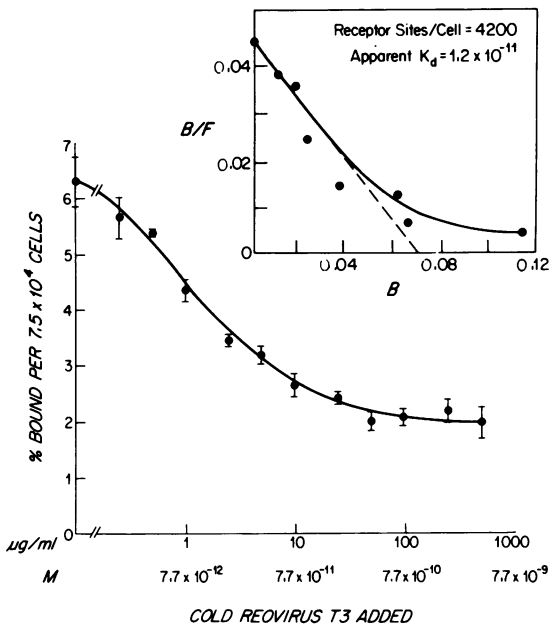

FIGURE 2 Inhibition of ^{125}I-reovirus type 3 binding by increasing amounts of unlabeled type 3 reovirus. 5×10^4 GH_4C_1 cells were suspended in 80 μl of binding buffer and unlabeled reovirus type 3 was added in 10-μl aliquots. Cells were incubated for 10 min at 22°C, and then placed in a 15°C waterbath. 5,000 cpm of labeled reovirus was then added in 10-μl aliquots and the incubation was continued for 90 min. Cells were then washed as described in Methods. Each point represents the mean of duplicate tubes and bars show the range. Similar results were obtained in three separate experiments.

affinity receptors/cell. Since the viral-cell interaction is much more complex than a simple ligand-ligand interaction, these estimates should only be taken as approximations and further studies will be required to determine the best method of data analysis in this system.

To better ascertain the specificity of viral binding, ligands other than unlabeled reovirus type 3 were tested for their ability to inhibit type 3 reovirus binding (Fig. 3 *A*). Of the two other naturally occurring serotypes of reovirus, type 1 at a concentration of 300 μg/ml had no effect on binding of labeled type 3, whereas type 2 at the same concentration produced about a 50% inhibition of that seen with type 3. VSV, another RNA virus, had no effect on reovirus binding. Similarly insulin, EGF, and concanavalin A, which have receptors on GH_4C_1 cells, had no effect.

The hemagglutinin on the viral particle has been shown to be responsible for its attachment to cells. Monoclonal antibodies directed against the viral hemagglutinin have been prepared and characterized by Burstin (16). Antibody F-4 is known to block the ability of type 3 virus to agglutinate human erythrocytes; antibody G-5 blocks the ability of type 3 virus to infect L cells; and antibody H-3 immunoprecipitates the purified hemagglutinin, but has no effect on either hemagglutinating capacity or infectivity. As can be seen in Fig. 3 *B*, both G-5 and F-4 blocked binding of iodinated type 3 reovirus to GH_4C_1 cells, whereas comparable concentrations of H-3 had no effect on viral binding. These data suggest that antibodies G-5 and F-4 interact with the portion of the hemagglutinin molecule that is responsible for binding of virus to the pituitary cell, whereas antibody H-3 does not.

DISCUSSION

Over the past twenty years, much indirect evidence has accumulated that suggests that the first step in viral infection is interaction of the virus with specific struc-

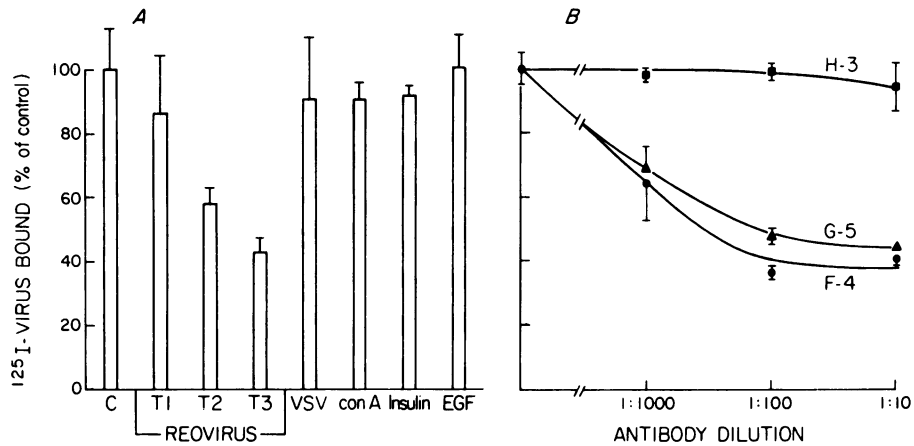

FIGURE 3 (*A*) Effect of types 1 and 2 reovirus, *VSV*, concanavalin A, insulin, and *EGF* ion binding of ^{125}I-reovirus type 3. Cells were incubated with various ligands for 10 min at 22°C, as described in Methods. Labeled reovirus was then added and a 90-min incubation at 15°C was performed. Cells were washed as described in Methods. Each bar shows the mean of two duplicates and bars show the range. Similar results were obtained in two separate experiments. (*B*) Effect of preincubation of ^{125}I-reovirus type 3 with antibodies directed against the viral hemagglutinin on viral binding. 5,000 cpm of labeled reovirus in 10 μl was incubated with 15 μl of diluted antibody for 20 min at 22°C. 7.5 × 10^4 GH_4C_1 cells were added to each sample and further incubation at 15°C for 90 min. Samples were then washed as described in Methods. Each point represents the mean of duplicates and bars show the range. Similar results were obtained in five separate experiments.

tures on the plasma membrane of the cell. For enteroviruses, it is known that cells that cannot adsorb virus are resistant to infection by intact virus (17), but remain susceptible to infection by extracted viral RNA (18). It has also been shown that treatment of cells with proteases will alter adsorption of virus in a specific manner (19), which suggests that a plasma membrane protein is involved in the adsorption process. More recently, evidence for the existence of specific viral receptors has come from studies using fluorescein-labeled viral particles. Fluorescein-labeled type 3 reovirus was shown to bind to neuronal cells, but it failed to bind to ependymal cells; using viral recombinants, it was possible to demonstrate that the binding was mediated by the hemagglutinin. These techniques, while helpful in cataloguing absence or presence of receptors on a given cell, generally require quantities of virus in excess to those used to establish infection. Also, when using such methods, it is not possible to study viral binding quantitatively or to characterize the cellular receptor in any detail.

McClintock and co-workers (20) have used ^{14}C- and ^{3}H-labeled encephalomyocarditis virus to study binding of virus to transformed human and mouse cells. With radioactively labeled virus, they were able to characterize viral binding at different temperatures and study some of its features. However, the low specific activity of the tracer prohibited extensive studies of the viral receptor. Similar studies performed with iodinated Sendai virus (21) and iodinated Epstein-Barr virus (22) have demonstrated binding of labeled virus that could be inhibited by large excess of unlabeled virus, but in neither case was further specificity demonstrated or quantitative analysis performed.

The data presented in this study demonstrate that whole viral particles can be labeled to a high specific activity using ^{125}I, and that the labeled virus will bind to cells. Furthermore, this binding can be studied in a quantitative manner similar to that used to study the interaction of other ligands with cells. Thus, we have shown that ^{125}I-reovirus, which is known to infect neural and endocrine cells, binds to an endocrine cell in a stable and saturable fashion, and this binding exhibits appropriate specificity consistent with other known facts. Ligands that interact with the plasma membrane of GH_4C_1 cells independently of the viral receptor, such as EGF, insulin, and concanavalin A, have no effect on viral binding. Likewise, type 1 reovirus did not affect binding of labeled type 3 virus, whereas type 2 virus produced partial inhibition. Tryptic mapping of hemagglutinin (σ1) polypeptide fragments of types 1, 2, and 3 (23) reovirus has revealed a greater degree of homology between types 2 and 3 reovirus than between types 1 and 3 reovirus. The ability of type 2 reovirus to inhibit binding of ^{125}I-type 3 reovirus suggests that this homology may occur

in the area of the σ1 protein that is responsible for cell attachment. In addition, blocking the capsid protein of the virus known to be responsible for adsorption of the virus to cells, i.e., the hemagglutinin, using monoclonal antibodies, blocked binding of labeled virus to GH_4C_1 cells; this rules out the possibility that the labeled virus is somehow denatured and adsorbing to cells in a nonspecific manner. Specific binding of iodinated reovirus types 1 and 3 to mouse L cell fibroblasts, lymphoma cells, and lymphocytes has also been obtained (H. L. Weiner and R. L. Epstein, personal communications), and suggests that this approach may be widely applicable.

These data not only extend the concept that viral binding to cells is mediated by specific plasma membrane receptors, but also demonstrate that there are a defined number of high affinity binding sites per cell and that the properties of the viral receptor are similar to those demonstrated for numerous peptide hormone receptors. Such receptors presumably mediate viral entry into cells. By comparing receptor properties such as number and affinity on different tissues, it should be possible to explain the role of the viral receptor in determining the tropism of viruses for various tissues, and to determine the importance of this interaction in the clinical manifestations of disease. In addition, the possibility that the number or affinity of viral receptors, and thereby cell susceptibility to infection, can be altered by treatment with hormones, growth factors, or other biologically active agents such as interferon or phorbol esters can be evaluated. Finally, the possibility that the viral receptor is meant to bind functional endogenous ligands can be addressed.

ACKNOWLEDGMENTS

We would like to acknowledge Alison Murray for helpful technical assistance and Valerie Abrahamsen for excellent secretarial assistance.

This work was supported in part by a grant from the Juvenile Diabetes Foundation.

REFERENCES

1. Lonberg-Holm, K. 1981. Attachment of animal viruses to cells. *In* Virus Receptors, Vol. 2. K. Lonberg-Holm and L. Philipson, editors. Chapman & Hall, London and New York. 3–20.
2. Incardona, N. L. 1981. The chemical nature of virus-receptor interactions. *In* Virus Receptors, Vol. 2. K. Lonberg-Holm and L. Philipson, editors. Chapman & Hall, London and New York. 157–168.
3. Dales, S. 1973. Early events in cell animal virus interactions. *Bacteriol. Rev.* 37:103–135.
4. Kahn, C. R. 1976. Membrane receptors for hormones and neurotransmitters. *J. Cell Biol.* 70:261–286.
5. Roth, J. 1975. Assay of peptide hormones using cell receptors. *Methods Enzymol.* 37:66–82.
6. Fields, B. N. 1982. Molecular basis of reovirus virulence. *Arch. Virol.* 71:95–107.
7. Weiner, H. L., K. A. Ault, and B. N. Fields. 1980. Interaction of reovirus with cell surface receptors. *J. Immunol.* 124:2143–2148.
8. Tardieu, M., and H. L. Weiner. 1982. Viral receptors on isolated murine and ependymal cells. *Science (Wash. DC).* 215:419–421.
9. Onodera, T., A. Toniolo, U. R. Ray, A. B. Jenson, R. A. Knazek, and A. L. Notkins. 1981. Virus-induced diabetes mellitus. *J. Exp. Med.* 153:1457–1473.
10. Yoon, J. W., S. Selvaggio, T. Onodera, J. Wheeler, and A. B. Jenson. 1981. Infection of cultured human pancreatic beta cells with reovirus type 3. *Diabetologia.* 20:462–467.
11. Hinkle, P. M., E. L. Woroch, and A. H. Tashjian, Jr. 1974. Receptor-binding affinities and biological activities of analogs of thyrotropin-releasing hormone in prolactin producing pituitary cells in culture. *J. Biol. Chem.* 249:3085–3097.
12. Schonbrunn, A., M. Krasnoff, J. M. Westendorf, and A. H. Tashjian, Jr. 1980. Epidermal growth factor and thyrotropin-releasing hormone act similarly on a clonal pituitary strain. *J. Cell Biol.* 85:786–797.
13. Schonbrunn, A., and A. H. Tashjian. 1978. Characterization of functional receptors for somatostatin in rat pituitary cells in culture. *J. Biol. Chem.* 253:6473–6483.
14. Maratos-Flier, E., D. Rubin, B. N. Fields, and A. H., Tashjian, Jr. 1981. Reovirus infection causes a decrease in EGF binding to rat pituitary cells. *Endocrine Soc. Prog.* 63:237. (Abstr.)
15. Scatchard, G. 1949. The attraction of proteins for small molecules and ions. *Ann. NY Acad. Sci.* 51:660–672.
16. Burstin, S. J., D. R. Spriggs, and B. N. Fields. 1982. Evidence for functional domains on the reovirus type 3 hemagglutinin. *Virology.* 117:146–155.
17. McLaren, L. C., J. J. Holland, and J. T. Sylverton. 1960. The mammalian cell-virus relationship. VI. Infection of naturally insusceptible cells with enterovirus nucleic acid. *J. Exp. Med.* 112:581–593.
18. Holland, J. J., L. C. McLaren, and J. T. Sylverton. 1959. The mammalian cell-virus relationship. V. Susceptibility and resistance of cells in vitro to infection by coxsackie A9 virus. *J. Exp. Med.* 110:65–80.
19. Smith, A. L., and G. H. Tignor. 1980. Host receptors for two strains of Sindbis virus. *Arch. Virol.* 66:11–26.
20. McClintock, P. R., L. C. Billups, and A. L. Notkins. 1980. Receptors for encephalomyocarditis virus on murine and human cells. *Virology.* 106:261–272.
21. McDonald, H. C., A. A. Luderer, D. M. Hess, and L. Credle. 1980. Preparation and characterization of a ^{125}I-labeled Sendai virus ligand. *Anal. Biochem.* 106:127–133.
22. Koide, N., A. Wells, D. J. Volsky, I. M. Shapiro, and G. Klein. 1981. The detection of Epstein-Barr virus receptors utilizing radiolabelled virus. *J. Gen. Virol.* 54:191–195.
23. Gentsch, J. R., and B. N. Fields. 1981. Tryptic peptide analysis of outer aopsid polypeptides of mammalian reovirus serotypes 1, 2, and 3. *J. Gen. Virol.* 38:208–218.