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Isolation of a Receptor Protein Involved in Attachment of Human Rhinoviruses

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Human rhinoviruses can be classified into major and minor groups on the basis of receptor specificity. Recently, a mouse monoclonal antibody was isolated which selectively blocked the attachment of the major group of human rhinoviruses to cells. Using this monoclonal antibody, the cellular receptor for the major group of human rhinoviruses was isolated. A radioimmunoassay was developed by using the receptor antibody to specifically detect rhinovirus receptor during isolation. Solubilized receptor from detergent-treated HeLa cell membrane extracts eluted from gel filtration columns with an apparent molecular weight of 440,000. A cellular receptor protein, which had a molecular weight of 90,000 when analyzed on sodium dodecyl sulfate-polyacrylamide gels, was purified from solubilized extracts on an immunoaffinity column containing receptor antibody. Polyclonal rabbit antiserum, resulting from immunization with the isolated receptor protein, specifically blocked the attachment of the major group of human rhinoviruses and indicated that the 90-kilodalton protein plays a functional role in attachment. Prolonged exposure of HeLa cell monolayers with the receptor antibody showed no inhibition of cell growth and division.

Cellular receptors play an important role in viral pathogenesis. As a first step in viral infection, viruses attach to specific receptors on the surface of cells. This specific interaction with cells determines, to a large extent, the host-range specificities and tissue tropisms of viruses (5, 7, 9). Characterization of the host-virus interaction ultimately requires isolation of the cellular receptor and the virion attachment protein. While several virus attachment proteins have been identified (6, 13, 19), little is known about the nature and identity of most cellular receptors for viruses.

In the past, isolation of receptors from host cells has been hampered by technological problems associated with detecting functional receptors during isolation and in purification of the membrane component(s) (14). Previous attempts to isolate cellular receptors for viruses have exploited the affinity of the viral ligand for identification and purification of the cellular receptor (9, 28). This approach has been difficult, presumably because viruses have lower association constants for receptor monomers after solubilization from cellular membranes. However, some virus-receptor interactions are stable in the presence of detergents since a putative receptor protein for the coxsackie B3 virus has been identified by using virus particles (16).

In this regard, antireceptor monoclonal antibodies may prove more useful as specific, high-affinity reagents for the isolation and characterization of viral receptors. The use of monoclonal antibodies as immunoaffinity probes has been a powerful tool in purifying detergent-solubilized receptors which are present in low quantities on cellular membranes. Monoclonal antibodies have been successfully utilized in the isolation and characterization of various receptor proteins such as the insulin, β-adrenergic, and transferrin receptors (22). A cellular glycoprotein, which appears to be the β-adrenergic receptor, was recently identified as the reovirus receptor by using an anti-idiotypic monoclonal antibody prepared against antibodies to the reovirus 3 hemagglutinin protein (4). In addition, monoclonal antibodies have been isolated which specifically block the attachment of poliovirus and coxsackie B virus to cells and which may be helpful in identifying the cellular receptors for these viruses (3, 18).

The human rhinoviruses (HRVs) are members of the picornavirus family and are the major causative agent of the common cold (8). Previous studies have shown that HRVs can be divided into major and minor groups on the basis of receptor specificity (1, 4a, 15). The majority (78 of 88) of HRV serotypes tested share a common receptor and belong to the major group. The remaining serotypes recognize a different receptor and belong to the minor group. Recently, a monoclonal antibody was isolated which specifically prevents attachment of the major group of HRVs to cellular receptors (4a). The receptor antibody was shown to protect cells from infection by coxsackie A viruses as well as from viruses in the major group of HRVs, whereas it was unable to protect cells against infection by the minor group of HRVs, other picornaviruses, or other DNA and RNA viruses (4a). The receptor antibody also demonstrated the same host and tissue specificities as the major group of HRVs in that it could bind only to cells of human or chimpanzee origin but not of mouse origin (4a). Using this receptor antibody, we now report the isolation of a receptor protein utilized by the major group of HRVs for attachment to susceptible cells.

MATERIALS AND METHODS

Cells, virus, and antibody. The growth of HeLa R-19, human laryngeal tumor (HEp-2), and mouse L fibroblast cells has been previously described (4a). The growth and purification of [^{35}S]methionine-labeled HRV-15, HRV-2, and poliovirus have been detailed previously (1). Cell protection assays have also been previously described (4a). Briefly, HeLa R-19 monolayers were pretreated with polyclonal serum for 1 h at 37°C before low multiplicity infection (1 to 5) with the indicated viruses. The cells were incubated for 24 h at 37°C and examined for cytopathic effect. Receptor monoclonal antibody was isolated from mouse ascites fluid and purified by chromatography on protein A-Sepharose and

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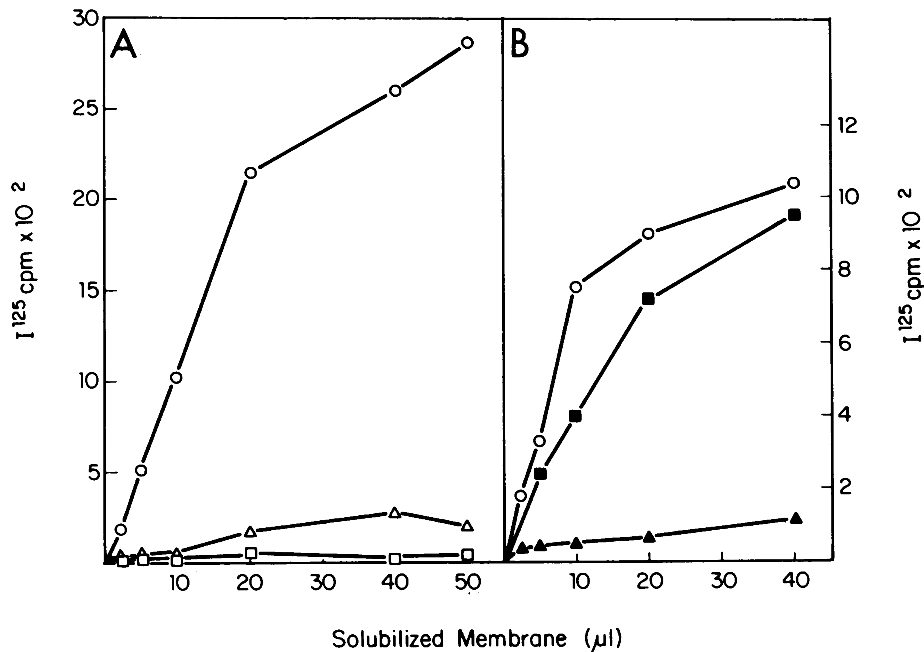

FIG. 1. RIA for receptor activity in membrane extracts. Solubilized membrane supernatants were prepared and assayed for receptor as described in Materials and Methods. (A) Increasing amounts of HeLa (○), HEp-2 (△), and mouse (□) cell membrane supernatants were bound to nitrocellulose, incubated with ^{125}I-labeled receptor antibody, and assayed for radioactivity. (B) Nitrocellulose-bound HeLa cell extracts were untreated (○) or preincubated with unlabeled receptor monoclonal antibody (▲) or HRV-neutralizing monoclonal antibody (■) before incubation with ^{125}I-labeled antibody.

Mono Q (Pharmacia, Inc., Piscataway, N.J.) fast protein liquid chromatography as described elsewhere (4a). Purified receptor antibody was iodinated by the chloramine-T procedure (W. M. Hurni, W. J. Miller, E. H. Wasmuth, and W. J. McAleer, submitted for publication).

Membrane preparation. Cell monolayers were treated with 50 mM EDTA in phosphate-buffered saline (PBS) for 10 to 15 min at 37°C to detach cells. Cells were pooled and then washed three times in PBS. Cells were swollen in 10 mM phosphate buffer and disrupted by Dounce homogenization. Cell debris and nuclei were removed by pelleting at 1,000 × *g* for 5 min, and membranes were pelleted from the resulting supernatant at 200,000 × *g* for 1 h at 4°C. The crude membrane pellet was resuspended in PBS to a final concentration of 8 mg of protein per ml and stored at −70°C.

Membrane binding assay. In vitro membrane binding assays have been described previously (4a). Briefly, HeLa cell membranes (20 µl) were incubated for 30 min with 10 µl of various dilutions of polyclonal serum in PBS before the addition of 10 µl (10^4 cpm) of [^{35}S]methionine-labeled HRV or poliovirus or 10 µl of ^{125}I-labeled receptor antibody. After incubation with virus for 1 h at 25°C or for 30 min with antibody, the membranes were pelleted at 12,000 × *g* for 2 min, radioactivity in the supernatants was counted. Pellets were suspended in 0.2 ml of 1% sodium dodecyl sulfate (SDS) and were also counted to determine percent binding.

Membrane solubilization. Membrane preparations were pelleted 10 min at 35,000 × *g* and suspended at 6.6 mg/ml in 0.3% sodium deoxycholate prepared fresh in 20 mM Tris hydrochloride (pH 7.4) plus 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.). After incubation at 4°C for 40 min, the extracts were centrifuged at 100,000 × *g* for 45 min in a Beckman Ti70.1 rotor at 4°C. Solubilized extracts (supernatants) were stored at 0°C.

Receptor radioimmunoassay (RIA). Nitrocellulose paper was equilibrated with PBS in a Schleicher & Schuell filtration manifold. Solubilized HeLa membrane supernatants were adsorbed to nitrocellulose for 2 to 24 h at 4°C. Unbound protein was removed with PBS, and the nitrocellulose paper was blocked with 20% fetal calf serum in 0.15 M NaCl–0.05 M Tris hydrochloride (pH 7.6)–0.01% sodium azide for 1 to 2 h at room temperature. The nitrocellulose paper was then incubated with ^{125}I-labeled receptor monoclonal antibody in PBS containing 0.02% bovine serum albumin for 2 h at room temperature. The unadsorbed material was removed by washing with PBS containing 0.05% Triton X-100, and the paper was assayed for radioactivity.

Gel filtration. Solubilized membrane supernatants (0.5 ml) were chromatographed on a Pharmacia Superose 12 column with a Bio-Rad model 1330 high-pressure liquid chromatography system. The column was run at 1 ml/min with 0.3% sodium deoxycholate in 20 mM Tris hydrochloride (pH 7.4) as a running buffer. Fractions (0.3 ml) were collected, and 80-µl aliquots were tested in the radioimmunoassay.

Affinity chromatography. Receptor monoclonal antibody (5 mg) was coupled to 1 ml of Bio-Rad Affi-Gel 10 in 0.1 M $NaHCO_3$ (pH 8.3) according to the Bio-Rad instructions, and the column was equilibrated with 0.3% sodium deoxycholate in 20 mM Tris hydrochloride (pH 7.4)–0.15 M NaCl. Solubilized membrane supernatants were chromatographed through the resin, and unadsorbed protein was washed from the column with 0.5% sodium deoxycholate in 20 mM Tris hydrochloride (pH 7.4). Adsorbed receptor was then eluted with 50 mM diethylamine (pH 11.5), lyophilized, and stored at 4°C.

Preparation of polyclonal antiserum. Antiserum was prepared by immunizing New Zealand White rabbits with 10 µg of immunoaffinity-purified receptor protein in complete Freund adjuvant administered intradermally. Four weeks later, an additional 10 µg of receptor protein in incomplete

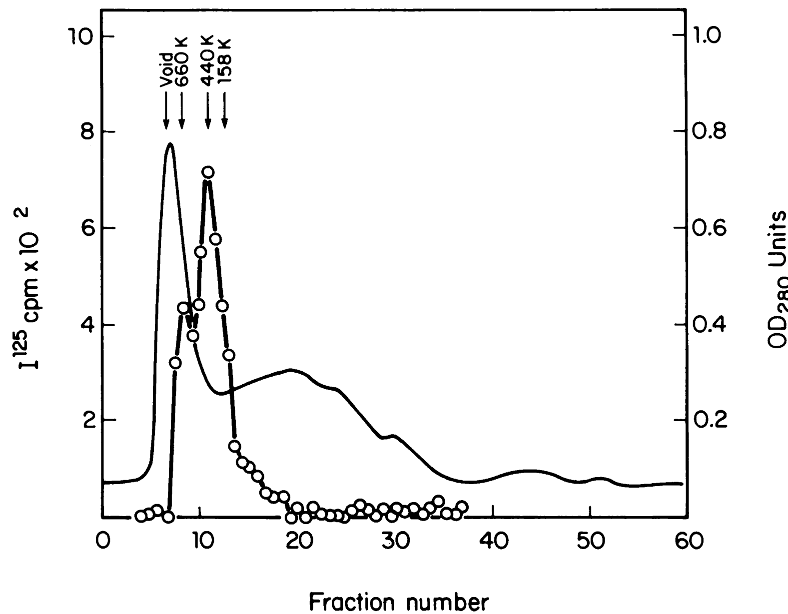

FIG. 2. Gel filtration of solubilized HeLa cell membranes. Solubilized HeLa cell membrane supernatant (0.5 ml) was fractionated on a Pharmacia Superose 12 column (10 by 300 mm) and eluted with 0.3% sodium deoxycholate in 20 mM Tris hydrochloride (pH 7.4)–0.15 M NaCl. Receptor was detected in column fractions by solid-phase RIA (O), and total protein was monitored at an optical density of 280 nm (——). The column was calibrated with blue dextran (void), thyroglobulin (660 kDa) (660K), ferritin (440 kDa), and aldolase (158 kDa).

Freund adjuvant was administered intradermally. A third intradermal immunization of 10 μg in incomplete Freund adjuvant was repeated 4 weeks later.

SDS-gel electrophoresis. Protein samples were analyzed on 10% polyacrylamide gels as described elsewhere (12). Gels were fixed and silver stained by the method of Merrill et al. (17).

Treatment of HeLa cells with receptor antibody. HeLa R-19 cells were distributed into 12 25-cm^2 flasks (2.5×10^5 cells per flask) and allowed to attach and grow overnight at 37°C. After a 1-day incubation, 165 μg of purified receptor antibody (4a) was added to each of six flasks. On day 3, cells from three treated and three untreated flasks were removed by EDTA treatment (see above) and counted. The remaining three antibody-treated flasks were given an additional 165 μg of receptor antibody without removing the old medium. After an additional 3-day incubation at 37°C, cells from all remaining flasks were removed and counted.

RESULTS

Solubilization and assay. Previous studies have indicated that functional HRV receptors are present on crude membrane preparations of HeLa cells (4a). Before performing initial detergent solubilization experiments, it was necessary to establish an assay to measure solubilization of the receptor. Receptor solubilization was indicated by the failure to pellet (100,000 × *g*) receptor from detergent-treated HeLa membranes. A variety of detergents were tested at various concentrations, including Triton X-100 (0.5 to 1.5%), 3-[3-cholamidopropyldimethyl-ammonio]-1-propane sulfate (0.6 to 2.4%), octylglucoside (0.3 to 1.2%), digitonin (0.3 to 1.2%), and sodium deoxycholate (0.1 to 1.2%), for various lengths of time (10 to 60 min). Treatment with 0.3% sodium deoxycholate for 40 min at 4°C was found to most effectively solubilize HRV receptors from HeLa membranes (data not shown).

Receptor was detected in a solid-phase RIA which measured specific binding of radiolabeled receptor antibody. The specificity of the assay was demonstrated by comparing the binding of ^{125}I-labeled antibody to receptor in high-speed supernatants of HeLa, HEp-2, and mouse L cell extracts (Fig. 1A). An increase in receptor antibody binding was observed when increasing amounts of HeLa cell membrane extract were used. The receptor antibody bound 14-fold more efficiently to HeLa cell extracts than to HEp-2 cell extracts, while showing no binding to mouse L cell extracts. HEp-2 cells are a human laryngeal tumor cell line having few, if any, receptors for the major group of HRVs, while mouse cells have no detectable receptors (4a).

The antibody binding could be reduced 10-fold by preincubation with unlabeled receptor antibody (Fig. 1B). Substitution of the unlabeled receptor antibody with HRV-14 neutralizing monoclonal antibody no. 30 (19a) of identical isotype (immunoglobulin G1) had little effect. Repeated attempts to use radiolabeled HRV in place of receptor antibody in the RIA gave inconclusive results owing to poor virus binding.

Gel filtration. The solubility of the cellular receptor in detergent extracts was further evaluated by gel filtration. HeLa cell high-speed cell supernatants were chromatographed on a Superose 12 gel filtration column, and fractions were assayed for the presence of receptor by the solid-phase RIA, using iodinated receptor antibody. Solubilized HeLa cell receptor eluted as a single peak with a molecular weight of approximately 440,000 as determined by protein standards run under identical conditions (Fig. 2). This result showed that a substantial solubilization of the HRV receptor from the membrane was achieved with 0.3% sodium deoxycholate. This receptor peak was not detected in column fractions of detergent-solubilized extracts of mouse L cells (data not shown).

Varying the concentration of deoxycholate used in the column running buffer resulted in a shift in the molecular

weight of the receptor peak. Without deoxycholate in the running buffer, the receptor peak eluted in the void volume of the column. With 0.1% deoxycholate present, three receptor peaks were detected: one in the void volume, one at 660 kilodaltons (kDa), and one at 440 kDa. Fractions representing each of the receptor peaks were pooled and analyzed on SDS-polyacrylamide gels. Results indicated that all detectable receptor peaks contained up to 12 proteins at this stage of purification, some of which were common to all the peaks (data not shown). This result suggested that the HRV receptor eluted from the gel filtration column as an aggregated complex or mini-micelle which varied with detergent concentration.

Affinity purification of receptor protein. Immunoaffinity purification was pursued to further purify the HRV receptor. Solubilized HeLa cell membranes were adsorbed to Affi-Gel 10 columns with or without coupled receptor antibody as described in Materials and Methods. After extensive washing, bound material was eluted with 50 mM triethylamine and analyzed by electrophoresis on SDS-polyacrylamide gels. A protein with a molecular weight of 90,000 was detected in HeLa cell extracts after adsorption to resin with receptor antibody, while no comparable protein eluted from control resin without coupled receptor antibody (Fig. 3). As evidenced by an undetectable amount of receptor protein present in the starting material (lane 2), a 4,000-fold purification of the putative receptor protein was achieved after a single pass through the immunoaffinity column. The 90-kDa protein was present in all the receptor peaks (discussed above). While a protein of similar molecular weight could be isolated from chimpanzee liver cells, no equivalent proteins were isolated from solubilized extracts of mouse L or HEp-2 cells by immunoaffinity chromatography (data not shown).

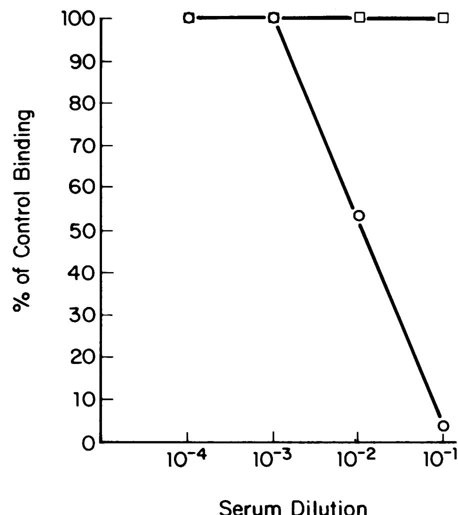

FIG. 4. Inhibition of HRV-15 binding by polyclonal rabbit antiserum. Membrane binding assays with radiolabeled HRV-15 were performed after a 30-min pretreatment of membranes with the indicated dilutions of control serum (□) or serum from rabbits immunized with the 90-kDa receptor protein (○). Results are expressed as the percentage of ^{35}S-labeled HRV-15 control binding to HeLa membranes without antiserum.

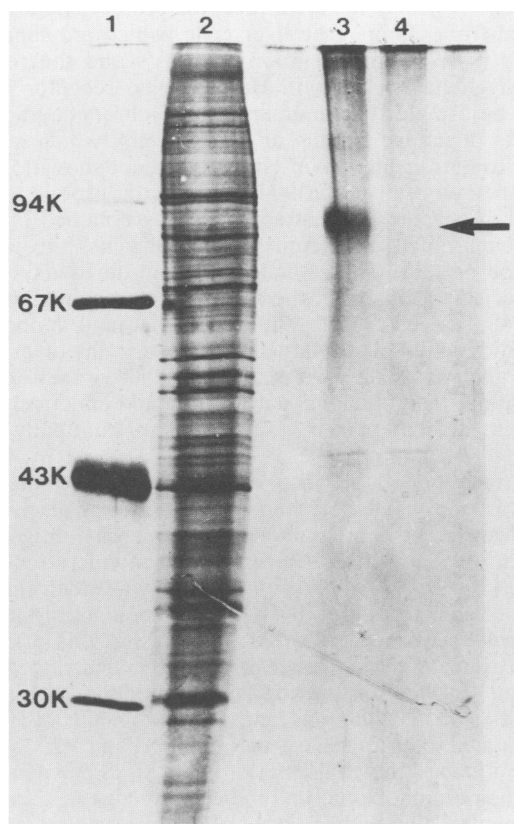

FIG. 3. Immunoaffinity isolation of receptor protein. Solubilized HeLa cell membrane supernatants were adsorbed to Affi-Gel 10 columns coupled with (lane 3) or without (lane 4) receptor antibody as described in Materials and Methods. Bound material was eluted with 0.2% SDS, analyzed on 10% SDS-polyacrylamide gels under reducing conditions (12), and silver stained (17). The arrow indicates the position of the 90-kDa receptor protein. Protein markers are shown in lane 1, and unfractionated HeLa cell membrane supernatant is shown in lane 2. K, kilodaltons.

Polyclonal antisera to purified HRV receptor. To prove that the isolated 90-kDa protein was the receptor protein for the major group of HRVs, rabbits were immunized with purified 90-kDa receptor protein to generate polyclonal antiserum. The ability of the polyclonal antiserum to selectively inhibit HRV virus attachment to susceptible cells was tested by both membrane binding and cell protection assays. Results showed that the addition of increasing amounts of receptor

TABLE 1. Effect of polyclonal antiserum on virus binding and infection of cells

Virus or antibody	Binding assay[a] (% inhibition)	Cell protection[b]
HRV-15	94	+
HRV-2	11	−
Poliovirus	0	−
Coxsackie A13 virus	ND[c]	+
HRV-14	ND	+
Receptor monoclonal antibody	83	ND

[a] Polyclonal antiserum was diluted 1:10 for binding assays and 1:250 for use in cell protection assays. [^{35}S]methionine-labeled HRV-15, HRV-2, and poliovirus and ^{125}I-labeled receptor antibody were assayed in a membrane binding assay described in Materials and Methods. Results are expressed as percent inhibition of control binding.

[b] Cell protection assays were done in 48-well cluster plates of HeLa cells as described previously (4a). +, Complete absence of viral infection; −, destruction of the cell monolayer within 24 h.

[c] ND, Not done.

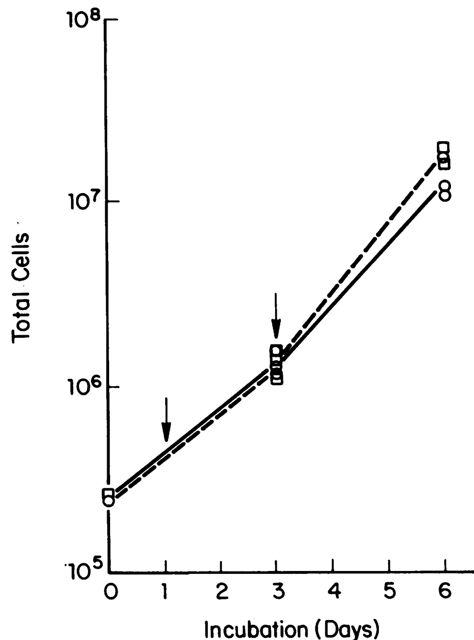

FIG. 5. Effect of receptor antibody on HeLa cell growth. Sparse HeLa cell monolayers in 25-cm^2 flasks were treated (□) or not treated (○) on days 1 and 3 (arrows) with 165 μg of purified receptor antibody and incubated at 37°C. On days 3 and 6, cells were removed from flasks, and cell numbers were determined as described in Materials and Methods.

antiserum corresponded to an increased inhibition of ^{35}S-labeled HRV-15 binding to HeLa membranes (Fig. 4). No inhibition of virus binding was observed with dilutions of control antiserum. Membrane binding assays (Table 1) involving HRV-2 (minor group) and poliovirus type 1 indicated that the polyclonal antiserum showed the same receptor specificity as the receptor monoclonal antibody in that it could not inhibit the binding of other picornaviruses. In addition, the polyclonal antiserum was capable of blocking the attachment of ^{125}I-labeled receptor monoclonal antibody to HeLa cell membranes (Table 1). Similar to the receptor antibody, the polyclonal antiserum was able to selectively protect HeLa cell monolayers from infection by HRV-14 and HRV-15 (major HRV group) and coxsackie A13 virus but not HRV-2 (minor HRV group) or poliovirus (Table 1). These results confirm that the 90-kDa protein is a receptor protein utilized by the major group of HRVs during attachment.

Cellular function of HRV receptor. To assess the importance of the HRV receptor protein to normal cell growth and division, sparse HeLa cell monolayers were treated with excess receptor antibody for 6 days, and cell counts were done on days 3 and 6 as described in Materials and Methods. The results (Fig. 5) demonstrated that the receptor antibody had no inhibitory effect on cell growth as measured by cell number. The 6-day incubation represented six cell doublings and a 60-fold increase in cell number. The amount of receptor antibody used was over 1,000 times the amount needed to protect cells from HRV infection.

DISCUSSION

A cellular receptor protein was identified which is utilized by the major group of HRV serotypes for attachment to susceptible cells. The receptor protein was isolated with an antireceptor monoclonal antibody which selectively blocks the attachment of the major group of HRVs to cells and exhibits the same host and tissue tropism as these viruses (4a). The HRV receptor was purified by using biochemical techniques typically applied to the isolation of other membrane receptors and proteins.

Critical for the successful isolation of the receptor protein was the development of a convenient and highly specific RIA to detect the receptor during purification. The RIA measured the binding of radiolabeled receptor antibody to solubilized receptor immobilized on nitrocellulose. A similar assay, in which solubilized membranes were adsorbed to microtiter plates, was used by Crowell and co-workers to characterize the coxsackie B virus receptor (10). However, it was our experience that the use of nitrocellulose provided a more efficient assay for binding receptor antibody. When HeLa cell membranes were solubilized with deoxycholate, the HRV receptor remained in the supernatants after centrifugation at 100,000 × g and eluted as a peak of ca. 440 kDa from gel filtration columns. The peak fractions contained a variety of proteins in addition to the 90-kDa receptor protein when analyzed on SDS-gels. The size of the receptor complex varied with detergent concentrations, suggesting that the receptor migrated as an aggregate or mini-micelle on the column. A single protein with a molecular weight of 90,000 was isolated after purification of membrane extracts on immunoaffinity columns containing cross-linked receptor antibody. A nearly identical receptor protein could also be isolated from chimpanzee liver cells, which are capable of binding both the major group of HRVs and the receptor antibody (data not shown). However, no receptor protein could be isolated by immunoaffinity chromatography of extracts of mouse L cells or HEp-2 cells, which lack the ability to bind major group HRVs (data not shown).

Further proof that the 90-kDa receptor protein was involved in specific HRV attachment was obtained by using polyclonal rabbit antiserum prepared against the isolated receptor protein. The polyclonal antiserum demonstrated the precise specificity of the receptor monoclonal antibody (Fig. 4; Table 1). Both the polyclonal and monoclonal antibodies inhibited the attachment of the major group of HRVs and coxsackie A virus, but not other viruses, to cells. In addition, the polyclonal antiserum could effectively compete with attachment of ^{125}I-labeled receptor antibody (Table 1).

The location of the virus attachment site on the HRV capsid is of considerable importance, since it represents a highly conserved region among the numerous antigenically distinct HRV serotypes. Recently, the atomic structure of HRV-14, a HRV major group virus, was determined to within 0.3-nm resolution by X-ray crystallography and nucleic acid sequencing (2, 18a, 21). Using this structural information and the sequence of naturally occurring variants resistant to numerous neutralizing antibodies, the precise location of each of the four neutralizing domains of HRV-14 was mapped to distinct areas in VP1, VP2, and VP3 that face outward toward the viral exterior (19a, 20). Since none of 18 neutralizing monoclonal antibodies tested were capable of cross-neutralizing with 23 other HRV serotypes tested (unpublished data), this result implies that the attachment site is distinct from these external neutralization regions of the virus. Instead, it has been postulated that the viral attachment site resides within a large canyon formed predominantly by VP1 and surrounding the vertices of fivefold symmetry on the capsid.

The canyon is 2.5 nm deep and too narrow to allow complete access of immunoglobulins (18a). The viral attach-

ment site would, therefore, represent a highly conserved region of the virus which is inaccessible to immunoglobulin response. This would correlate well with the clinical findings that infection with one HRV serotype fails to result in immunity against infection by other HRV serotypes (8). Additional circumstantial evidence that the canyon contains the viral attachment site can be found in the repeated failure to generate neutralizing idiotypic antiserum against our receptor antibody. If the canyon receptor hypothesis is correct, such an antibody would be unable to attach to intact virions.

Clearly, the 90-kDa receptor protein reported here plays a functional role in HRV attachment to susceptible cells. The size of the solubilized receptor complex (440 kDa) in HeLa membrane extracts suggests that it may exist as a multicomponent complex of which the 90-kDa protein is one component. The complex may consist of additional components or multimers of this protein which in its entirety may be necessary for HRV attachment. Since the HRV canyon encircles VP1 and the axis of the fivefold symmetry, it is quite tempting to speculate that a pentamer of the 90-kDa receptor protein is needed for a functional receptor complex. This would correlate well with the 440-kDa receptor peak obtained by gel filtration and the inability to isolate a 90-kDa receptor protein capable of binding virus.

The normal cellular function of the HRV receptor protein remains unknown. Previous cell culture studies have indicated that the HRV receptor is ubiquitous in the human body (4a). It is, therefore, logical to assume that this receptor plays some role in cell growth or maintenance. However, attachment of the receptor antibody does not inhibit cell growth and division in cell culture (Fig. 5). This result suggests either that this human receptor is not functional in cell culture or that attachment of the receptor antibody does not interfere with its function. This latter possibility would be more likely if the receptor function involved channeling small molecules rather than attachment of a ligand.

Further characterization and sequencing of the receptor protein will inevitably aid in determining its normal cellular function and understanding its interaction with HRVs.

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