Mice Immunized with Measles Virus Develop Antibodies to a Cell Surface Receptor for Binding Virus

DAVID L. KRAH[†]* AND PURNELL W. CHOPPIN[‡]

The Rockefeller University, New York, New York 10021

Received 5 October 1987/Accepted 27 January 1988

Mice were immunized with measles virus to determine whether an auto-anti-idiotypic antireceptor response could be generated as a probe for measles virus receptors. Mice initially responded to viral antigens (days 11 to 18) and subsequently developed antibodies to a putative measles virus receptor (peak at day 30 to 35) by three criteria: the sera (1) agglutinated erythrocytes which virus agglutinates, (2) reacted with Vero cells, and (3) inhibited virus attachment to Vero cells. Additionally, select sera inhibited virus infection of Vero cells. The cell-reactive activity was identified as immunoglobulin G antibody and was neutralized by sera reacting with virus (idiotypic). The application of this anti-idiotypic antibody to identify measles virus-binding sites on Vero cells was revealed by the ability of sera to immunoprecipitate 20- and 30.5-kilodalton proteins from metabolically labeled (³⁵S)methionine) Vero cells.

Measles virus (MV) is an enveloped virus, in the *Morbivirus* genus of the paramyxovirus family, which causes acute febrile illness as well as persistent central nervous system infection in humans (36). The virus envelope contains two well-characterized glycoproteins, the hemagglutinin and fusion proteins (6). The 79,000- to 80,000-*M_r* hemagglutinin protein (79-80K protein) mediates binding to receptors on host cells and erythrocytes, whereas the fusion protein (F1, 40K; F2, 20-25K) is involved in the fusion of the viral and cellular membranes during infection.

The cell surface requirements for MV binding and fusion have not been well characterized. However, a few general features of the receptors for binding virus have been identified (13, 19). The binding site on both tissue culture cells and erythrocytes has a glycoprotein component (19; Krah and Choppin, manuscript in preparation). In contrast to other paramyxoviruses, MV does not require sialic acid residues on receptors for efficient binding to cells (19).

Recent work in this laboratory has centered on characterizing the binding sites of host cells (Vero) and erythrocytes for MV, including purification of receptors from solubilized membranes by using intact virus as an affinity support (Krah and Choppin, in preparation). During these studies, it was reasoned that an antireceptor antibody would provide a chemically stable alternative reagent for the purification and identification of MV receptors. Early attempts to obtain monoclonal antireceptor antibodies were not successful.

The recent use of anti-idiotypic antibodies (a-ids), prepared against antibodies to the viral attachment protein, to bind to and identify receptors for reovirus type 3 (22, 27, 28), leukemogenic retroviruses (1), and polyomaviruses (26) prompted us to investigate this approach for the study of MV receptors. We adopted the auto-anti-idiotypic method used by Shechter et al. (33, 34) for the preparation of anti-idiotypic anti-insulin receptor antibodies. The rationale for this approach is that ligand will stimulate the production of antiligand antibodies (idiotypes) and, in turn, a-ids. The

resulting a-ids can recognize the ligand-binding site of the receptor.

In this report, we show that mice immunized with MV develop antibodies 30 to 35 days after immunization which react with cell surface receptors for binding MV by several criteria: these antibodies (1) bind to Vero cells and a panel of erythrocytes which are agglutinated by virus, (2) inhibit virus attachment to Vero cells, and (3) inhibit virus plaque formation in Vero cells. The characteristics and specificity of the antireceptor antibodies were determined. Cell-reactive sera immunoprecipitated Vero cell proteins of 20 and 30.5 kilodaltons from Vero cells.

MATERIALS AND METHODS

Virus. The MV used in this study was the attenuated Edmonston strain (20). Virus was grown in Vero cells, infected at 0.01 PFU/cell (1 h of attachment at 37°C) in Dulbecco medium. Cells were harvested when the monolayer was >90% fused by scraping into the overlay medium with a rubber policeman. The extract was frozen at -70°C, thawed twice at 23°C, and clarified (1,800 × *g*, 20 min). Virus was purified by banding twice in 20 and 60% (wt/wt) discontinuous sucrose gradients (70,000 × *g*, 2 h). The virus band was collected from the side of the tube, diluted sevenfold in phosphate-buffered saline (PBS) devoid of Ca and Mg, and pelleted (70,000 × *g*, 45 min). The final pellet was suspended in PBS (1 ml per 10⁹ infected cells) and dispersed with the aid of a 25-gauge needle. Virus was stored at -70°C. A typical preparation contained 250 µg of protein per ml as measured with a dye-binding assay (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin (BSA) as a standard.

Cells. Vero cells were grown in Dulbecco medium supplemented with 10% heat-inactivated fetal calf serum. Erythrocytes were from Hazleton/Dutchland (Denver, Pa.) and included African green monkey erythrocytes (MRBC); cynomolgus monkey erythrocytes (CMRBC); adult chicken erythrocytes (CRBC); sheep erythrocytes (SRBC); and rabbit erythrocytes (RRBC). Mouse erythrocytes (mouse RBC) were collected from ether-anesthetized BALB/c mice into Alsever solution (Flow Laboratories, McLean, Va.). Erythrocytes were washed four times in at least 10 volumes of PBS (1,000 × *g* for 5 min for CRBC; 10 min for MRBC, CMRBC, SRBC, RRBC, and mouse RBC) and resuspended

* Corresponding author.

[†] Present address: Department of Virus and Cell Biology, Merck Sharp and Dohme Research Laboratories, West Point, PA 19486.

[‡] Present address: Howard Hughes Medical Institutes, 6701 Rockledge Drive #2, Bethesda, MD 20817.

to 20% (vol/vol) in PBS, 4°C. Cells were used within 48 h after washing.

HA assays. Hemagglutination (HA) assays were performed in 96-well U-bottomed polystyrene microtiter plates (Dynatech, Alexandria, Va.). Serial twofold dilutions of virus or sera were made in PBS and mixed with an equal volume of 1% MRBC or CMRBC or 0.5% CRBC, SRBC, RRBC, or mouse RBC. Plates incubated at 37°C were shaken at 45 and 90 min. Titers, represented as the reciprocal of the endpoint dilution (HA units [HAU]), were read at 4 h or after overnight incubation and represented the highest dilution of sample which produced complete HA.

Immunizations. Female BALB/c mice (Jackson Laboratories, Bar Harbor, Maine), approximately 10 weeks old, were immunized intraperitoneally with 50 µg of viral protein in 200 µl of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) or a defined mix of monophosphoryl lipid A and trehalose dimycolate (MPL-TDM emulsion; RIBI Immunochem Research, Inc., Hamilton, Mont.). In some experiments, virus was solubilized in 1.5% octyl glucoside (Boehringer Mannheim, Indianapolis, Ind.) for 1 h at 4°C before injection. Detergent-treated virus was clarified for 45 min at 165,000 × *g* in an Airfuge (A-100/30 rotor; Beckman Instruments, Somerset, N.J.), and the supernatant was dialyzed against 1,000 volumes of PBS for 16 h at 4°C to remove detergent before mixing with adjuvant. Blood samples were collected from the retro-orbital plexus following ether anesthesia of the mice, and the serum was allowed to express from the clots for 1 h at 23°C and 16 h at 4°C. Sera were stored at -20°C.

Screening of antibodies to measles virus. Antibodies to virus were identified by radioimmunoassay (RIA) or by HA inhibition (HAI) assay. In the RIA, virus was solubilized in 1.5% octyl glucoside, diluted to 2 µg of protein and 0.015% octyl glucoside per 50 µl, and coated overnight in 50-µl volumes in Immulon 1 plates (Dynatech). Plates were washed three times with PBS and then reacted for 2 h at 23°C with dilutions of mouse sera in PBS-5% BSA. Wells were washed three times in PBS and incubated for 2 h at 23°C with 50-µl volumes of rabbit anti-mouse antiserum (anti-immunoglobulin G [IgG], -IgM, and -IgA) (Cooper Biomedical, West Chester, Pa.), radiolabeled with ¹²⁵I (1.48 × 10⁵ cpm/µg of protein). After being washed five times, cells were solubilized in 50 µl of 5% sodium dodecyl sulfate (SDS), and ¹²⁵I counts were measured in a Packard Gamma counter (40% counting efficiency). In some experiments, a peroxidase-conjugated anti-mouse immunoglobulin serum (Cappel) was substituted for the radioiodinated antibody. Bound antibody was detected by addition of a peroxidase substrate solution containing 1 mg of *o*-phenylene diamine in 10 ml of pH 5.0 phosphate-citrate buffer, with 0.06% H₂O₂. The enzyme reaction was stopped by addition of 25 µl of 4 M H₂SO₄, and color development was measured in a microplate reader at 490 nm.

Serum HAI activity was determined by incubating 2 HAU of virus with an equal volume of serial serum dilutions, in PBS, for 1 h at 23°C, followed by assay of remaining MRBC-HA activity. The resulting serum HAI titer was assigned to the highest dilution of serum which prevented virus agglutination of MRBC.

A third measurement of mouse antibodies to MV was in an immunoprecipitation assay, with 5 µl of mouse serum, octyl glucoside (1.5%)-solubilized ¹²⁵I-MV, and protein A-Sepharose beads, preloaded with rabbit anti-mouse antibodies (see Immunoprecipitations).

Reactivity of mouse sera with Vero cells. Confluent mono-

layers of Vero cells in 96-well microtiter plates (2 × 10⁴ cells per well) were washed twice in PBS and incubated with 50-µl volumes of mouse sera diluted in PBS-5% BSA for 2 h at 23°C. Wells were washed three times and incubated with a 1:1,000 dilution of ¹²⁵I-labeled rabbit anti-mouse antibodies (in buffer with 1:50 dilution of unlabeled rabbit anti-mouse antibodies) for 2 h at 23°C. Wells were washed five times, and bound ¹²⁵I counts were determined. Similar results were found at a 4°C incubation temperature during the antibody reactions.

Virus attachment inhibition. Vero cells in 96-well plates were incubated with serial dilutions of sera, as described for the detection of antibodies to Vero cells, and incubated with 30 µl of ¹²⁵I-labeled MV (5.61 × 10⁵ cpm/µg of protein), diluted 1:100 in PBS-1% BSA, for 30 min at 23°C. Attached virus was estimated from the ¹²⁵I counts bound.

Plaque inhibition assays. Confluent monolayers of Vero cells in six-well cluster plates (Falcon Plastics, Oxnard, Calif.), 10⁶ cells per well, were treated with 2.5 µl of test serum in 100 µl of Dulbecco medium for 30 min at 23°C and washed three times in PBS. Cells were infected with 150 PFU of virus in 100 µl of Dulbecco medium for 45 min at 23°C and overlaid with 3 ml of 0.5% Bacto-agar (Difco) in Dulbecco medium with 2% fetal calf serum. Plaques were visualized by staining the cells for 6 to 8 h with 3 ml of 0.005% neutral red in Dulbecco medium 3 to 4 days after infection.

Serum absorptions. Sera diluted in PBS were absorbed for 4 h at 4°C with cells, sera, protein A-Sepharose, or virus. Cells or precipitates were pelleted (10 s, 15,000 × *g*), and residual MRBC HA activity remaining in the supernatant was measured. Virus was pelleted by centrifugation (20 min, 15 lb/in²) in the A-100/30 rotor of an Airfuge (Beckman). Additionally, sera were incubated with MRBC, the cells were washed three times with PBS, and the fraction of antibodies bound to the cells was eluted with 100 µl of 50 mM glycine hydrochloride-150 mM NaCl, pH 1.5 (1 min, 4°C). The pH was adjusted to neutral by 10-fold dilution in PBS-50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.5.

Radiolabeling. Samples were iodinated with ¹²⁵I-monoiodo Bolton-Hunter reagent (NEX-120; New England Nuclear Corp., Boston, Mass.) by the procedure recommended by the manufacturer. Samples were labeled for 1 h on ice and then for 16 h at 4°C and used without further purification (antibody preparations) or pelleted and washed in PBS (viruses). Vero cells were metabolically labeled for 72 h with L-[³⁵S]methionine (NEG-009H; New England Nuclear), 450 µCi and 3 × 10⁶ cells, and solubilized for 1 h at 4°C in 1.0 ml of PBS-1.5% octyl glucoside with 2 mM phenylmethylsulfonyl fluoride (PMSF) and 100 µg of aprotinin per ml. The extract was clarified (30 min at 15,000 × *g*) and stored at -70°C for use in immunoprecipitations.

Immunoprecipitations. Samples for immunoprecipitation were diluted into 250 µl of buffer containing PBS, 1.5% octyl glucoside, 2 mM PMSF, 100 µg of aprotinin per ml, and a sample of test serum and incubated for 16 h at 4°C. A 50-µl volume of a 50% suspension of protein A-Sepharose CL-4B was added and incubated with shaking for 2 h on ice. The beads were washed five times for 10 s each, 15,000 × *g*, and solubilized in disruption buffer, in preparation for counts per minute determination and gel analysis.

SDS-PAGE. Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) with a discontinuous buffer system (23), with an acrylamide-bisacrylamide ratio of 30:0.8. The disruption buffer contained 5% SDS and 5%

2-mercaptoethanol. Samples were heated at 100°C for 5 min and clarified for 1 min at 15,000 × *g* before loading. Gels (13-cm resolving gel, 2.5-cm spacer gel) were electrophoresed for 16 to 18 h at 6 mA constant current. Gels were fixed for 2 h in 50% methanol–7% acetic acid, washed in 10% methanol–10% acetic acid, treated with En³Hance (New England Nuclear) fluorography enhancer, and dried under heat and vacuum. Gels were exposed to Kodak XAR-5 X-ray film at –70°C and developed in an automatic X-Omat processor. Molecular weights were determined by using high- and low-range molecular weight markers (Bio-Rad), visualized by staining with Coomassie blue R-250.

Protein determinations. Amounts of protein were measured in a dye-binding assay (Bio-Rad) with BSA as a standard.

Chemicals and reagents. All chemicals were of reagent quality and were from Sigma Chemical Co. (St. Louis, Mo.), unless otherwise indicated.

RESULTS

A series of experiments were designed to obtain antibodies recognizing MV receptors on Vero cells to facilitate the characterization and identification of the protein(s) involved in the virus-binding site. Our initial approach was based on the observation that monoclonal antibodies (idiotypes) with specificity for the receptor-binding site of the virus attachment protein could be used to elicit production of a-ids, which are homologous to the virus attachment protein and bind to receptors (27; reviewed in reference 15).

A monoclonal anti-MV hemagglutinin antibody, designated C1H (originally obtained from E. Norrby), was used to immunize mice, and the resulting sera were tested for their ability to inhibit binding of ¹²⁵I-labeled MV and Sendai (negative control) virus to Vero cells and agglutinate MRBC or CRBC (negative control) as indicators of MV receptor-binding activity. A polyclonal anti-MV serum (horse; Cappel Laboratories) was also used as a source of multiple antihemagglutinin idiotypes.

Although anti-C1H sera inhibited MV attachment (42 to 56%, with 1 µl of serum) in three of five mice (Table 1), no

FIG. 1. Reactivity of sera from mice immunized with MV in virus or virus receptor screens. Sera were collected at the indicated times after immunization and tested for the ability to inhibit virus HA (HAU; ●), agglutinate MRBC (HAU; ▲), bind to Vero cells (¹²⁵I-rabbit anti-mouse IgG [RAM]; Δ), or inhibit ¹²⁵I-MV (MS) attachment to Vero cells (○), as described in Materials and Methods.

agglutination of MRBC was observed and no Vero cell proteins were specifically immunoprecipitated with these sera (not shown). Additional monoclonal antihemagglutinin antibodies with a different idio type were not available for comparison. In contrast, anti-Vero cell antibodies both inhibited MV attachment and agglutinated MRBC. In preliminary experiments, anti-horse anti-MV sera also agglutinated MRBC.

These pilot experiments led to a second-generation approach for the production of antibodies to an MV binding receptor, using virus to generate anti-MV antibodies (idiotypes) and auto-anti-idiotypic antibodies, with methods described by Elias et al. and Shechter et al. (11, 33, 34).

Figure 1 shows the results of a representative experiment, in which the antibody responses of mice to a single immunization with MV were monitored. Sera were collected (two mice per time point) and screened in four different assays designed to identify antibodies to MV and MV receptors: (i) anti-MV activity, measured in an HAI assay; (ii) anti-Vero cell activity, measured by RIA with ¹²⁵I-labeled rabbit anti-mouse IgG; (iii) MV attachment inhibition activity, measured from the binding of ¹²⁵I-MV to Vero cells; and (iv) anti-MRBC activity, measured by agglutination of MRBC. Similar results were obtained with intact virus and detergent-extracted (1.5% octyl glucoside) virus and with Freund complete adjuvant versus a defined adjuvant mix (MPL-TDM emulsion). Results similar to those presented in Fig. 1 were obtained in four separate experiments, with peak MRBC HA titers occurring at 30 to 32, 35, 30 to 35, and 32 to 35 days, respectively, after immunization. None of the sera agglutinated CRBC, SRBC, or RRBC, which are not agglutinated by MV, to indicate a cell specificity of the serum HA activity.

TABLE 1. Ability of antisera to monoclonal and polyclonal anti-MV sera to inhibit virus attachment and agglutinate MRBC

Immunizing agent and mouse no. ^a	% Inhibition of ¹²⁵ I-MV attachment ^b	MRBC agglutination ^c (HAU/ml)
C1H		
5-1	56	<400
5-2	55	<400
2-1	42	<400
2-2	<1	<400
2-3	<1	<400
Vero cells	62	800
Horse anti-MV		
1	NT ^d	1,200
2	NT	600
3	NT	≤400
4	NT	1,200
5	NT	≤400

^a Mice were immunized with 50 µg of C1H monoclonal antibody or horse anti-MV serum or with 10⁶ Vero cells and boosted twice at 1-month intervals.

^b Vero cells, in 96-well plates, were incubated with 1 µl of serum, and attachment of ¹²⁵I-MV was determined. No inhibition of ¹²⁵I-Sendai virus attachment was observed with any of the sera.

^c None of the sera agglutinated CRBC.

^d NT, Not tested.

TABLE 2. Neutralization of serum HA activity

Treatment ^a	HAU (% of control)
Mock (PBS)	100
Horse anti-MV serum	31
Normal horse serum	100
C1H	31
Anti-MV serum (11 day)	≤50
Anti-MV serum (18 day)	44
Preimmune mouse serum	100
Rabbit anti-mouse IgG	28

^a Treatments consisted of 25 μ l of horse serum (anti-MV or normal horse serum) or 10 μ l of other sera and 8 HAU of test serum (from days 32 to 35 after immunization) for 1 h at 23°C. No HAU reduction was found with 10 μ l (10 μ g of protein) of mouse IgG or goat anti-rabbit immunoglobulins or an antiactin monoclonal antibody (from ascites) (kindly provided by Eugenia Wang). Results are the average of three experiments.

Mice first developed antibodies reactive with MV, which peaked at 11 to 18 days after immunization. Putative anti-MV receptor antibodies (a-ids) reactive with Vero cells or MRBC were detectable by day 18 and peaked by day 35. Sera from mice at 32 to 42 days after immunization inhibited ¹²⁵I-MV attachment to Vero cells, but did not affect ¹²⁵I-Sendai virus attachment (not shown). The specificity of the response for virus as the immunogen was revealed from the absence of a positive reaction in any of the four screens with sera from mice immunized with adjuvant alone or a mock virus preparation, i.e., an uninfected Vero cell extract adjusted to the same protein concentration as the virus for immunization.

The serum HA activity paralleled the Vero binding and virus attachment inhibition activities (Fig. 1). Therefore, to simplify the screening of sera, the MRBC HA assay was chosen for routine measurement of putative antireceptor activity. The MRBC HA activity of the sera was reduced (50 to 56%) following absorption with sera from days 11 to 18 (Table 2), the time of antiviral antibody production, but not with preimmune sera or sera from mice immunized with a mock MV preparation. Additionally, the serum HA activities were partially neutralized by polyclonal horse anti-MV serum (69%), monoclonal antihemagglutinin antibody (C1H) (69%), and rabbit anti-mouse IgG antibody (72%) (Table 2). The neutralizing titers of these sera (HAU units [HAU]) were 600 HAU/ml (35 mg of protein per ml) for horse anti-MV, 200 HAU/ml (1 mg/ml) for C1H, and 2,400 HAU/ml (1 mg/ml) for rabbit anti-mouse IgG sera. Normal horse serum and a monoclonal antiactin antibody did not detectably neutralize serum HA activity.

The specificity of the putative a-ids was evaluated by comparing the range of erythrocytes agglutinated by sera and MV. The pattern and extent of HA by these two reagents were essentially identical (Table 3), providing further evidence that the antibodies recognized an MV receptor. MRBC were most strongly agglutinated by both a-id serum and MV, followed by neuraminidase-treated CMRBC and untreated CMRBC. Chymotrypsin-treated MRBC were not detectably agglutinated by MV, but were weakly agglutinated by a-id serum. No detectable HA with a-id serum or virus was seen with CRBC, RRBC, SRBC, or mouse RBC. However, in contrast to the temperature dependence of MV HA (8,000 HAU at 4°C; 64,000 HAU at 23°C; and 512,000 HAU at 37°C), the MRBC HA activity of a-id sera was not affected by temperature.

An additional characterization of a-id sera is presented in

TABLE 3. Agglutination of different erythrocytes by mouse sera and MV

Cells	Serum ^a HA activity (HAU/ml)	MV HA activity (HAU/ml)
MRBC	7,467	102,400
CMRBC	1,467	1,600
Neuraminidase-treated CMRBC ^b	2,933	25,600
CRBC	<400	<1,600
RRBC	<400	<1,600
SRBC	<400	<1,600
Mouse RBC	<400	<1,600
Chymotrypsin-treated MRBC ^c	800	<12,800

^a Serum was collected from mice 35 days after immunization with MV.

^b Cells (100 μ l, 20% suspension) were incubated 1 h at 37°C with 5 U of *Vibrio cholerae* neuraminidase (Gibco) per ml, washed three times with PBS, and suspended to 20% (vol/vol) in PBS.

^c Cells (100 μ l, 20% suspension) were diluted to 1%, treated with 1% chymotrypsin for 30 min at 37°C, washed three times with PBS, and suspended to 20% (vol/vol) in PBS–0.5 mM PMSF–100 μ g of aprotinin per ml.

Table 4, where the ability of different erythrocytes, Vero cells, protein A-Sepharose, and MV to absorb MRBC HA activity from sera was determined. As expected, Vero cells and erythrocytes which were agglutinated by both MV and a-id sera effectively removed HA activity from solution, in a cell dose-dependent reaction. In contrast, MV-insensitive erythrocytes did not detectably bind MRBC HA activity. Protein A-Sepharose bound the activity, indicating that the HA reaction involved IgG antibodies. This confirmed the sensitivity of HA activity to anti-mouse IgG treatment (Table 2). It is important to note that MV (20 μ g of protein), the original immunization antigen, did not reduce MRBC HA titers, indicating that the cell-reactive antibodies were not simply directed to contaminating Vero cell proteins in the virus preparation. An approximation of the purity of the MV preparations was given from the finding that approximately 37% of the total counts in radioiodinated purified virus bound specifically to Vero cells or MRBC (not shown).

The biological activity of the sera was evaluated by measuring their ability to agglutinate MRBC, bind to Vero cells, inhibit MV attachment to Vero cells, and inhibit MV plaque production (infectivity) on Vero cells. Included in this experiment were sera from mice that were boosted with 50 μ g of MV at 35 and 56 days and bled at 81 days after the initial immunization in an attempt to obtain sera with increased cell-reactive titers. The MRBC HA, Vero binding,

TABLE 4. Absorption of serum HA activity

Treatment ^a	Relative HA activity (% of control HAU/ml)
None	100
Erythrocytes	
MRBC	<25
MRBC (5 μ l)	50
CMRBC	100
CRBC	100
SRBC	100
RRBC	100
Protein A-Sepharose	<25
Vero cells	38
MV	100

^a Mouse sera from the peak MRBC HA response (days 32 to 35) were incubated with 50 μ l of a 20% suspension of the indicated erythrocytes, 50% suspension of protein A-Sepharose CL-4B, 2×10^6 Vero cells, or 20 μ g of MV, and MRBC HA activity remaining in the supernatant was determined.

and virus attachment inhibition activities were closely linked (Table 5; also see Fig. 1). In addition, three sera (a-MV13, a-MVBoost/A, and a-MVBoost/B) with the highest MRBC HA titers also inhibited MV plaque formation. These three sera, and three additional sera corresponding to a peak HA titer, inhibited formation of virus-induced syncytia in Vero cells (2.5 μ l of serum per 2×10^4 cells) by 63 to 70% (data not shown). Receptor specificity was shown from the failure of these sera to inhibit plaque formation in Vero cells by simian virus 5 (SV5), which uses a receptor different from that used by MV. No decrease in activity of sera from mice boosted with virus was noted following absorption with intact MV (20 μ g).

A-ids were affinity purified from pooled sera from days 32 to 35 (Fig. 1) by absorption with MRBC and elution with 50 mM glycine buffer, pH 1.5, with no detectable loss of activity (320 HAU input and recovered), permitting an estimation of the antibody amounts. The amount of IgG antibody recovered, measured by enzyme immunoassay, was 45.4 μ g/ml, corrected for the amount of IgG recovered from sera of adjuvant-treated mice (5.6 μ g/ml). A pooled preparation from three separate experiments provided 23.8 μ g of specific antibody per ml. In the enzyme immunoassay, dilutions of antibody were adsorbed on Immulon 1 microtiter plates, and mouse IgG was detected with rabbit anti-mouse IgG and peroxidase-labeled goat anti-rabbit Ig. The IgG concentration in the affinity-purified samples was determined by using normal mouse IgG as a standard. The ability of this affinity-purified antibody, from the experiment described in Fig. 1, to inhibit virus attachment or infectivity was not determined. However, treatment of Vero cells in six-well plates with the second pooled preparation, containing 23.8 μ g of cell-reactive antibody per ml, produced a 45% reduction in MV plaque formation with 2.7 μ g of IgG and a 7% reduction with 0.14 μ g of IgG. A pooled serum preparation from mock-virus-immunized mice was used as a control.

In total, the data were consistent with the presence of antibodies in the a-id sera which recognized an MV receptor on Vero cells and erythrocytes. An evaluation was next made of the usefulness of such sera in identifying the virus receptors. Octyl glucoside was selected to prepare cell extracts, as this detergent solubilizes active MV receptors from Vero cells and MRBC (Krah and Choppin, in preparation). 35 S labeling was chosen over iodination (Bolton-Hunter reagent or oxidative labeling) because iodinated preparations did not provide consistent results in immunoprecipitations. A 1.5% octyl glucoside extract of [35 S]methionine-labeled (metabolically) Vero cells was immunoprecipitated with sera from a representative immunization experiment, performed as in Fig. 1, and the resulting proteins were identified by SDS-PAGE. Sera containing MRBC HA activity immunoprecipitated major 20K and 30.5K proteins (Fig. 2). Minor bands (faintly staining) at 12.5K, 49K, and 140K were also consistently precipitated. Additional 37K and 55-60K proteins were inconsistently observed in immunoprecipitates. These two major and three minor protein bands were also found with sera from MV-boosted mice (lane i). None of these protein bands were found with serum from mock-virus-immunized mice (lane j) or a preimmune serum (lane k). In a single experiment with affinity-purified a-ids, no specific reaction with any Vero cell proteins was observed. No specific immunoprecipitation of 125 I-labeled MRBC protein was found with any of these sera.

No viral proteins ([35 S]methionine or 125 I labeled) were immunoprecipitated by any of these sera (not shown). The failure of sera from days 11 to 18, which reacted with virus

TABLE 5. Reactivity of mouse sera in MV receptor assays^a

Mouse serum ^a	MRBC HA titer (HAU/ml)	Vero cell binding ^b (cpm)	Virus attachment inhibition ^c (% of control cpm)	Virus plaque reduction (% of control)
MV day 1	2,000	156	22	6
MV day 3	1,000	180	23	11
MV day 11	4,000	0	<1	11
MV day 13	16,000	3,521	72	38
MVBoost/A ^d	16,000	1,339	70	97
MVBoost/B ^d	16,000	2,489	44	94
Mock infected	<1,000	0	<1	15
Vero cells	$\leq$ 1,000	0	<1	13
Complete Freund adjuvant	<1,000	0	<1	8

^a Sera from mice immunized with MV, Vero cells, or adjuvant were screened for ability to agglutinate MRBC, bind to Vero cells, inhibit virus attachment to Vero cells, and inhibit virus plaque formation in Vero cells, as described in the text, with 2.5 μ l of serum. Results are the average of duplicate samples for each time point.

^b Measured from the binding of 125 I-rabbit anti-mouse IgG to Vero cells in 96-well microtiter plates.

^c 125 I-MV attachment to Vero cells in 96-well plate. No effect on 125 I-Sendai virus attachment to Vero cells was observed with any of the sera.

^d Mice were boosted 35 and 56 days after the initial immunization with MV, and serum was collected on day 81.

in an HAI assay, to immunoprecipitate viral proteins may be due to a low antibody titer or the probability that these antibodies were IgM (primary response) and would not be detected by protein A.

DISCUSSION

The immunological response to antigens has been exploited in several systems to facilitate the study of cell surface receptors. In these studies, a primary antibody, specific for the receptor-binding site of a ligand (hormone, drug, virus), was used to elicit production of secondary a-ids (21) which mimicked the original ligand (12, 14, 15, 32). The a-ids served as a structural homolog to a site on the original ligand and could bind to appropriate receptors. In some systems, including insulin (11), substance P (8), and alprenolol (17, 31), a-ids could mimic the action of ligand binding.

The resulting a-ids have been proposed as a more chemically defined, stable probe for receptors, which avoids unfavorable characteristics of the original ligands, including low affinity constants, instability, and multivalency (14, 15). Furthermore, a-ids can be generated in the absence of purified or well-characterized receptor proteins. These antibodies have proven useful in the identification of receptors for factor H, retinol-binding protein, insulin, thyrotropin, alprenolol, acetylcholine, mammalian reovirus type 3, leukemogenic retroviruses, and polyomaviruses (26; reviewed in reference 15), and chemotactic formyl peptide (25). Additionally, a-ids have been proposed as a potential alternative vaccine source, for agents including poliovirus type 2, reovirus 3, Sendai virus, and rabies virus (10, 15) and hepatitis B virus and trypanosomes (10).

Recent work in this laboratory considered the early interactions of MV with cellular receptors. One focus of this work involved the development of an antireceptor antibody, to facilitate the identification of receptors for binding MV. Preliminary attempts to obtain monoclonal antibodies specific for an MV receptor, as used with group B coxsackieviruses (4, 9) and rhinoviruses (7), were not successful.

We chose to exploit the natural immune network response to purified MV to generate a-ids specific for the receptor,

FIG. 2. Immunoprecipitation of Vero cell extract with mouse sera. An octyl glucoside extract of [35 S]methionine-labeled Vero cells was immunoprecipitated with 2.5 μ l of serum (2 h on ice) and 50 μ l of a 50% suspension of protein A-Sepharose (1 h, on ice). Immunoprecipitates were washed in PBS-1.5% octyl glucoside and solubilized in SDS-PAGE disruption buffer, containing 5% SDS and 5% 2-mercaptoethanol. Samples were analyzed by SDS-PAGE on 12.5% acrylamide gels. Lanes a to h and i to k represent two separate immunoprecipitations with mouse sera collected at the indicated times after immunization with MV (lanes a to h) or a mock virus preparation (serum M, lane j). Lane i, Serum (serum A) obtained from mice receiving two booster immunizations with virus. Lane k, Preimmune serum. Sera collected from replicate mice at 28 or 31 days are shown in lanes c and d and lanes e and g, respectively. Small arrows on the far left indicate the positions of molecular weight (MW) markers (in thousands). Large arrows on the right represent positions of protein bands immunoprecipitated specifically by sera exhibiting HA activity. At the bottom are presented the time (in days [d]) after immunization at which serum was collected, and the serum MRBC HA titer (in 10^3 HAU per milliliter).

patterned after the work of Shechter et al. in an insulin-receptor system (33, 34). A limitation to this approach is that the immunizing antibody (idiotype) must recognize the receptor-binding site of the ligand. The MV hemagglutinin protein contains at least three epitopes, two partially overlapping and one nonoverlapping, as shown by mapping with monoclonal antibodies (5, 16, 30, 35). However, the site(s) involved in the receptor-binding site of the hemagglutinin have not been identified. Monoclonal (C1H) and polyclonal (horse anti-MV) antibodies apparently recognized a determinant of this binding site, as both of these antibodies reacted with a-ids specific for MV receptors (Table 2). Additionally, immunization of mice with C1H or horse anti-MV preparations generated antibodies which inhibited MV attachment but did not consistently agglutinate MRBC (Table 1).

An immune network response following immunization with virus was selected to ensure that antibodies to all MV hemagglutinin epitopes were available to generate a-ids. A single dose of virus (50 μ g) reproducibly generated serum antibodies 30 to 35 days after immunization which (i) agglutinated erythrocytes susceptible to MV agglutination, (ii) bound to Vero cells, (iii) inhibited MV attachment to Vero cells, and (iv) in several sera, inhibited virus infectivity (plaque formation). The kinetics of antibody development closely paralleled that seen for the development of antibodies to insulin and insulin receptors following immunization of mice with insulin (11, 33, 34).

A close correlation was noted between the ability of the sera to agglutinate MRBC and inhibit plaque formation. Only sera with the highest HA titers effectively inhibited virus infectivity in Vero cells, indicating a threshold value of antibodies needed to inhibit plaque formation. For example, 0.11 μ g of a-id antibody, from day 32 to 35 sera, did not significantly inhibit MV plaque formation in Vero cells (11% plaque reduction; data not shown), but did inhibit virus attachment by 34% (Fig. 1). An affinity-purified pooled

serum preparation provided a 45% reduction in virus plaque formation with 2.7 μ g of a-id IgG and a 7% reduction with 0.14 μ g of IgG. Similarly, Marriott et al. (26) showed that 0.01 to 1 μ g of a-id antibody inhibited polyomavirus infection of BHK cells by 20 to 50%, while 10 μ g or more was needed to produce >90% protection.

The development of serum activity in MV receptor screens was not dependent on the adjuvant preparation or physical state of the virus. Comparable results were obtained with Freund complete adjuvant versus a defined mixture (MPL-TDM emulsion) or intact virus versus octyl glucoside-solubilized virus preparations. The response was specific for MV as the immunogen, as mice immunized with adjuvant alone or an equivalent amount of uninfected Vero cell protein (mock infection) did not develop antibodies reactive in any of the receptor screens. The activity was not due to antibodies to host cell proteins in the virus preparation (2), as MRBC HA titers were not reduced following absorption with excess MV. The composite data support the hypothesis that mice immunized with MV develop antibodies (IgG; Table 4) which recognize an MV-binding site on both host cells (Vero) and erythrocytes (MRBC).

During the preparation of this manuscript, Richert et al. (J. Richert, J. Gheuens, H. McFarland, and D. McFarlin, Fed. Proc. 46:776, 1987) reported on the use of monoclonal anti-MV hemagglutinin antibodies to produce monoclonal a-ids which inhibited MV infection of Vero cells (45 and 72% inhibition, respectively, with two a-ids), confirming that antireceptor antibodies could be generated in the MV system by using a-id methods. An earlier report described the appearance in mice of a-ids which neutralized the virus-binding activity of monoclonal antihemagglutinin antibodies following six immunizations with the monoclonals (16). However, no interaction of these a-ids with MV receptors was investigated.

A primary application of the a-ids derived in the present

study was to permit identification of MV receptors. To this end, sera consistently immunoprecipitated putative 20K and 30.5K receptor proteins from octyl glucoside-solubilized Vero cells, as well as minor 12.5K, 49K, and 140K bands. An increase in the amount of the 12.5K protein coincided with a decrease in the 30.5K protein (not shown), suggesting that the 30.5K protein may be degraded into the smaller protein. However, the relationships between the proteins identified in immunoprecipitates and their ability to bind MV were not investigated further.

The observation that antibodies to an MV receptor develop following a single immunization with MV in an auto-anti-idiotypic system (11, 24) raises the possibility that MV may induce an autoimmune response against MV receptors in humans after the initial virus infection (29). A-ids directed against normal receptors on the cell surface have been implicated in several autoimmune diseases, including myasthenia gravis (acetylcholine receptors), Grave's disease (thyroid-stimulating hormone receptors), insulin-resistant diabetes mellitus (insulin receptors), asthma (β -adrenergic receptors), and pernicious anemia (gastrin receptors) (reviewed in references 15, 18, and 28). Additionally, a correlation was recently reported between the presence of antibodies to the Epstein-Barr virus/C3d receptor and rheumatoid arthritis (3). A second role of a-ids in enhancing disease by neutralizing the protective effects of anti-MV antibodies has been proposed (16).

It would be of interest to test human sera for reactivity with MV receptors to determine whether a potential autoimmune response is induced by MV. The appearance of a biological marker for an antireceptor immune response would lead to improved understanding of virus pathology, as well as provide insight into the role that virus receptors serve in the host cell.

ACKNOWLEDGMENTS

We gratefully acknowledge the expert technical assistance of Elisa Stone and Karen Winkel.

This work was supported by Public Health Service grant AI-07233 from the National Institutes of Health.

LITERATURE CITED

- Ardman, B., R. H. Khirya, and R. S. Schwartz. 1985. Recognition of a leukemia-related antigen by an anti-idiotypic antiserum to an anti-gp 70 monoclonal antibody. *J. Exp. Med.* 161:669-686.
- Armstrong, M. A., K. B. Fraser, and P. V. Shirodaria. 1984. Recognition of host-membrane antigens in the envelope of measles virus. *Intervirology* 21:210-220.
- Barel, M., A. Kahan, C. Charriat-Marlangue, A. Kahan, and R. Frade. 1986. Autoantibodies against gp140, the Epstein-Barr virus and C3d receptor in sera from rheumatoid arthritis patients. *Eur. J. Immunol.* 16:1357-1361.
- Campbell, B. A., and C. E. Cords. 1983. Monoclonal antibodies that inhibit attachment of group B coxsackieviruses. *J. Virol.* 48:561-564.
- Carter, M. J., M. M. Willcocks, S. Löffler, and V. Ter Meulen. 1982. Relationships between monoclonal antibody-binding sites on the measles virus hemagglutinin. *J. Gen. Virol.* 63:113-120.
- Choppin, P. W., and A. Scheid. 1980. The role of viral glycoproteins in adsorption, penetration, and pathogenicity of viruses. *Rev. Infect. Dis.* 2:40-61.
- Colonna, R. J., P. L. Callahan, and W. J. Long. 1986. Isolation of a monoclonal antibody that blocks attachment of the major group of human rhinoviruses. *J. Virol.* 57:7-12.
- Couraud, J.-Y., E. Escher, D. Regoli, V. Imhoff, B. Rossignol, and P. Pradelles. 1985. Anti-substance P anti-idiotypic antibodies. Characterization and biological activities. *J. Biol. Chem.* 260:9461-9469.
- Crowell, R. L., A. K. Field, W. A. Schleif, W. J. Long, R. J. Colonna, J. E. Mapoles, and E. A. Emimi. 1986. Monoclonal antibody that inhibits infection of HeLa and rhabdomyosarcoma cells by selected enteroviruses through receptor blockade. *J. Virol.* 57:438-445.
- Dreesman, G. R., and R. C. Kennedy. 1985. Anti-idiotypic antibodies: implications of internal image-based vaccines for infectious diseases. *J. Infect. Dis.* 151:761-765.
- Elias, D., R. Maron, I. R. Cohen, and Y. Shechter. 1984. Mouse antibodies to the insulin receptor developing spontaneously as anti-idiotypes. II. Effects on glucose homeostasis and the insulin receptor. *J. Biol. Chem.* 259:6416-6419.
- Farid, N. R., and T. C. Y. Lo. 1985. Anti-idiotypic antibodies as probes for receptor structure and function. *Endocrine Rev.* 6:1-23.
- Fenger, T. W., and C. Howe. 1979. Isolation and characterization of erythrocyte receptors for measles virus. *Proc. Soc. Exp. Biol. Med.* 162:299-303.
- Gaulton, G. N., M. S. Co, H.-D. Royer, and M. I. Greene. 1985. Anti-idiotypic antibodies as probes of cell surface receptors. *Mol. Cell. Biochem.* 65:5-21.
- Gaulton, G. N., and M. I. Greene. 1986. Idiotypic mimicry of biological receptors. *Annu. Rev. Immunol.* 4:253-280.
- Gheuens, J., D. E. McFarlin, K. W. Rammohan, and W. J. Bellini. 1981. Idiotypes and biological activity of murine monoclonal antibodies against the hemagglutinin of measles virus. *Infect. Immun.* 34:200-207.
- Guillet, J. G., S. V. Kaveri, O. Durieu, C. Delavier, J. Hoebeke, and A. D. Strosberg. 1985. β -Adrenergic agonist activity of a monoclonal anti-idiotypic antibody. *Proc. Natl. Acad. Sci. USA* 82:1781-1784.
- Hansen, B. L. 1986. Why do some individuals produce autoreactive antibodies against receptors and/or their ligands? A possible answer to the question. A review with implications. *Scand. J. Immunol.* 24:363-370.
- Howe, C. 1979. Measles virus proteins and receptors, p. 63-79. In A. G. Bearn and P. W. Choppin (ed.), *Receptors and human diseases*. Josiah Macy, Jr. Foundation, New York.
- Hull, J. D., D. L. Krah, and P. W. Choppin. 1987. Resistance of a measles virus mutant to fusion inhibitory oligopeptides is not associated with mutations in the fusion peptide. *Virology* 159:368-372.
- Jerne, N. K., J. Roland, and P.-A. Cazenave. 1982. Recurrent idiotypes and internal images. *EMBO J.* 1:243-247.
- Kauffman, R. S., J. H. Noseworthy, J. T. Nepom, R. Finberg, B. N. Fields, and M. I. Greene. 1983. Cell receptors for the mammalian reovirus. II. Monoclonal anti-idiotypic antibody blocks viral binding to cells. *J. Immunol.* 131:2539-2541.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
- Ludwig, D. S., R. A. Finkelstein, A. E. Karu, W. S. Dallas, E. R. Ashby, and G. K. Schoolnik. 1987. Anti-idiotypic antibodies as probes of protein active sites: application to cholera toxin subunit B. *Proc. Natl. Acad. Sci. USA* 84:3673-3677.
- Marasco, W. A., and E. L. Becker. 1982. Anti-idiotypic antibody against the formyl peptide chemotaxis receptor of the neutrophil. *J. Immunol.* 128:963-968.
- Marriott, S. J., D. J. Roeder, and R. A. Consigli. 1987. Anti-idiotypic antibodies to a polyomavirus monoclonal antibody recognize cell surface components of mouse kidney cells and prevent polyomavirus infection. *J. Virol.* 61:2747-2753.
- Nepom, J. T., H. L. Weiner, M. A. Dichter, M. Tardieu, D. R. Spriggs, C. F. Gramm, M. L. Powers, B. N. Fields, and M. I. Greene. 1982. Identification of a hemagglutinin-specific idiotypic associated with reovirus recognition shared by lymphoid and neural cells. *J. Exp. Med.* 155:155-167.
- Noseworthy, J. H., B. N. Fields, M. A. Dichter, C. Sobotka, E. Pizer, L. L. Perry, J. T. Nepom, and M. I. Greene. 1983. Cell receptors for the mammalian reovirus. I. Syngeneic monoclonal

- anti-idiotypic antibody identifies a cell surface receptor for reovirus. *J. Immunol.* **131**:2533–2538.
29. **Plotz, P. H.** 1983. Autoantibodies are anti-idiotypic antibodies to antiviral antibodies. *Lancet* **ii**:824–826.
 30. **Sato, T. A., A. Fukuda, and A. Sugiura.** 1985. Characterization of major structural proteins of measles virus with monoclonal antibodies. *J. Gen. Virol.* **66**:1397–1409.
 31. **Schreiber, A. B., P. O. Couraud, C. Andre, B. Vray, and A. D. Strosberg.** 1980. Anti-alprenolol anti-idiotypic antibodies bind to β -adrenergic receptors and modulate catecholamine-sensitive adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **77**:7385–7389.
 32. **Sege, K., and P. A. Peterson.** 1978. Use of anti-idiotypic antibodies as cell-surface receptor probes. *Proc. Natl. Acad. Sci. USA* **75**:2443–2447.
 33. **Shechter, Y., D. Elias, R. Maron, and I. R. Cohen.** 1983. Mice immunized to insulin develop antibody to the insulin receptor. *J. Cell. Biochem.* **21**:179–185.
 34. **Shechter, Y., D. Elias, R. Maron, and I. R. Cohen.** 1984. Mouse antibodies to the insulin receptor developing spontaneously as anti-idiotypes. I. Characterization of the antibodies. *J. Biol. Chem.* **259**:6411–6415.
 35. **Sheshberadaran, H., and E. Norrby.** 1986. Characterization of epitopes on the measles virus hemagglutinin. *Virology* **152**: 58–65.
 36. **Ter Meulen, V., and M. J. Carter.** 1982. Morbillivirus persistent infections in animals and man, p. 97–132. *In* B. W. J. Mahy, A. C. Minson, and G. K. Darby (ed.), *Virus persistence*. Cambridge University Press, Cambridge.