Evidence for at Least Two Dominant Neutralization Antigens on Human Rhinovirus 14

BARBARA SHERRY AND ROLAND RUECKERT*

Biophysics Laboratory and Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

Received 2 July 1984/Accepted 14 September 1984

A collection of 28 mutants of human rhinovirus 14, selected for resistance to 10 individual neutralizing monoclonal antibodies, was used to identify two major neutralization antigens, N-Ag I and N-Ag II. Isoelectric analysis showed that all 16 of the N-Ag I mutants analyzed were charge altered in VP1; 8 of 12 N-Ag II mutants were altered in VP3. These results suggest that N-Ag I resides on VP1, whereas N-Ag II lies on VP3. The frequency of charge alterations was much higher than predicted by the genetic code, suggesting that charged amino acids on the antigenic sites play an important role in interaction with neutralizing antibody. Antibodies against N-Ag I and N-Ag II neutralize with widely different efficiencies.

Rhinoviruses are a predominant cause of the common cold in humans (4, 10). These riboviruses belong to the family *Picornaviridae* (18), which includes, among others, poliovirus and foot-and-mouth disease virus. The protein shell of these nonenveloped viruses is composed of 60 protein subunits, each containing four polypeptide chains, VP1 through VP4 (23).

The serology of human rhinoviruses has been studied extensively and has revealed at least 89 serotypes (4, 10, 11), but there have been no reports of the use of monoclonal antibodies to study their antigenic substructure. Studies on poliovirus have revealed the value of monoclonal antibodies for studying the location of neutralization antigens, revealing that most, if not all, murine monoclonal antibodies stimulated by intact virions are directed against a single antigen located on VP1 (20).

We now report the isolation of a panel of 10 monoclonal antibodies, also raised against intact virions, and we describe use of the antibodies to identify two major neutralization antigens on human rhinovirus 14 (HRV-14). One of the antigens was located on VP1, as reported for poliovirus. The second antigen was located on VP3, raising the possibility that the neutralization antigens on rhinoviruses may differ significantly in number from those on enteroviruses.

MATERIALS AND METHODS

Cells and media. H1-HeLa cells, a cloned line of HeLa cells selected for the ability to grow HRV-14, were propagated in monolayers with medium A or in suspension with medium B (19). The growth media for HeLa cells were routinely supplemented with 10% bovine serum. SP2/0 myeloma cells, a gift of Bill Sugden, University of Wisconsin, were propagated in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.; made 0.2% NaHCO₃ and adjusted to pH 7.2 with HCl) supplemented with 1 mM pyruvate, 2 mM glutamine, 2 mM oxaloacetic acid, and 20% bovine serum (containing penicillin and streptomycin for a final medium concentration of 100 U of each per ml). Hybridoma cells were selected in HAT medium (13.6 µg of hypoxanthine, 0.175 µg of amethopterin, and 3.9 µg of thymidine per ml) and then propagated in the myeloma cell medium. Human fibroblast cells, a gift of Bill Sugden, were propagated in

Dulbecco medium supplemented with 10% fetal bovine serum.

Medium P6, used for plaque assay, consisted of medium A supplemented with 40 mM MgCl₂ and 0.1% bovine serum albumin. Phosphate-buffered saline consisted of 137 mM NaCl, 2.7 mM KCl, 1.47 mM KH₂PO₄, 4.86 mM Na₂HPO₄, 0.68 mM CaCl₂ · 2H₂O, and 0.49 mM MgCl₂ · 6H₂O. PBSA consisted of phosphate-buffered saline supplemented with 0.1% bovine serum albumin.

High-multiplicity-of-infection (MOI) virus stocks. HRV-14 strain 1059 was plaque purified three times after receipt from V. V. Hamparian of Ohio State University, and the serological identity of the resulting stock was confirmed by neutralization with specific antiserum from the American Type Culture Collection.

Stock (high-MOI) virus was propagated at 35°C with a multiplicity of 10 to 20 PFU per cell unless otherwise specified. Virus was harvested 8 h postinfection by sedimenting the cells, which contained at least 95% of the infectivity, and freeze-thawing three times in medium A or AL (24) at a concentration of 4×10^7 cells per ml. Cell debris was removed by low-speed centrifugation to yield a crude extract.

Low-MOI virus stocks. Virus was plaque purified as follows. Agar plugs, selected from plates with one plaque each, were freeze-thawed in 1 ml of PBSA to release virus, typically 10^4 to 10^5 PFU, and the supernatant was used to infect monolayers containing about 4×10^6 cells. The progeny virus was then amplified by infecting suspension cultures at low MOI (<0.1 PFU per cell). Virus was harvested after a single growth cycle (8 h) to minimize the incidence of "mosaic" virus, which might otherwise result from simultaneous infection with mutant and wild-type virus.

Viral antigen. Crude extract (30 ml) was supplemented with Sarkosyl to 1% and with 2-mercaptoethanol to 0.1%, underlaid with 2 ml of a 30% sucrose solution in buffer IV (24), and centrifuged in an SW25 rotor for 4 h at 25,000 rpm and 15°C. The viral pellet was suspended in LS buffer (10 mM NaCl, 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.5], 0.01% bovine serum albumin), layered on two linear 5-ml gradients (7.5 to 45% sucrose in LS buffer), and centrifuged in an SW50 rotor for 75 min at 45,000 rpm and 15°C. The viral bands were removed in 1.25-ml volumes with a Pasteur pipette, diluted

* Corresponding author.

twofold with LS buffer, layered on two preformed 5-ml 20 to 45% CsCl gradients, and centrifuged in an SW50 rotor for 8 h at 45,000 rpm and 6°C. The viral bands were removed in 1.5-ml volumes and desalted by gel filtration (Bio-Gel A-5m, 100-200 mesh; Bio-Rad Laboratories, Richmond, Calif.), and the virus concentration was determined spectrophotometrically (24). The virus was diluted to a final concentration of 50 µg/ml in phosphate-buffered saline containing a final concentration of 0.02% bovine serum albumin and stored at -70°C in working samples.

Radiolabeled virus preparations. Wild-type HRV-14 was radiolabeled with [³⁵S]methionine to a specific activity of 10⁴ to 10⁵ cpm/µg of virus, by a modification of the method of Rueckert and Pallansch (24). Briefly, H1-HeLa cells were infected with HRV-14 at an MOI of 10 to 20 PFU per cell in medium A (100 µM methionine), and [³⁵S]methionine (0.5 mCi/10⁸ cells) was added 2 h postinfection. Virus was harvested 8 h postinfection by freeze-thaw lysis and sucrose gradient centrifugation as described above, followed by fractionation and pooling of the viral peak fractions. Approximately 1% of the added radiolabel was recovered in purified virions.

HRV-14 mutants to be analyzed by isoelectric focusing (IEF) were radiolabeled as described above at an MOI of approximately 1 PFU per cell. The sedimented cells were lysed with 0.5% Nonidet P-40 (NP-40), rather than multiple freeze-thaws. Cell debris was removed by low-speed centrifugation, the supernatant was made 1% in Sarkosyl and 0.1% in 2-mercaptoethanol, and the virus, in a 10-ml volume, was centrifuged through a 1-ml cushion of 30% sucrose in buffer IV with an SW41 rotor for 2 h at 40,000 rpm and 15°C. The virus pellet was suspended in 100 µl of LS buffer for storage at -70°C.

Pactamycin mapping. Two suspensions (2 × 10⁷ cells in 0.5 ml) were infected at room temperature with 50 PFU/cell in medium AL (medium A without amino acids, except glutamine, and supplemented with 25 mM HEPES, pH 7.4) supplemented with 5 µg of actinomycin D per ml and then diluted with 9 volumes of the same medium 30 min later. Four hours postinfection, 25 µl of pactamycin (10⁻⁴ M pactamycin in 1 mM acetic acid) or 1 mM acetic acid only (control) was added, followed by the immediate addition of 100 µCi of [³⁵S]methionine. Eight hours postinfection, virus was harvested by NP-40 cell lysis and purified by sucrose gradient fractionation as described above.

Monoclonal antibodies. Ten hybridoma cell lines were selected for their ability to secrete monoclonal antibodies that neutralize HRV-14, by a modification of the method of Kohler and Milstein (15). Briefly, BALB/c mice were injected intraperitoneally with an emulsion of Freund adjuvant and approximately 5 µg of purified HRV-14 per injection according to the following schedule: day 0, in complete Freund adjuvant; days 7, 14, and 42 or 73, in incomplete Freund adjuvant. Three days after the final injection, spleen cells were harvested and used in a polyethylene glycol-mediated fusion with SP2/0 myeloma cells. The fused cells were selected on HAT medium, screened for HRV-14 neutralizing activity in microneutralization assays, cloned three times by limiting dilutions with irradiated human fibroblast feeder cells, amplified, and injected intraperitoneally into mineral oil-primed BALB/c mice to produce ascites fluid. The ascites fluids were harvested after 6 to 10 days and clarified by low-speed centrifugation, and the supernatant fluids were stored at -70°C. Sterile filtered dilutions (0.22-µm membrane filters; Millipore Corp., Bedford, Mass.) of the ascites fluids were used in these studies.

Plaque assay. H1-HeLa cell monolayers were prepared by seeding 60-mm culture dishes with 4 × 10⁶ to 5 × 10⁶ cells

suspended in medium A. After 3 to 5 h of incubation at 37°C in 5% CO₂, the medium was removed, the cell monolayer was washed with phosphate-buffered saline, and 200 µl of virus was applied. Virus was allowed to attach to cells for 30 min at room temperature, and then an overlay of 2.5 ml of agar (0.8% Noble agar in medium P6) followed by 2.5 ml of medium P6 (supplemented with 0.2% glucose, 2 mM pyruvate, 4 mM glutamine, and 4 mM oxaloacetic acid to enhance survival of the cell monolayer) was applied. Plates were incubated at 35°C in 5% CO₂ for 48 h, the overlays were removed, and the monolayers were stained for >5 min with 0.1% crystal violet in 20% ethanol. After the plates were rinsed with distilled water, plaques were scored. All samples were plated in duplicate.

Isolation of antibody-resistant mutants. For determination of the frequency of monoclonal antibody-resistant mutants, 250 µl of low-MOI cloned virus at 10⁷ PFU/ml was incubated in PBSA for 1 h at room temperature with 250 µl of a 10⁻² dilution of sterile ascites fluid in PBSA. In all cases, this represented enough antibody to give maximal neutralization, with residual infectivity ranging from 1 × 10⁻² to 4 × 10⁻⁶ (see Table 4). The treated virus was then plated by the standard plaque assay procedure with a 5 × 10⁻⁴ dilution of ascites fluid in the overlay to allow plaque development by mutant virus, but not by surviving wild-type virus. "Resistant" plaques, isolated by this method, were never found to be wild type.

Nonneutralized fraction. For determination of the frequency of the nonneutralized fraction, 250 µl of high-MOI virus was incubated in PBSA for 1 h at room temperature with 250 µl of a 10⁻² dilution of sterile ascites fluid in PBSA. This treated virus was plated by the standard plaque assay procedure. The cell monolayers were washed after virus attachment to ensure the absence of free antibody, which might inhibit the plaque development of the nonneutralized fraction.

Microneutralization assay. To screen for hybridomas secreting HRV-14-neutralizing antibody, 100-µl samples of medium from hybridoma cultures in 96-well multititer plates were aliquoted to matching wells of an empty 96-well plate. Virus, 10⁴ PFU in 50 µl of medium P6, was then added to each well, and the plates were incubated for 1 h at 35°C in 5% CO₂. H1-HeLa cells (5 × 10⁴) in 100 µl of medium P6 were then added, and the plates were incubated as above. After 48 h, the plates were stained with 0.1% crystal violet in 20% ethanol.

For cross-neutralization tests, 50 µl of ascites fluid diluted in PBSA, followed by 50 µl of virus (wild type or mutant) in medium P6, was aliquoted into the appropriate wells of a 96-well culture plate. The plate was incubated for 1 h at 35°C in 5% CO₂, cells were added, and the assay was incubated and developed as described above.

IEF. IEF was carried out by a modification of the procedure of O'Farrell (21). The gel mix consisted of 3.78% acrylamide, 0.22% N,N'-methylenebisacrylamide, 9.2 M urea, 2% (wt/vol) NP-40, 3.13% each of pH 3 to 10 and pH 5 to 8 ampholines, 0.1% N,N,N',N'-tetramethylethylenediamine, and 0.02% ammonium persulfate.

To prepare slab gels, 40 ml of gel mix was poured in a 13-by-15-by-0.2-cm chamber and fitted with a slot-forming comb (0.8-cm teeth), overlaid with water-saturated isobutanol, and allowed to polymerize for at least 2 h. The wells were washed with distilled water, and the gel was placed in a standard vertical slab gel apparatus. The upper and lower reservoirs were filled with 0.01 M H₃PO₄ and 0.02 M NaOH, respectively. Samples (in 9.5 M urea, 2% [wt/vol] NP-40, ampholines as above, and 5% 2-mercaptoethanol) were loaded by underlaying, and the gels were run toward the

cathode at 400 V for 15 to 17 h followed by 800 V for 1 h. After removal from the glass plates, gels were fixed in two changes of gel soak (25% methanol, 10% acetic acid, 1% glycerol) for >1 h, soaked in En³Hance for 1 h and then soaked in distilled water for 1 h. Gels were dried on Whatman 3MM paper and subjected to autoradiography with Kodak XAR-5 film at -70°C.

To prepare cylindrical gels, a 0.6- by 13-cm column gel was poured, overlaid, and allowed to polymerize as described above. Samples were applied and overlaid with 9 M urea (in half-concentrations of ampholines with respect to the gel mix), and the samples were electrophoresed as described above. The gels were fractionated into 2-mm fractions in 0.5 ml of distilled water by an automatic gel crusher (Gilson Medical Electronics). For scintillation counting, each fraction was dissolved in 0.3 M NaOH for 2 h at 37°C and suspended in Tt21 (18).

Material sources. Bovine serum (HyClone) was from Sterile Systems, Inc. (Logan, Utah), and fetal bovine serum was from KC Biological, Inc. (Lenexa, Kans.). Penicillin, streptomycin, hypoxanthine, amethopterin, and thymidine were purchased from Sigma Chemical Co., St. Louis, Mo.; (nos. PEN-NA, S-6051, H-9377, A-6770, and T-9250, respectively). Polyethylene glycol was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J., (PEG 1540, no. 8-U220), or Eastman Kodak Co., Rochester, N.Y. (PEG 1450, no. 15414). [³⁵S]methionine and En³Hance are products from New England Nuclear Corp., Boston, Mass. (nos. NEG-009T and NEF-966, respectively). Pactamycin was purchased from The Upjohn Co., Kalamazoo, Mich. (no. U-15,800), and actinomycin D was from Sigma (no. A-5505). Acrylamide (ultrapure), *N,N'*-methylenebisacrylamide (ultrapure), and NP-40 were purchased from Bethesda Research Laboratories, Inc., Rockville, Md. Urea was purchased from Columbus Chemical Industries (ACS crystals), and pharmalytes were from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.

RESULTS

Selection and frequency of antibody-resistant HRV-14 mutants. Ten mouse hybridoma lines were developed for their ability to secrete monoclonal antibodies that neutralize HRV-14. Each was then used to select viral mutants resistant to neutralization by that antibody. When stocks prepared at a high MOI (10 PFU per cell) were used for this selection, the apparent mutation frequencies ranged from 10⁻⁷ to 10⁻⁵ (data not shown). On the other hand, stocks prepared at a low MOI (0.1 PFU per cell) resulted in frequencies ranging from 2 × 10⁻⁶ to 1 × 10⁻⁴ (Table 1). We attribute the lower mutation frequencies obtained with the high-MOI stocks to the presence of mosaic virions, i.e., mutant RNA genomes packaged in protein coats containing antibody-reactive as well as mutant subunits. Accordingly, to avoid underestimating the true mutation frequencies, low-MOI stocks were always used for determining the frequencies of antibody-resistant mutants.

The results summarized in Table 1 represent an average of three separate experiments, each using four plaque-purified virus stocks. The highest mutation frequencies (10⁻⁴) probably correspond to substitutions that have little, if any, effect on the ability of the mutant to multiply. Lower mutation frequencies may reflect the additional effects of silent mutations or the size or essentiality of the antigenic site. Similar frequencies of antibody-resistant mutants have been reported for other RNA viruses (6, 20, 22).

The standard deviation of the mean frequency of mutants for each monoclonal antibody ranged from 20% to approxi-

TABLE 1. Frequency of antibody-resistant HRV-14 mutants

Monoclonal antibody	Frequency of resistant mutants ^a (%SD)
1	1 × 10 ⁻⁵ (40)
3	8 × 10 ⁻⁵ (50)
4	3 × 10 ⁻⁵ (20)
6	9 × 10 ⁻⁵ (40)
7	9 × 10 ⁻⁵ (40)
2	2 × 10 ⁻⁶ (50)
5	1 × 10 ⁻⁵ (60)
8	8 × 10 ⁻⁶ (60)
9	3 × 10 ⁻⁵ (70)
10	8 × 10 ⁻⁶ (60)

^a Low-MOI cloned virus stocks were prepared as described in the text. In three separate experiments, each of four cloned stocks was incubated with individual monoclonal antibodies, and the frequencies of monoclonal antibody-resistant mutants were determined by plaque assay. The frequencies, rounded to the nearest integral figure, were calculated as the number of plaques in the presence of antibody divided by the number of PFU plated.

mately 70%. This large deviation was not, for the most part, due to experimental error and most likely reflects variation in the time at which the antibody-resistant mutations occurred during propagation from a single virion.

Antibody resistance patterns define at least seven neutralization epitopes. Each monoclonal antibody was used to select several HRV-14 mutants. The mutants were then incubated with each of the 10 antibodies in a microneutralization assay. Of 50 mutants, 30 behaved reproducibly in these assays, whereas the remaining 20 were unstable or gave ambiguous results and therefore were not studied further.

Each of the 30 mutants was classified by its resistance or sensitivity to neutralization by each of the monoclonal antibodies, thus defining a series of characteristic antibody resistance patterns (Fig. 1). For example, the mutants defining pattern A were resistant to antibodies 1, 3, 4, 6, and 7, but were neutralized by antibodies 2, 5, 8, 9, and 10. In all, the 30 mutants exhibited seven antibody resistance patterns, illustrated by classes A through F (Fig. 1). The epitopes that defined these antibody resistance patterns established that at least 7 of the 10 antibodies (1, 2, 3, 4, 5, 6, and 8) were unique. Antibody 3 was distinguished from antibody 6 by a single mutant that was only partially neutralized; we have

FIG. 1. Antibody resistance patterns of HRV-14 mutants. Antibody-resistant mutants or wild-type HRV-14 (WT) were assayed for resistance to neutralization by each of the 10 monoclonal antibodies. Neutralization is indicated by closed circles, absence of neutralization (resistance) is indicated by blanks, and partial neutralization is indicated by stippling. HRV-14 mutants were selected for resistance to monoclonal antibodies as described in the text. Any of the nonneutralizing antibodies appeared to be suitable for selecting mutants defining the resistance patterns. For example, mutants exhibiting pattern E were obtained with antibodies 5, 8, 9, or 10 (data not shown). Class C' represents a single isolate exhibiting a reproducible partial resistance to monoclonal antibody 3.

TABLE 2. Predicted effect of mutation on amino acid charge

Amino acid side chain	Charge	Charge of amino acid after missense mutation ^a			No. of ways in which indicated charge shift can occur ^b				
		-	0	+	2-	1-	0	1+	2+
Phe	0	0/16	16/16	0/16	0	0	16	0	0
Leu	0	0/33	27/33	6/33	0	0	27	6	0
Ile	0	0/21	20/21	1/21	0	0	20	1	0
Met	0	0/9	8/9	1/9	0	0	8	1	0
Val	0	4/24	20/24	0/24	0	4	20	0	0
Pro	0	0/24	18/24	6/24	0	0	18	6	0
Thr	0	0/24	20/24	4/24	0	0	20	4	0
Ala	0	4/24	20/24	0/24	0	4	20	0	0
Tyr	0	2/12	8/12	2/12	0	2	8	2	0
His	+	2/16	12/16	2/16	2	12	2	0	0
Gln	0	2/14	4/14	8/14	0	2	4	8	0
Asn	0	2/16	8/16	6/16	0	2	8	6	0
Lys	+	2/14	10/14	2/14	2	10	2	0	0
Asp	-	4/16	10/16	2/16	0	0	4	10	2
Glu	-	4/14	8/14	2/14	0	0	4	8	2
Cys	0	0/14	12/14	2/14	0	0	12	2	0
Trp	0	0/7	5/7	2/7	0	0	5	2	0
Arg	+	0/38	30/38	8/38	0	30	8	0	0
Ser	0	0/37	31/37	6/37	0	0	31	6	0
Gly	0	4/23	13/23	6/23	0	4	13	6	0

^a Single nucleotide substitutions resulting in an amino acid of the indicated charge/the total number of possible missense mutations predicted by the genetic code. Silent and termination mutations were excluded.

^b The number of single nucleotide substitutions which result in the indicated charge shift, computed from the missense mutation data. The following percentages (of all 396 missense mutations) were obtained: 2- shifts, 1%; 1-, 18%; 0, 63%; 1+, 17%; 2+, 1%.

not yet been able to distinguish antibody 7 from 6, 9 from 5, or 10 from 8.

The seven epitopes fall within two neutralization antigens.

Of the 30 mutants investigated, every one selected with antibodies of one group was neutralized by any member of the other group. Thus, mutants that were resistant to some or all of the monoclonal antibodies 1, 3, 4, 6, and 7 were always neutralized by the monoclonal antibodies 2, 5, 8, 9, and 10 (Fig. 1). Conversely, mutants resistant to all or some of the antibodies 2, 5, 8, 9, and 10 were completely neutralized by the antibodies 1, 3, 4, 6, and 7. These 30 mutants thus defined at least two neutralization antigens.

Here we adopt the definitions proposed by Wimmer et al. (28). The neutralization antigen represents that region of a capsid protein, composed of contiguous or noncontiguous amino acids, that induces and binds neutralizing antibodies. An epitope is the subset of those amino acids recognized by a particular neutralizing antibody. The epitopes that together define an antigen may or may not overlap, that is, genetic variants of a virus resistant to neutralization by one monoclonal antibody may or may not escape neutralization by others reacting with the same antigen.

That the 10 antibodies defined only two neutralization antigens is suggested by the high frequency of mutants resistant to all five antibodies within one group or the other. That is, 10 of 16 mutants (data not shown) selected with antibodies 1, 3, 4, 6, or 7 were resistant to all five of those antibodies (pattern A, Fig. 1). Similarly, 4 of 14 mutants (data not shown) selected with antibodies 2, 5, 8, 9, or 10 were resistant to all five of those antibodies (pattern D, Fig. 1). The seven epitopes thus segregated into two antigens. The first antigen, hereafter designated neutralization antigen I (N-Ag I), was detected by antibodies 1, 3, 4, 6, and 7, whereas the second antigen, N-Ag II, was detected by antibodies 2, 5, 8, 9, and 10.

Identification of HRV-14 coat proteins by IEF. The mutations in the antibody-resistant viruses define sites involved in HRV-14 neutralization. These mutations were mapped to the individual viral coat proteins by comparing the IEF

patterns of the mutant coat proteins to those of wild-type virus. This strategy was based upon the expectation that about 37% of all amino acid substitutions would involve a charge alteration detectable as a shift in isoelectric point of the protein (Table 2).

The first task, to develop a convenient and reproducible IEF system for separating all of the coat proteins, was solved empirically by a series of trials. By loading virions in 9.5 M urea-2% NP-40-5% mercaptoethanol on the acid side of an IEF gel and electrophoresing toward the cathode, the dissociated virion products were resolved into well-separated peaks (Fig 2A). The identities of the four peaks of wild-type virions were established in two steps: comparison of proteins in virions with those in natural top component and ordering on the translational map with the drug pactamycin.

The first step, comparing patterns of virions and natural top component, exploited the fact that picornaviruses are rich in VP1, VP2, VP3, and VP4, whereas natural top component is rich in VP0, VP1, and VP3, where VP0 is a cleavage precursor of VP2 and VP4 (23). Peaks 2 and 4 were tentatively identified as VP2 and VP4 by their relatively greater proportion in virions relative to natural top component (Fig. 2, compare peaks 2 and 4). Peak 2, in the pattern of natural top component, is not apparent because it focuses as an unresolved shoulder on the left side of VP0.

The second step used in assigning the IEF bands to individual HRV-14 coat proteins was to determine their relative order on the genetic map with the aid of the drug pactamycin. Pactamycin inhibits initiation of translation, but does not inhibit completion of previously initiated polypeptide chains. The drug acts almost instantly. Thus, when [³⁵S]methionine is added to infected cells simultaneously with pactamycin, proteins encoded at the 5' end of the genome incorporate less radiolabel than those at the 3' end, i.e., establish a gradient of radiolabel in the order VP4 < VP2 < VP3 < VP1 (Fig. 3). The IEF pattern of the viral protein showed the greatest inhibition in peak 4 (compare top and bottom panels, Fig. 4). This identifies peak 4 as VP4.

FIG. 2. IEF of proteins from native virions and natural top component. HRV-14 was radiolabeled and fractionated on a sucrose gradient as described in the text. Native virus and natural top component were electrophoresed in parallel IEF tube gels and fractionated, and the amount of radioactivity in each fraction was determined by scintillation counting. The rightmost peak probably represents an aggregate, which was present in variable amounts from one experiment to another.

Assignment of the peaks 1, 2, and 3 as VP1, VP2, and VP3, respectively, was similarly based on the gradient of ratios computed as indicated in Table 3.

Evidence that N-Ag I and N-Ag II lie on VP1 and VP3, respectively. To identify the mutated coat proteins, each of the 30 stable HRV-14 mutants was metabolically labeled with [^{35}S]methionine, purified, and electrophoresed on IEF gels. The resulting patterns could be assigned to seven different classes (Fig. 5, lanes a through c and e through h). All mutants mapping to N-Ag I by microneutralization assays had an altered VP1 isoelectric point (Fig. 5, compare lanes a through c with the wild-type pattern in lane d). Only 1 of the 16 N-Ag I mutants had a detectable charge shift in any other coat protein (Fig. 5, lane c). This suggests that N-Ag I is located on VP1. Similarly, all but 1 (see below) of the 12 N-Ag II mutants had an altered VP3 isoelectric point (Fig. 5, compare lanes e and f with lane d) or no detectable charge-shift (Fig. 5, lane g), suggesting that N-Ag II is located on VP3. There were no other obvious correlations between the mutant classes established by IEF and those identified by microneutralization pattern (Fig. 1).

The anomalous N-Ag I mutant (Fig. 5, lane c) had an altered VP3 in addition to the characteristic VP1 mutation. The anomalous N-Ag II mutant (Fig. 5, lane h), which presumably carried a neutral VP3 mutation, also had an unexpectedly altered VP1. These second mutations occurred with a much higher frequency, 2 of 28, than the first mutations, which ranged from 2×10^{-6} to 1×10^{-4} . This suggests strong selection for the second mutation, perhaps

FIG. 3. Genetic map of picornaviral coat protein-encoding region. Top, common nomenclature; bottom, systematic nomenclature (25).

FIG. 4. IEF of HRV-14 coat proteins radiolabeled in the presence and absence of pactamycin. Infected cells were radiolabeled in the presence or absence of pactamycin, the virions were purified, and the dissociated coat proteins were electrophoresed on IEF gels.

because it compensates for a debilitating effect that sometimes accompanies the first mutation.

Two of the 16 N-Ag I mutants involved a double positive charge shift in VP1; this shift may correspond to single mutations of glutamate to lysine or aspartate to histidine (Table 2). However, in view of the unexpectedly high frequency of double mutants illustrated in Fig. 5 (lanes c and h), the possibility that the double charge shifts reflect two mutations cannot be ruled out.

Nonneutralized fraction correlates with N-Ag II. When virus is incubated with increasing amounts of antibody, the infectivity decreases until it reaches a plateau where higher concentrations of antibody achieve no further neutralization (data not shown). This nonneutralized fraction has been observed previously by others and has been attributed to aggregation (27). This residual infectivity consists mainly of infectious wild-type virions whose plaque development was prevented only by including antibody in the overlay. The fraction of nonneutralized virus was characteristic of the antibody and was strongly correlated with the antigenic site (Table 4). Antibodies recognizing N-Ag II induced a non-neutralized fraction of about 1%, whereas antibodies recognizing N-Ag I (with the exception of antibody 1) neutralized the virus much more completely. The nonneutralized fraction induced by antibodies 3, 4, 6, and 7 was substantially lower than the corresponding frequencies (Table 1) of mu-

TABLE 3. Identification of coat proteins by pactamycin mapping

Peaks ^a	% of total counts ^b		Pactamycin ratio ^c	Identity ^d
	- Pactamycin	+ Pactamycin		
1	16.5	39.1	2.37	VP1
2	23.9	10.6	0.44	VP2
3	21.3	20.9	0.98	VP3
4	8.0	2.0	0.25	VP4

^a The fractions (Fig. 4) were selected to exclude the small unidentified peak flanking 1 on the right (Fig. 4B) and to exclude VP0, present as a shoulder on the right side of peak 2 (Fig. 4A). These corrections did not change the identification.

^b (Sum of counts per minute recovered in peak/total counts per minute recovered in gel) $\times 100$.

^c Ratio of results with pactamycin to results without pactamycin.

^d See Fig. 3.

FIG. 5. IEF patterns of HRV-14 mutants. Of the 30 mutants, 28 were successfully radiolabeled, and these exhibited seven different IEF patterns. Lanes a through c and e through h contain mutants representative of these seven patterns. Lane d contains wild-type HRV-14. The frequency with which the mutants were isolated is indicated beneath each lane.

tant virus. This illustrates how the presence of mosaic virions in high-MOI stocks reduces the neutralization-resistant fraction to levels less than the frequency of resistant mutations. In several experiments repeated with low-MOI virus stocks, the nonneutralized fraction resulting from antibodies 3, 4, 6, and 7 corresponded to the mutation frequencies reported in Table 1.

DISCUSSION

Do rhinoviruses carry neutralization antigens not found on enteroviruses? Recent studies on poliovirus indicate that when intact virions are used as an immunogen in mice, most, if not all, monoclonal antibodies are directed against an antigen located on VP1 (20). This is not to say that neutralizing antibodies are never directed against other sites. It has been shown that individually isolated poliovirus protein chains VP1, VP2, and VP3 (3), as well as synthetic peptides (2, 5), are also able to stimulate neutralizing antibodies. However, these isolated proteins and synthetic peptides are orders of magnitude less effective than intact virions in stimulating a neutralizing response. Thus, whereas protein

subunits on the poliovirion possess many potential neutralization antigens, a VP1 antigen, called "immunodominant," appears to be much more immunogenic than any of the others on poliovirus.

The microneutralization assays reported here show that, of 10 neutralizing monoclonal antibodies raised against intact HRV-14 virions, half are directed against N-Ag I and half are directed against N-Ag II. Preliminary studies with hybridomas isolated in this laboratory (A. Mosser, personal communication) as well as another (R. Colonno, Merck Laboratory) indicate that 10 of 18 new antibodies recognize at least one additional neutralization antigen. Thus HRV-14 has several immunodominant neutralization sites, whereas poliovirus appears to have only one. In addition, this is the first report for any picornavirus of an immunodominant site on VP3.

These differences may reflect a looser packing of protein subunits in rhinoviruses already indicated by a greater permeability of rhinovirus capsids to cesium ions (17; K. C. Medappa and R. R. Rueckert, *Abstr. Annu. Meet. Am. Soc. Microbiol.* 1974, V43, p. 207). It is therefore of great interest to determine whether other enteroviruses, which are probably impermeable to cesium ions, have immunodominant sites in locations different from those on cesium-permeable viruses such as rhinoviruses and aphthoviruses.

Location of immunodominant antigens on poliovirus and HRV-14. The thorough cross-neutralization studies of Minor et al. (20), involving 16 monoclonal antibodies of 101 antibody-resistant mutants, detected only a single neutralization antigen on type 3 poliovirus, and all except one group of the mutants defining this antigen were mapped to VP1 amino acid residues 93 through 100 (9). On the other hand, Emini et al. (6) suggested that there are two antigens on type 1 poliovirus, because their monoclonal antibodies, also raised against the intact virus, bound to one or the other of synthetic peptides representing VP1 amino acid residues 70 through 75 and 93 through 103. These two sequences thus clearly represent dominant epitopes. It is, however, still unclear whether these epitopes reside on the same antigen or two different ones, because they have not yet been sufficiently analyzed by cross-neutralization of mutants with these antibodies. Significantly, Minor et al. (9, 20) have not excluded the possibility that their one group of type 3 poliovirus mutants lacking a mutation in the region of amino acid residues 93 through 100 contains a mutation in the region encoding residues 70 through 75. Thus it remains to be determined whether there is a single antigen composed of two noncontiguous amino acid sequences, two independent neutralization antigens, or a difference in the antigenic structure of poliovirus types 1 and 3.

Sequencing studies will now be required to locate the positions of the mutations in VP1 and VP3 in HRV-14 and to determine whether the VP1 mutations on HRV-14 occur in a region homologous to the positions 70 through 75 (5) and 93 through 100 (9) in poliovirus. Competition studies with synthetic peptides representing the mutated sites should resolve whether the mutations associated with each of the antigenic regions map to the antibody-binding site or to some distal location that changes the antigenic structure. The successful crystallization of poliovirus (12) and HRV-14 (1, 8) opens the way for comparing the three-dimensional structure of the two viruses. In this way it should be possible to determine precisely the location of the surface antigens and to discern the structural features that determine immunodominance.

Important role of surface charge in the interaction of antibody with virus. The high frequency of charge shifts in the coat proteins of the antibody-resistant mutants of HRV-14

TABLE 4. Correlation of the nonneutralized fraction with neutralization antigen

Neutralization antigen	IEF shift	Monoclonal antibody	Plaques/PFU in nonneutralized fraction ^a
N-Ag I	VP1	1	5×10^{-2}
		3	4×10^{-6}
		4	4×10^{-6}
		6	9×10^{-6}
		7	4×10^{-6}
N-Ag II	VP3	2	1×10^{-2}
		5	1×10^{-2}
		8	5×10^{-3}
		9	9×10^{-3}
		10	5×10^{-3}

^a The nonneutralized fraction was determined using high-MOI (10 to 20 PFU per cell) virus. Data (number of plaques/number of PFU plated) represent the average of six experiments with each of the 10 antibodies. Similar results were obtained with two other high-MOI virus stocks (data not shown).

is unusual. A tabulation of all possible missense mutations indicates that only one-third should involve a change in amino acid charge (Table 2). This predicted distribution has been substantiated in studies on coat proteins of mutants in foot-and-mouth disease virus (14) and poliovirus (9). Yet, in HRV-14, 24 of 28 mutants examined have undergone a charge-shifting substitution, revealed by IEF (Fig. 5). In fact, every N-Ag I mutant investigated had a VP1 charge shift, and every one of these charge shifts was in the positive direction. This suggests an unusually important role of ionic bonds in the interaction of neutralizing antibodies with HRV-14.

Mechanisms of neutralization. Antibodies associated with N-Ag II generally neutralized the virus incompletely, whereas neutralization of virions by antibodies against N-Ag I was very efficient. Indeed, the nonneutralized fraction was limited only by the frequency of mutation to resistance (Table 4). This suggests that antibodies against the two antigens utilize different mechanisms for neutralization. It is already clear that several different mechanisms are involved in the neutralization of poliovirus. These include oligomerization without changing the infectivity of individual particles (1a), cross-linking of pentamers with decrease in infectivity of individual particles (13), and configurational change (7, 16), which may or may not lead to isoelectric aggregation (26). Further studies will be required to determine which mechanisms are involved in HRV-14 neutralization and whether they are characteristically associated with particular antigenic sites.

ACKNOWLEDGMENTS

We thank Bill Sugden for valuable discussions on hybridoma technology and Colleen Hayes and Rex Risser for use of animal facilities.

This work was supported by grant MV-331 from the American Cancer Society. B.S. was supported by Public Health Service training grant 5-T32-GM07215 from the National Institutes of Health.

ADDENDUM IN PROOF

Mutant sequencing studies carried out in collaboration with A. Mosser and R. Colonna revealed substitutions in VP1 at positions 95 to 98 (N-Ag I), in VP3 at 72 to 78 (N-Ag II), and in VP2 at 161 to 162 (N-Ag III).

LITERATURE CITED

1. Arnold, E., J. W. Erickson, G. S. Fout, E. A. Frankenberg, H.-J. Hecht, M. Luo, M. G. Rossmann, and R. R. Rueckert. 1984. Virion orientation in cubic crystals of the human common cold virus HRV-14. *J. Mol. Biol.* **177**:417-430.
- 1a. Brioen, P., D. Dekegel, and A. Boeye. 1983. Neutralization of poliovirus by antibody-mediated polymerization. *Virology* **127**:463-468.
2. Brown F. 1983. Neutralizing site of poliovirus. *Nature (London)* **304**:395-396.
3. Dernick, R., J. Heukeshoven, and M. Hilbrig. 1983. Induction of neutralizing antibodies by all three structural poliovirus polypeptides. *Virology* **130**:243-246.
4. Dick, E. C., and P. J. Chesney. 1981. Rhinoviruses, p. 1167-1186. In R. D. Feigin and J. D. Chesney (ed.), *Textbook of pediatric infectious diseases*. The W. B. Saunders Co., Philadelphia.
5. Emini, E. A., B. A. Jameson, and E. Wimmer. 1983. Priming for the induction of anti-poliovirus neutralizing antibodies by synthetic peptides. *Nature (London)* **304**:699-703.
6. Emini, E. A., S. Y. Kao, A. J. Lewis, R. Crainic, and E. Wimmer. 1983. Functional basis of poliovirus neutralization determined with monospecific neutralizing antibodies. *J. Virol.* **46**:466-474.
7. Emini, E. A., P. Ostapchuk, and E. Wimmer. 1983. Bivalent attachment of antibody to poliovirus leads to conformational alteration and neutralization. *J. Virol.* **48**:547-550.
8. Erickson, J. W., E. A. Frankenberg, M. G. Rossmann, G. S. Fout, K. C. Medappa, and R. R. Rueckert. 1983. Crystallization of a common cold virus, human rhinovirus 14: isomorphism with poliovirus crystals. *Proc. Natl. Acad. Sci. U.S.A.* **80**:931-934.
9. Evans, D. M. A., P. D. Minor, G. S. Schild, and J. W. Almond. 1983. Critical role of an eight amino acid sequence of VP1 in neutralization of poliovirus type 3. *Nature (London)* **304**:459-462.
10. Gwaltney, J. M., Jr. 1982. Rhinoviruses, p. 491-517. In A. S. Evans (ed.), *Viral infections of humans; epidemiology and control*, 2nd ed. Plenum Publishing Corp., New York.
11. Halfpapp, L. M., and M. K. Cooney. 1983. Isolation of rhinovirus intertypes related to either rhinoviruses 12 and 78 or 36 and 58. *Infect. Immun.* **40**:213-218.
12. Hogle, J. 1982. Crystallographic studies on type 1 poliovirus. *J. Mol. Biol.* **160**:663-668.
13. Icenogle, J., H. Shiwen, G. Duke, S. Gilbert, R. Rueckert, and J. Anderegg. 1983. Neutralization of poliovirus by a monoclonal antibody: kinetics and stoichiometry. *Virology* **127**:412-425.
14. King, A. M. Q., and J. W. I. Newman. 1980. Temperature-sensitive mutants of foot-and-mouth disease virus with altered structural polypeptides. I. Identification by electrofocusing. *J. Virol.* **34**:59-66.
15. Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (London)* **256**:495-497.
16. Mandel, B. 1976. Neutralization of poliovirus: a hypothesis to explain the mechanism and the one-hit character of the neutralization reaction. *Virology* **69**:500-510.
17. Mapoles, J. E., J. W. Anderegg, and R. R. Rueckert. 1978. Properties of poliovirus propagated in medium containing cesium chloride: implications for picornaviral structure. *Virology* **90**:103-111.
18. Matthews, R. E. F. 1982. Classification and nomenclature of viruses. *Intervirology* **17**:129-132.
19. Medappa, K. C., C. McLean, and R. R. Rueckert. 1971. On the structure of rhinovirus 1A. *Virology* **44**:259-270.
20. Minor, P. D., G. C. Schild, J. Bootman, D. M. A. Evans, M. Ferguson, P. Reeve, M. Spitz, G. Stanway, A. J. Cann, R. Hauptmann, L. D. Clarke, R. C. Mountford, and J. W. Almond. 1983. Location and primary structure of a major antigenic site for poliovirus neutralization. *Nature (London)* **301**:674-679.
21. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**:4007-4021.
22. Portner, A., R. G. Webster, and W. J. Bean. 1980. Similar frequencies of antigenic variants in Sendai, vesicular stomatitis, and influenza A viruses. *Virology* **104**:235-238.
23. Rueckert, R. R. 1976. On the structure and morphogenesis of picornaviruses, p. 131-213. In H. Fraenkel-Conrat and R. R. Wagner (ed.), *Comprehensive virology*, vol. 6. Plenum Publishing Corp., New York.
24. Rueckert, R. R., and M. A. Pallansch. 1981. Preparation and characterization of EMC virus. *Methods Enzymol.* **78**:315-325.
25. Rueckert, R. R., and E. Wimmer. 1984. Systematic nomenclature of picornavirus proteins. *J. Virol.* **50**:957-959.
26. Vrijen, R., B. Rombaut, and A. Boeye. 1983. pH-dependent aggregation and electrofocusing of poliovirus. *J. Gen. Virol.* **64**:2339-2342.
27. Wallis, C., and J. L. Melnick. 1967. Virus aggregation as the cause of the non-neutralizable persistent fraction. *J. Virol.* **1**:478-488.
28. Wimmer, E., B. A. Jameson, E. A. Emini. 1984. Poliovirus antigenic sites and vaccines. *Nature (London)* **308**:19.