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Use of Monoclonal Antibodies to Identify Four Neutralization Immunogens on a Common Cold Picornavirus, Human Rhinovirus 14

BARBARA SHERRY,[1] ANNE G. MOSSER,[1] RICHARD J. COLONNO,[2] AND ROLAND R. RUECKERT[1]*

Biophysics Laboratory and Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706,[1] and Merck, Sharp & Dohme Research Laboratories, West Point, Pennsylvania 19486[2]

Received 19 July 1985/Accepted 28 August 1985

A collection of 35 mouse monoclonal antibodies, raised against human rhinovirus 14 (HRV-14), was used to isolate 62 neutralization-resistant mutants. When cross-tested against the antibodies in a neutralization assay, the mutants fell into four antigenic groups, here called neutralization immunogens: NIm-IA, -IB, -II, and -III. Sequencing the mutant RNA in segments corresponding to serotype-variable regions revealed that the amino acid substitutions segregated into clusters, which correlated exactly with the immunogenic groups (NIm-IA mutants at VP1 amino acid residue 91 or 95; NIm-II mutants at VP2 residue 158, 159, 161, or 162; NIm-III mutants at VP3 residue 72, 75, or 78; and NIm-IB mutants at two sites, either VP1 residue 83 or 85, or residue 138 or 139). Examination of the three-dimensional structure of the virus (M. G. Rossmann, E. Arnold, J. W. Erickson, E. A. Frankenberger, J. P. Griffith, H.-J. Hecht, J. E. Johnson, G. Kamer, M. Luo, A. G. Mosser, R. R. Rueckert, B. Sherry, and G. Vriend, Nature [London], 317:145–153, 1985) revealed that each of the substitution clusters formed a protrusion from the virus surface, and the side chains of the substituted amino acids pointed outward. Moreover, four of the amino acid substitutions, which initially appeared to be anomalous because they were encoded well outside the cluster groups, could be traced to surface positions immediately adjacent to the appropriate viral protrusions. We conclude that three of the four antigens, NIm-IB, -II, and -III, are discontinuous. Thus, the amino acid substitutions in all 62 mutants fell within the proposed immunogenic sites; there was no evidence for alteration of any antigenic site by a distal mutation.

Picornaviruses are attractive models for basic immunostructural analysis because many of these economically important viruses have been thoroughly catalogued with respect to serotype (26) and because the architecture of the protein shell, 60 subunits organized as 12 pentamers, is particularly simple (31). The segmented nature of the protein subunit, which consists of four polypeptide chains (VP1 through VP4), offers additional advantages for genetic analysis. Moreover, the three-dimensional shell structure of one picornavirus, human rhinovirus serotype 14 (HRV-14), is now known in atomic detail (29a), and similar studies on poliovirus, a related picornavirus, are well under way (17).

Human rhinoviruses are a predominant cause of common colds in humans (8, 9, 16, 38). With 89 serotypes now officially recognized (21) and at least 20 more waiting for assignment (6, 8), the rhinoviruses constitute the single largest genus in the picornavirus family (24). Recent studies on the crystal lattice (14) and genome sequence (4, 36) of HRV-14 have revealed a relationship to poliovirus much closer than previously suspected. One of the striking differences, however, is the much greater serotypic variety of the rhinoviruses (>100 serotypes), most of which use one cellular receptor (1) as compared with the polioviruses (3 serotypes), which use another (27).

We and others have set out to identify and map the location of polio- and rhinoviral antigens with the aid of neutralizing monoclonal antibodies raised against native virions. Such antibodies identify the most highly immunogenic neutralization antigens, i.e., those most likely to confer immune protection after vaccination or natural infection. Identification of neutralizing immunogens (NIm) also affords a useful framework for studying mechanisms of neutralization. Such studies, in conjunction with detailed structural and functional comparisons, may also provide insight into the reason for the great difference in antigenic variation between polio- and rhinoviruses, which belong to different receptor families and occupy different biological niches.

We have previously identified two neutralization immunogens in HRV-14 with the aid of 10 neutralizing monoclonal antibodies raised against intact virions (34). We now report the identification of two new neutralization immunogens by using 25 additional monoclonal antibodies raised against native virions. Our findings, when analyzed in the context of the three-dimensional structure of the virus, suggest that mutational analysis is an effective tool for mapping immunogenic neutralization antigens in nonenveloped viruses.

MATERIALS AND METHODS

Cells and media. H1-HeLa cells are a cloned line of H-HeLa cells. The origin of H-Hela cells and methods for their propagation in medium A or medium B have been described previously (25). SP2/0 cells were propagated in supplemented RPMI 1640 medium, and human fibroblast cells were propagated in supplemented Dulbecco medium as described previously (34). Hybridoma cells were selected in hypoxanthine-aminopterin-thymidine medium as described previously (fusions 1 through 4 were described by Sherry and Rueckert [34]; fusion 5 was described by W. J. Long, W. R. McGuire, A. Palombo, and E. A. Emini, [Immun. Methods, in press]). Medium P6 and phosphate-buffered saline were as described previously (34). PBSA consisted of phosphate-buffered saline supplemented with 0.1% bovine serum albumin.

* Corresponding author.

Virus stocks. HRV-14 strain 1059, originally obtained from V. V. Hamparian of Ohio State University, was plaque purified three times after receipt. The serological identity of the resulting stock was confirmed by neutralization with specific antiserum.

Stock was passaged several times at 35°C at a multiplicity of infection (MOI) of 10 to 20 PFU per cell; a low-MOI stock was then produced by infecting an H1-HeLa suspension culture at <0.1 PFU per cell, and 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 (34). This low-MOI stock was used for most mutant selections and all plaque reduction assays.

Viral antigen. Virus for fusions 1 through 4 was pelleted from an infected cell lysate, suspended, fractionated on a 7.5 to 45% sucrose gradient, further purified on a 20 to 45% CsCl gradient, and desalted by gel filtration (34). For fusion 5, virus was polyethylene glycol precipitated from an infected cell lysate, pelleted, suspended, fractionated on a 40 to 60% metrizamide gradient, and then repelleted (1).

Monoclonal antibodies. The 10 neutralizing monoclonal antibodies generated from fusions 1 and 2 were selected as described previously (34). The 17 monoclonal antibodies generated from fusions 3 and 4 were selected as above, but 5 μg of poliovirus type 1 was included (for reasons not relevant to this study) in each injection. Injection schedules were as follows. For fusion 3, on day 0, 5 μg of each virus in Freund complete adjuvant was injected; on days 14, 28, 84, and 111, 5 μg of each virus in Freund incomplete adjuvant was injected. For fusion 4, day 0, 5 μg of each virus in Freund complete adjuvant was injected; on day 24, 5 μg of each virus (aqueous) was injected. Spleen cells were fused with SP2/0 myeloma cells 3 days after the final injections, as described (34). The eight neutralizing monoclonal antibodies generated from fusion 5 were selected as follows. BALB/c mice were injected with approximately 5 μg of purified HRV-14 per injection according to the following schedule: day 0, 2.5 μg of virus in each rear footpad in Freund complete adjuvant; day 21, 2.5 μg of virus (aqueous) in each front footpad; day 47, 5 μg of virus intravenously in the tail. Spleen cells were fused with SP2/0 myeloma cells 3 days after the final injection, as described previously (Long et al., in press).

Microneutralization assay. For hybridoma screening (fusions 1 through 4) and microneutralization cross-tests, 100 μl of hybridoma culture fluid or 50 μl of ascites fluid diluted in PBSA was incubated with 50 μl of wild-type (10^4 PFU) or mutant virus in a 96-well culture plate for 1 h at 35°C under a 5% CO_2 atmosphere. H1-Hela cells (5×10^4 in 100 μl of medium P6) were added, and the plates were incubated as above. After 48 h, the plates were stained with 0.1% crystal violet in 20% ethanol.

Plaque reduction assay. Approximately 200 PFU of wild-type or mutant virus in 250 μl of PBSA and an equal volume of PBSA or a 10^{-1} or 10^{-2} dilution of ascites fluid in PBSA were incubated for 1 h at room temperature. The antibody-virus mix was applied to H1-HeLa cell monolayers, and the plaque assay was performed as described previously (34).

Isolation of antibody-resistant mutants. Low-MOI stock virus was incubated with diluted ascites fluid, and plated on H1-HeLa cell monolayers, and resistant plaques were harvested as described previously (34). In certain cases, the virus harvested from plaques was replaqued one to three times more. The number of cloning rounds has been designated by a letter in the mutant name (no letter indicates no further cloning; B indicates one additional round, etc.).

Radiolabeled virus preparations. Mutant and wild-type virus preparations were radiolabeled with [^{35}S]methionine and purified as described by Sherry and Rueckert (34). H1-HeLa cells were generally infected at an MOI of 10 to 20 PFU per cell (or approximately 1 PFU per cell in the case of many mutants for which virus titers were low). Infected cells were cultivated in medium AL (medium A lacking all amino acids except glutamine), and [^{35}S]methionine was added 2 h postinfection. Virus was harvested 8 h postinfection by freeze-thaw lysis (wild type) or lysis with 0.5% Nonidet P-40 (NP-40) (mutant preparations), followed by sucrose gradient centrifugation and fractionation (wild type) or pelleting through a 30% sucrose cushion and suspension in LS buffer (mutant preparations).

IEF. Isoelectric focusing (IEF) polyacrylamide slab gels were prepared and samples prepared and electrophoresed as described previously (34) except that samples were diluted 1:9 in a solution containing 9.5 M urea, 2% NP-40, 3.13% each of pH 3 to 10 and pH 5 to 8 ampholines, and 5% 2-mercaptoethanol. Samples were electrophoresed toward the cathode on a 4% acrylamide gel containing 3.78% acrylamide, 0.22% *N,N'*-methylenebisacrylamide, 9.2 M urea, 2% NP-40, 3.13% each of pH 3 to 10 and pH 5 to 8 ampholines, 0.1% *N,N,N',N'*-tetramethylethylenediamine, and 0.04% ammonium persulfate at 400 V for 18 h and then at 800 V for 1 h. Gels were fixed in several changes of gel soak (25% methanol, 10% acetic acid, 1% glycerol) for 1 to 2 h, soaked in En^{3}Hance for 1 h, and then soaked in distilled H_2O for 1 h. Gels were dried on Whatman 3MM paper and subjected to autoradiography with Kodak XAR-5 film at −70°C.

Viral RNA preparations. H1-HeLa cells were infected with wild-type or mutant virus as described for the radiolabeled preparations, pelleted ($630 \times g$ for 5 min) 8 h postinfection, and suspended in a small volume of medium AL/H (Medium A lacking amino acids except glutamine and made 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.2]). The infected cells were lysed in 0.5% NP-40, and the nuclei were pelleted ($12,060 \times g$ for 20 min). The decanted supernatant was made 1% sarcosyl and 0.1% 2-mercaptoethanol and then layered on a preformed three-step cushion of 1 ml each of 10, 20, and 30% sucrose in buffer IV/A (buffer IV [32] supplemented with 0.01% bovine serum albumin). After centrifugation in an SW50 rotor at 45,000 rpm and 16°C for 90 min, the liquid was decanted, and the pellet was resuspended in NHE buffer (100 mM NaCl, 50 mM HEPES [pH 7.4], 1 mM EDTA). Typically, virus from 10^8 cells was suspended to a volume of 2.5 ml.

The virus solution was made 1% sodium dodecyl sulfate, and 1 volume of pre-equilibrated phenol (phenol equilibrated three times with an equal volume of NHE buffer) was added. The mixture was vortexed vigorously for 30 s, one-half volume of chloroform-isoamyl alcohol (24:1) was added, and the mixture was vortexed as above. The phases were separated by a brief centrifugation ($1,120 \times g$), and the aqueous phase was removed to a fresh corex tube. The phenol-chloroform extraction was repeated and then followed by two chloroform-isoamyl alcohol washes of the aqueous phase (1 volume of chloroform-isoamyl alcohol was used each time). The volume of the final aqueous phase was estimated, 2.5 volumes of absolute ethanol (−20°C) was added, and the mixture was vortexed vigorously. After 1 h at −70°C, the precipitate was pelleted ($12,060 \times g$, 10 min, 4°C), and the supernatant was decanted. The pellet was

washed with chilled 70% ethanol, incubated at −70°C for 45 to 60 min, centrifuged as above, and then vacuum dried. The pellet was suspended in a small volume (50 to 200 μl) of deionized distilled H_2O and stored at −20°C. Analysis on an agarose gel revealed heavy contamination with ribosomal RNA.

Dideoxynucleotide sequencing. Wild-type and mutant RNAs were sequenced by a modification of the Sanger dideoxynucleotide sequencing method (33, 41). The primer (0.16 to 0.5 μg) and RNA template (estimated by band intensity on acridine orange-stained gels to be 0.1 to 1 μg) were annealed in a 10-μl volume containing 45 mM Tris (pH 8.3), 9 mM $MgCl_2$, 45 mM NaCl, and 1 mM dithiothreitol for 3 to 5 min at 67°C. The mix was allowed to cool slowly to 42°C and then was ice cooled. Chain synthesis was initiated by making a reaction mixture containing 0.7 μM [^{32}P]dATP (3 μCi per reaction), 0.1 mM dCTP, 0.1 mM dGTP, 0.1 mM dTTP, 0.4 U of reverse transcriptase per μl (2 U per reaction), 37 mM Tris (pH 8.3), 7 mM $MgCl_2$, 46 mM NaCl, and 3 mM dithiothreitol. Each reaction mixture also contained one of the dideoxynucleotides, ddATP (0.17 μM), ddCTP (0.035 mM), ddGTP (0.017 mM), or ddTTP (0.017 mM). In certain cases, dITP (1 mM) was used instead of dGTP to alleviate band compression; the ddGTP concentration was then dropped to 0.009 mM. The reaction mixture was incubated at 42°C for 15 min, chased with a fresh charge of reverse transcriptase and dNTPs, and incubated an additional 15 min. The reaction was terminated by the addition of one-half volume of FDE (90% deionized formamide, 20 mM Na_2EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue). The samples were boiled for 5 min, ice cooled, and then loaded on 6% acrylamide gels (5.7% acrylamide, 0.3% *N,N'*-methylenebisacrylamide, 8.3 M urea, 45 mM Tris, 1.4 mM Na_2EDTA, 45 mM boric acid, 0.05% ammonium persulfate, 0.0012% *N,N,N',N'*-tetramethylethylenediamine) which had been prerun for a minimum of 30 min at 60 W. The gels were electrophoresed at 60 to 75 W and then fixed in 10% methanol–10% acetic acid and dried for autoradiography.

Material sources. Most reagents used in these studies were purchased as described previously (34). Oligonucleotides were prepared at Merck, Sharp & Dohme on a DNA synthesizer (model 380A; Applied Biosystem, Inc.) as described by the manufacturer. Phenol was purchased from Matheson Coleman and Bell, Gibbstown, N.J.; chloroform was purchased from Mallinckrodt, Inc., St. Louis, Mo.; isoamyl alcohol was from J.T. Baker Chemical Co., Phillipsburg, N.J.; and ultrapure urea was purchased from Schwarz/Mann, Orangeburg, N.Y. Deoxynucleotide and dideoxynucleotide triphosphates were purchased from P-L Biochemicals Inc., Piscataway, N.J. (catalog no. 27-5500, 5600, 5700, and 5800 and 27-4817, 4819, 4823, and 4831, respectively). Reverse transcriptase (no. AMV-002) was purchased from Life Sciences Inc., St. Petersburg, Fla. [α-^{32}P]dATP was purchased from Amersham Corp., Arlington Heights, Ill. (no. PB.10204).

RESULTS

Two new neutralization immunogens suggested by microneutralization cross-tests. We previously identified two NIm by using 10 neutralizing monoclonal antibodies raised against intact virus. Each antibody was used to select several resistant mutants. The mutants were then cross-tested against all 10 antibodies in a microneutralization assay, and in many cases, a single mutant was resistant to several antibodies, suggesting that those antibodies recognized a common antigen. In fact, one class of mutants was resistant to half of the antibodies, while another class was resistant to the other half. In addition, every mutant selected with the first group of antibodies was neutralized by the second group, and vice versa. Together, the data suggested two independent NIm on the virus. Isoelectric focusing of the dissociated coat proteins revealed that all mutants associated with one immunogen were altered in VP1, while most mutants associated with the other were altered in VP3. These immunogenic sites were previously reported (34) as N-Ag I and N-Ag II, but have been renamed NIm-IA and NIm-III, respectively, to identify them as neutralization immunogens (NIm) and to indicate their major coat protein locations.

We have now isolated 25 additional neutralizing monoclonal antibodies. These were screened for their ability to neutralize the previously selected NIm-IA and NIm-III mutants (Fig. 1, mutant classes A through F). Of the 25 new antibodies, 9 (Fig. 1, antibodies 17, 27, 34, 11, 15, 19, 23, 12, and 14) were unable to neutralize certain NIm-IA mutants (virus classes A through C'), suggesting that they recognized NIm-IA for neutralization. Antibodies 12, 14, and 20 still strongly neutralized some NIm-IA mutants. The latter was not recognized as NIm-IA specific until a new set of mutants, class G, selected for resistance to that antibody, proved to be resistant to other NIm-IA antibodies. Note that neutralization of certain NIm-IA mutants does not conflict with assignment of these antibodies to NIm-IA, because antibodies recognizing a given antigen may interact with it in different ways. Thus, two antibodies may recognize an overlapping set of amino acids, but one may be sensitive to a particular substitution while the other is not. The two antibodies are then said to recognize different epitopes in the same antigen.

Of the remaining 15 new antibodies, 6 (Fig. 1, antibodies 21, 22, 24, 33, 25 and 31) were unable to neutralize certain NIm-III mutants (virus classes D through F), indicating their recognition of that antigen. As expected, antibodies recognizing NIm-IA were able to neutralize all NIm-III mutants, and vice versa. The remaining nine new antibodies (13, 16, 18, 26, 28, 29, 30, 32, and 35) neutralized all NIm-IA and NIm-III mutants tested (Fig. 1), indicating that they recognized new epitopes, possibly in new antigens. Antibody 16 behaved anomalously in the microneutralization assays, in that it only partially neutralized NIm-IA mutants, and the extent of neutralization was variable. This antibody expressed an unusually low titer in both the microneutralization and plaque reduction assays and was not necessarily present in excess. If the NIm-IA mutants had a slightly higher particle-to-PFU ratio than the wild type or other mutants, then the antibody could have been at too low a concentration to fully neutralize these mutants.

To determine whether any of the new epitopes were located in new antigens, six of the nine remaining antibodies were used individually to select 24 new mutants, and the mutants were tested for resistance to NIm-IA and NIm-III antibodies. All of the mutants were completely neutralized by every NIm-IA and NIm-III antibody tested (data not shown), suggesting the presence of at least one new antigen.

When the 24 mutants were tested for resistance to the nine unassigned antibodies, the results suggested at least two new antigenic groups, NIm-II and NIm-IB (Fig. 2). Mutants selected with antibodies 16, 18, 28, and 35 (virus classes H through L) were completely neutralized by antibodies 13, 26, 29, 30, and 32. Conversely, mutants selected with antibodies 13 and 26 (virus classes M and N) were completely neutral-

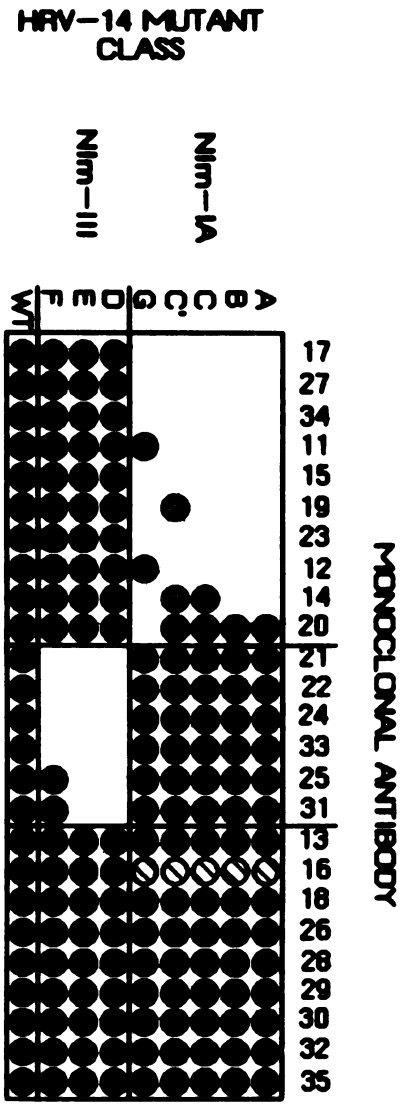

FIG. 1. Microneutralization screen for resistance of NIm-IA and NIm-III mutants to the 25 new monoclonal antibodies. Antibody-resistant mutants or wild-type (WT) HRV-14 were assayed for resistance to neutralization by each of the 25 monoclonal antibodies. Neutralization is indicated by closed circles, absence of neutralization (resistance) is indicated by blanks, partial neutralization is indicated by stippled circles, and variable resistance is indicated by hatched circles. HRV-14 mutants were selected for resistance to monoclonal antibodies as described in the text. All NIm-IA and NIm-III mutant classes have previously been shown to be altered in different epitopes based on their patterns of resistance against the first 10 antibodies (34), although these differences are not always evident here (for example, mutant classes A and B seem identical, as do classes D and E).

ized by antibodies 16, 18, 28, and 35. However, the mutants were often only partially resistant to their selecting antibody, and the extent of resistance varied from one assay to the next. Thus it was not clear whether all of the antibodies in either group (NIm-II or NIm-IB) recognized a common antigen. To deal with this problem, a more quantitative assay (plaque reduction assay) was undertaken.

Plaque reduction assays support the linkage of antibodies within each of the two new immunogens. To determine

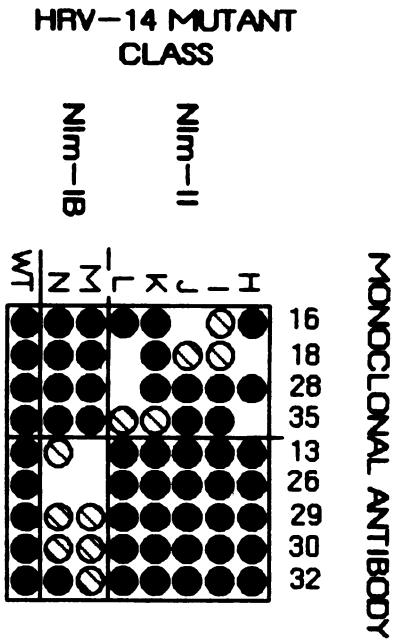

FIG. 2. Microneutralization screen for resistance of newly selected mutants to the nine antibodies unassigned to NIm-IA or NIm-III. Six of the nine antibodies (13, 16, 18, 26, 28, and 35) were used to select 24 mutants; the remaining three antibodies were too low in titer for use in selection. The 24 mutants were then screened for their resistance to each of the nine antibodies, resulting in seven new patterns of antibody-resistance (mutant classes H through N). Symbols are as indicated in the legend to Fig. 1.

whether antibodies 16, 18, 28, and 35 recognized a single antigen, mutants selected with the four antibodies were tested for antibody resistance in a plaque reduction assay (Table 1). Consistent with the microneutralization results, mutants were often only partially resistant to their selecting antibody. Such partial resistance could reflect a lowered affinity of the antibody for the antigen, through the disruption of only one of the numerous interactions that presumably stabilize an antibody-antigen interaction. A mutant selected with antibody 16 (mutant 16.1B) was approximately equally resistant to antibodies 16 and 18, suggesting that these two antibodies recognized the same antigen.

Similarly, a mutant selected with antibody 18 (mutant 18.1) was completely resistant to antibody 35, although only

TABLE 1. Survival of NIm-II mutants after treatment with monoclonal antibody[a]

HRV-14	% Survival after treatment with the following antibody:			
	16	18	28	35
Mutants				
16.1B	35	25	<1	0
18.1	3	20	3	112
28.1D	0	92	92	18
35.1B	4	0	4	87
Wild type	1	0	0	0

[a] Mutants were selected for resistance to one of six antibodies, as described in the legend to Fig. 2. Representative mutants were incubated with each of the indicated antibodies to assay antibody resistance and plated in a plaque reduction assay as described in Materials and Methods. The percentage of PFU remaining was scored relative to that for incubation with PBSA alone.

TABLE 2. Survival of NIm-IB mutants after treatment with monoclonal antibody[a]

HRV-14	% Survival after treatment with the following antibody:				
	13	26	29	30	32
Mutants					
13.2	114	84	64	85	21
13.5	73	69	46	37	16
26.1C	71	52	65	69	15
26.5C	67	91	26	24	8
Wild type	0	0	20[b]	19[b]	3[b]

[a] Conditions and procedures were as described in footnote *a* of Table 1.
[b] Extremely small plaques, relative to those of control, were observed.

partially resistant to its selecting antibody, indicating that these two antibodies recognized a common antigen. Finally, a mutant selected with antibody 28 (mutant 28.1D) was equally resistant to antibodies 28 and 18, indicating their common antigen recognition. Thus, these four antibodies recognized a common antigen. In addition, their differing abilities to neutralize the four mutants suggest that they recognized distinct epitopes within this antigen.

A second plaque reduction assay was used to determine whether antibodies 13, 26, 29, 30, and 32 recognized a common antigen (Table 2). Again, mutants were often only partially resistant to their selecting antibody. In addition, antibodies 29, 30, and 32 expressed very low titers and, in fact, failed to completely neutralize the wild-type virus, although the remaining plaques were very small and thus were inhibited in their development. A mutant selected with antibody 13 (mutant 13.2) demonstrated increased resistance to antibodies 26, 29, 30, and 32, in both the number and size of the plaques remaining. A mutant selected with antibody 26 (mutant 26.1C) also demonstrated resistance to all five antibodies, suggesting that they recognized a common antigen.

NIm-II and NIm-IB mutants have altered VP2 and VP1 coat proteins, respectively. It was shown previously, by IEF of dissociated radiolabeled mutant virions, that mutants in NIm-IA were always charge altered in VP1, while mutants in NIm-III were usually altered in VP3. In all, we have characterized 62 neutralization-resistant mutants by their IEF patterns (Fig. 3). There was a good correlation between the coat protein altered and the antigenic group defined by the neutralization assays. In addition to the correlations already described for NIm-IA and NIm-III, we now find that NIm-II mutants were sometimes altered in VP2, while NIm-IB mutants were often altered in VP1.

Thus, four of the 14 NIm-II mutants had charge shifts in VP2 (one shifted +2 [Fig. 3, lane k], and three shifted +1 [lane 1]) and, as expected, in the VP2 precursor, VP0, while

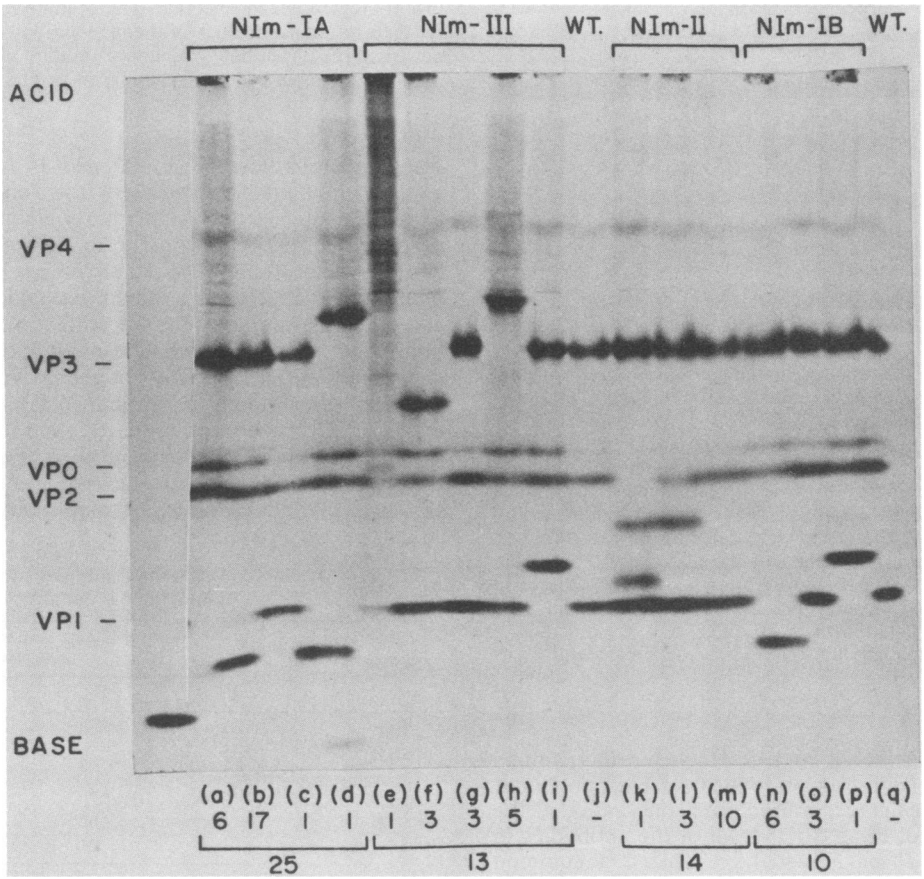

FIG. 3. IEF patterns of the 62 HRV-14 mutants. The 62 HRV-14 mutants exhibited 15 different IEF patterns, shown in lanes a through d (NIm-IA mutants), lanes e through i (NIm-III mutants), lanes k through m (NIm-II mutants), and lanes n through p (NIm-IB mutants). Wild-type HRV-14 (WT) is shown in lanes j and q. The frequency with which the mutants displayed these patterns is indicated beneath each lane, and the total number of mutants isolated for each NIm is indicated beneath each bracket.

TABLE 3. High correlation between immunogen and site of mutation

Immunogen	Total no. of mutants	No. of mutants with mutation in the following cluster:				
		VP1			VP2	VP3
		91–95	83–85	138–139	158–162	72–78
NIm-IA	25	24	0	—[a]	—	—
NIm-IB	10	0	3	7	—	—
NIm-II	14	—	—	—	13	—
NIm-III	13	—	—	—	—	11

[a] —, Not determined (see Fig. 4).

none had charge shifts in any other coat protein. This suggested that NIm-II was located on VP2. Seven of the 10 NIm-IB mutants had charge shifts in VP1 (6 shifted +1, [Fig. 3, lane n], and 1 shifted −1, [lane p], while none were altered in any other coat protein. This suggested that NIm-IB was located on VP1.

In all, 24 of 25 NIm-IA mutants were altered in VP1, 9 of 13 NIm-III mutants were altered in VP3, 4 of 14 NIm-II mutants were altered in VP2, and 7 of 10 NIm-IB mutants were altered in VP1. On the average, only one-third of missense mutations should result in charge-shifting amino acid substitutions; however, mutation from a charged amino acid is very likely to result in a charge shift, while mutation from a neutral amino acid is not (see Table 2 in reference 34). The relative frequencies of charge-shifting substitutions suggested that NIm-IA, -III, and -IB mutants were predominantly mutated at charged wild-type residues, while NIm-II mutants were predominantly mutated at neutral wild-type residues, and this is in fact true (see below).

Nucleotide sequencing reveals immunogen-specific mutation clusters. Mutant virus RNAs were harvested and sequenced in limited regions as described below. As will be shown, 58 of the 62 mutants encoded a mutation within one of five small regions (Table 3). One mutation cluster was associated with NIm-IA, one was associated with NIm-II, and one was associated with NIm-III. Two of these clusters were associated with NIm-IB and unexpectedly flanked the cluster associated with NIm-IA.

The immunodominant site on poliovirus type 3 is located in the region of VP1 amino acids 93 through 100 (15), and we anticipated that an HRV-14 VP1 immunogen might be located in an analogous position. The 25 NIm-IA mutants were sequenced for a minimum of 100 bases, and most were sequenced for more than 200 bases, in the region of VP1 amino acid residues 30 through 105 (Fig. 4, NIm-IA mutants). Of the 25, 24 had a single-nucleotide mutation encoding an amino acid substitution at either residue 91 or 95 (the exception, 1.16, is described below). Eighteen of the 24 mutants were altered at amino acid residue 91. These 18 cases included all amino acid substitutions possible from a single-nucleotide mutation (Fig. 5). This included the substitution of glutamate for the wild-type aspartate, implying that the charge of the side chain is not the sole recognition determinant.

The remaining 6 of the 24 clustered NIm-IA mutants were altered at amino acid residue 95, and 5 of these (independent isolates selected with three different antibodies) substituted lysine for the wild-type glutamate (Fig. 5). The sixth mutant (14.2), which contained a substitution to glycine, encoded a second VP1 mutation (evidenced by its VP1 +2 shift on IEF gels) which is not yet identified. The observations that five of the six mutants encoded lysine at this position and that the sixth was a double mutant suggest a limitation on the substitutions that can occur here, contrasting the case described for residue 91. This limitation may reflect either interaction with the antibody or an essential structural feature of the virion.

When the VP3 amino acid sequences of the three poliovirus serotypes are aligned for optimal homology, or when any of the three poliovirus serotypes are aligned with HRV-14, most of VP3 is highly conserved with the exception of a large region spanning approximately amino acids 60 through 95 (Fig. 6). This suggests that this region is important in defining both the antigenicity of the poliovirus serotypes and the differences between poliovirus and HRV-14. Thus, the 13 NIm-III mutants were sequenced for a minimum of 150 bases, and most were sequenced for more than 200 bases, in the region of VP3 amino acid residues 20 through 100 or 50 through 100 (Fig. 4, NIm-III mutants). Of the 13 mutants, 11 had a single-nucleotide mutation encoding an amino acid substitution at residue 72, 75, or 78 (Fig. 5). Thus, the mutations defining NIm-III were clustered in this VP3 region. Two exceptions, mutants 9.6 and 10.3, will be discussed below.

When the VP2 amino acid sequences of the three poliovirus serotypes were aligned with each other or with HRV-14, the result was similar to that of the VP3 alignment (Fig. 6). Two large, adjacent divergent regions were apparent, spanning amino acid positions 135 through 175. Eleven of the 14 NIm-II mutants (the remaining 3, selected with antibody 28, are discussed below) were sequenced for approximately 150 bases in the region of VP2 amino acid residues 135 through 180 (Fig. 4, NIm-II mutants). All but one (the exception, 16.4B, is described below) had a single-nucleotide mutation encoding an amino acid substitution at residue 158, 159, 161, or 162. Six of these 10 mutants (independent mutants selected with two different antibodies) substituted methionine for the wild-type valine at position 162 (Fig. 5). These results suggest both a preference for substitutions at this position and a strong limitation on acceptable substitutions.

Seven of the 10 NIm-IB mutants demonstrated a VP1 charge shift on IEF gels, yet the microneutralization assays defined NIm-IB as distinct from NIm-IA. Having previously correlated neutralization immunogens with nonconserved regions in poliovirus and HRV-14 alignments, we noted a large nonconserved region at the amino terminus of VP1 and sequenced five NIm-IB mutants for approximately 100 bases through this region (Fig. 4, NIm-IB mutants). All five mutants were wild type, suggesting that this region did not represent NIm-IB. We then sequenced two mutants through 90% of VP1, hoping to locate a site where both were mutated. To our surprise, one was mutated at amino acid residue 85 and the other was mutated at residue 138. When all 10 NIm-IB mutants were sequenced for a minimum of 100 bases through each region, 7 mutants were found to have a single mutation at amino acid residue 138 or 139, while the remaining 3 mutants had a single mutation at residue 83 or 85 (Fig. 5). This suggests that amino acid substitutions in either region (83 through 85 or 138 through 139) could confer antibody resistance in NIm-IB.

Outlying mutations encode substitutions in the proposed immunogenic sites. As noted above, 4 of the 62 mutants were anomalous, in that they were wild type when sequenced through the appropriate antigen-specific mutation clusters. These mutants were sequenced further to identify distal mutations that might determine antibody resistance. A mutation was identified in three of the four cases, and, as will be

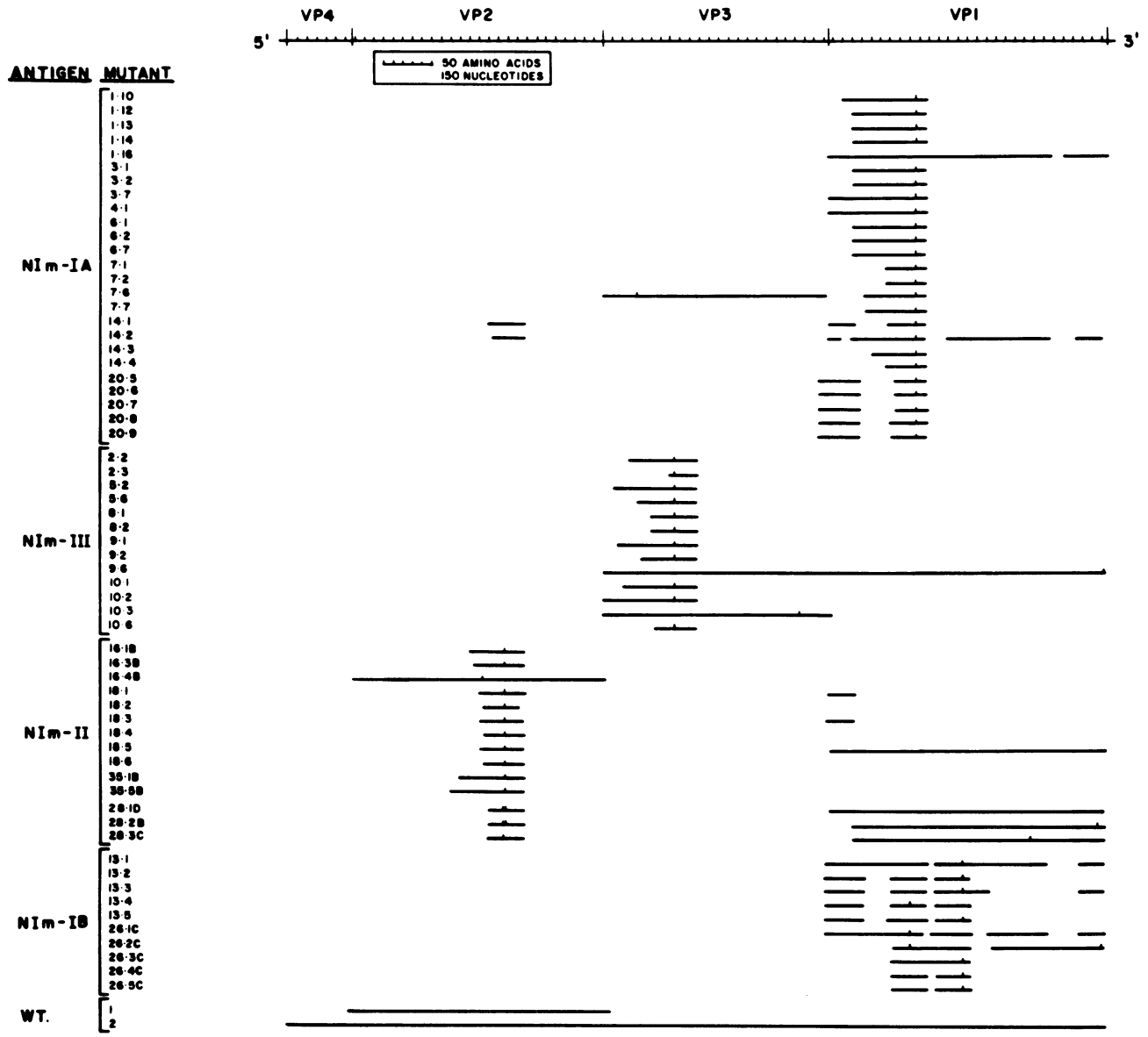

FIG. 4. Coat protein-encoding regions sequenced in mutant virus RNAs. RNA was extracted from the 62 mutants, wild-type HRV-14 (WT 2), and a plaque-purified preparation of WT (WT 1) derived from WT 2. Oligonucleotide primers were used for dideoxynucleotide sequencing as described in Materials and Methods. The region sequenced in each case is represented by a horizontal line, and the site(s) where a mutation was found is indicated by a vertical mark. The nucleotide sequence of the coat protein-encoding region of WT virus was identical to that of Stanway et al. (36), although our sequencing method left 3% of the bases undefined.

discussed later, each of the resulting amino acid substitutions was subsequently found to lie in the proposed immunogen on the virus surface.

NIm-IA mutant 1.16 was wild type in the NIm-IA cluster, yet isoelectric focusing confirmed a VP1 charge shift. Subsequent sequencing of most of VP1 (Fig. 4) failed to detect a mutation. The mutation either occurred in the small unsequenced region or was obscured by the sequencing method, which left approximately 5% of the bases undefined.

NIm-III mutant 10.3 was sequenced through all of VP3 (Fig. 4), and a single mutation (glycine to aspartate) was detected at amino acid position 203 (Fig. 7).

NIm-III mutant 9.6 appeared to be wild type throughout VP3 (5% of the bases being undefined) (Fig. 4). In fact, this mutant had no VP3 charge shift on IEF gels and was the only NIm-III mutant that produced a charge shift in another coat protein, VP1. The mutant was sequenced throughout VP1, and a single-nucleotide mutation was observed at VP1 amino acid position 287 (lysine to isoleucine), just two amino acids away from the carboxy terminus of that coat protein (Fig. 7).

NIm-II mutant 16.4B was wild-type in the NIm-II cluster, yet it demonstrated a VP2 +1 charge shift on IEF gels. By subsequent sequencing through all of VP2, a single mutation (glutamate to glycine) at position 136 was identified (Fig. 7). The two adjacent, divergent regions on VP2 are separated by a well-conserved five-amino acid sequence (Fig. 6), and this substitution at position 136 implies a role for both regions in NIm-II.

Mutants carrying a second substitution. Three mutants

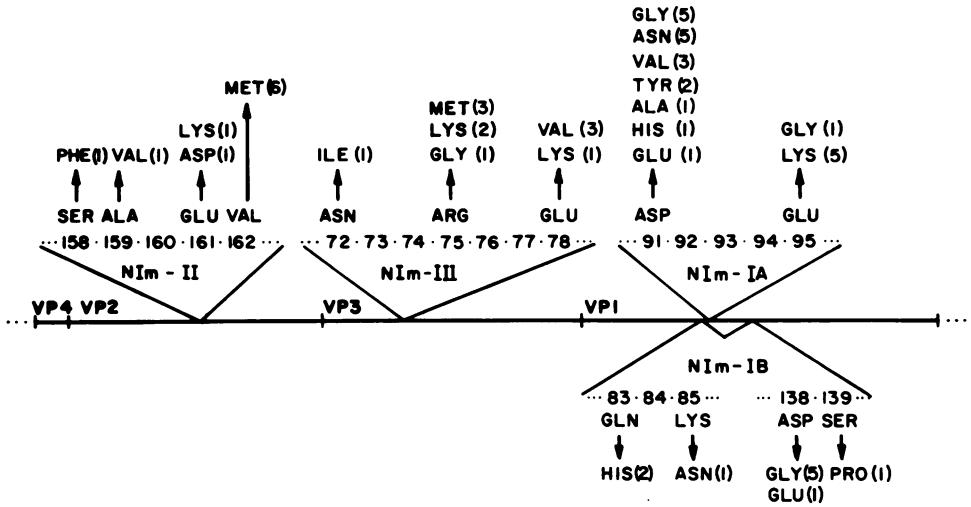

FIG. 5. Frequency of amino acid substitutions conferring antibody-resistance. The amino acid substitutions that occurred within the mutation clusters, excluding those that occurred in the three mutants selected with antibody 28, are summarized. The frequency with which each substitution was observed is indicated in parentheses.

demonstrated coat protein charge shifts inconsistent with the single mutation described.

One of these, NIm-IA mutant 14.2, which had a glycine substituted for the wild-type glutamate in the NIm-IA cluster, demonstrated a +2 VP1 charge shift. The mutant was sequenced through approximately 80% of VP1 (Fig. 4), but the second mutation remained unidentified.

A second NIm-IA mutant, 7.6, encoded a mutation in the VP1 cluster consistent with its VP1 charge shift but exhibited an altered VP3 as well. Sequencing through all of VP3 (Fig. 4) revealed a single mutation, the substitution of tyrosine for the wild-type histidine at VP3 position 35 (Fig. 7). This residue lines the inner cavity with the side chain pointing toward the RNA (M. G. Rossmann, personal communication).

In the third case, NIm-IB mutant 26.2C substituted histidine for the wild-type glutamine at VP1 position 83 in the NIm-IB cluster. Yet 26.2C exhibited a +1 VP1 charge shift on IEF gels, while 13.4, identically substituted at this residue, exhibited no charge shift. Histidine should behave neutrally charged on VP1 but positively charged on VP3 (as in mutant 7.6), because the pK_a of histidine is approximately 6.5, and VP1 focuses at the alkaline end of the gel, while VP3 focuses at the acid end. To explain the anomalous 26.2C charge shift, the mutant was sequenced through most of VP1 and only one other mutation was observed, the substitution of glycine for the wild-type aspartate at VP1 position 285 (Fig. 7). This residue resides on the surface with the side chain pointing up (M. G. Rossmann, personal communication), but the mutation did not appear to affect the phenotype relative to mutant 13.4.

These three mutants all encoded substitutions within the proposed antibody-binding sites. Their second amino acid substitutions appear to be random.

Antibody 28 selects double mutations in NIm-II. Antibody 28 behaved unusually in two ways. Firstly, resistant plaques (occurring at a low frequency, approximately 10^{-6}) were very small and yielded very few normal-sized plaques when replated under antibody selection. This suggests that the small plaques originated from only partially resistant mutants, which subsequently underwent an additional mutation(s) upon further propagation. Secondly, although this antibody was known to recognize NIm-II (Table 1), it neutralized every one of the 11 NIm-II mutants selected with the other antibodies. The three mutants selected with antibody 28 were sequenced through the region encoding NIm-II (Fig. 4). Two (28.1D and 28.2B) encoded double mutations in the cluster, both substituting valine for the wild-type glutamate at residue 161 and alanine for the wild-type valine at residue 162. Neither of these substitutions was observed in any other NIm-II mutant. The third mutant (28.3C) encoded a single mutation in the cluster, the substitution of aspartate for the wild-type glutamate at VP2 residue 161. This substitution was identical to that in mutant 18.6, yet the latter was

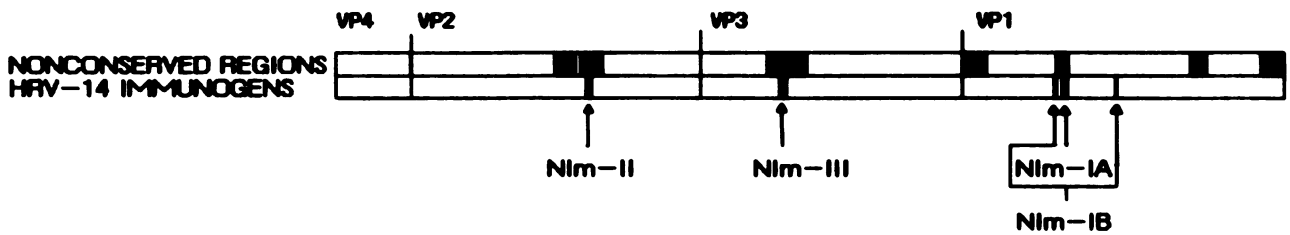

FIG. 6. HRV-14 immunogens correlate with regions nonconserved relative to poliovirus. The amino acid sequence of HRV-14 (4) was aligned with that of Sabin type 1 poliovirus by the method of Needleman and Wunsch (29). The adjusted ("gapped") HRV-14 sequence was then compared with alignments of poliovirus types 1, 2, and 3 (see Fig. 4 in reference 37). Nonconserved regions (black) were defined as segments in which few if any of the homologous amino acids were conserved.

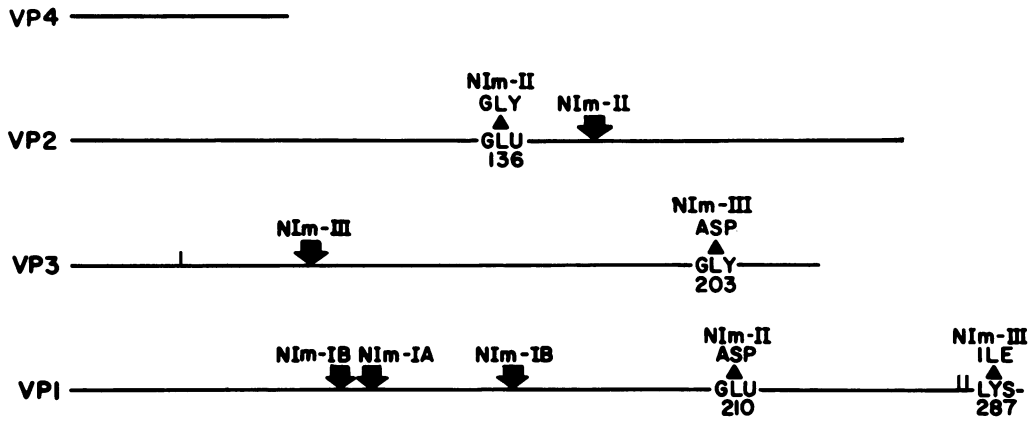

FIG. 7. Amino acid substitutions outside the major clusters. The mutation clusters corresponding to the four immunogens are indicated by thick arrows. The four amino acid substitutions that lie outside these clusters on the primary sequence but fall within the proposed immunogens on the virus surface are indicated. Unlabeled vertical lines denote additional mutations found in mutants with previously identified substitutions within the appropriate cluster (see the text).

neutralized by antibody 28. The mutants were sequenced through most of VP1 (Fig. 4), and 28.3C encoded a mutation substituting aspartate for the wild-type glutamate at residue 210 (Fig. 7). Examination of the viral structure reveals that the side chain of this VP1 residue is located adjacent to VP2 residue 162 (29a). Thus, it appears that two residues in NIm-II must be altered to confer resistance to antibody 28. In two mutants, both residues were located on VP2, but in the third case, one residue was located on VP2 while the other was on VP1. Again, this provides evidence for a noncontiguous antigen composed of sequences from two different coat proteins. Additional sequencing revealed that 28.2B encoded a mutation substituting arginine for the wild-type lysine at VP1 residue 280 (Fig. 7). Again, a substitution in addition to those in the antigen (as for mutants 7.6 and 26.2C) is of unknown significance.

Distribution of immunogen recognition in the five fusions. The 35 monoclonal antibodies used for these studies were generated in five independent fusions (Table 4). The first four fusions generated many more antibodies recognizing NIm-IA than antibodies recognizing NIm-IB, but the opposite was true of the fifth fusion. This may reflect a bias towards one immunogen or the other, depending on the method of immunization or the particular mouse inoculated. Both fusion 3 and fusion 5, which involved different virus purification techniques and immunization schedules, generated antibodies representing all four immunogens, suggesting that the immunogenicity of these virus sites was not an artifact of the method or of the particular mouse inoculated. Finally, it is worth noting that fusions 1, 2, and 4 did not generate representatives of NIm-IB or NIm-II, probably reflecting the small number of antibodies.

DISCUSSION

Validity of mutational analysis as a tool for mapping dominant immunogens. The ease of obtaining mutants resistant to neutralization by monoclonal antibodies and the simplicity of isoelectric and sequencing analyses makes analysis of resistance mutations an attractive general approach for detection of dominant immunogenic sites. The most serious criticism of this approach has been lack of any guarantee that the amino acid sites, which are substituted in neutralization-resistant mutants, actually reside in the antibody-binding site. It might equally well be argued, for example, that configurational alteration of the antibody-binding site is triggered by a distal mutation (20). Intensive investigations on the four neutralization immunogens on the hemagglutinin of influenza virus have revealed little, if any, evidence for such long-range conformational effects (39, 40). However, the degree to which such findings on a membrane protein can be applied to nucleocapsids, particularly those of nonenveloped viruses with tightly packed coat proteins, has not been clear. This may be especially true of picornavirions in which a variety of conditions, including pH and binding of neutralizing antibodies (13, 22, 23), cause large changes in the isoelectric point which are thought to result from concerted rearrangements on the surface of identical protein subunits (30).

Our results indicate that analysis of antibody-resistant mutants is, in fact, also a reliable method for identifying antibody-binding sites in a picornavirus, HRV-14. Thus, with the exception of three apparently random second mutations, every one of the mutations in our 62 neutralization-resistant mutants was located in a surface cluster with the side-chain of the affected residue pointing outward (29a). Moreover, these mutations were, without exception, correlated with the antigen identified by the cross-neutralization tests (Fig. 1 and 2). We have not found a single case of a distal mutation which alters antigenic structure. These findings suggest that if viable mutations causing long-range conformational effects occur at all, they are liable to be rare.

Nature and location of the four immunogens. Three of the four immunogens, NIm-IB, NIm-II, and NIm-III, are com-

TABLE 4. Distribution of immunogen specificities[a]

Fusion	No. of antibodies recognizing the following NIm:			
	NIm-IA	NIm-IB	NIm-II	NIm-III
1	1	0	0	1
2	4	0	0	4
3	8	2	2	4
4	1	0	0	0
5	1	3	2	2

[a] The 35 antibodies were generated in five hybridoma fusions, as described in Materials and Methods.

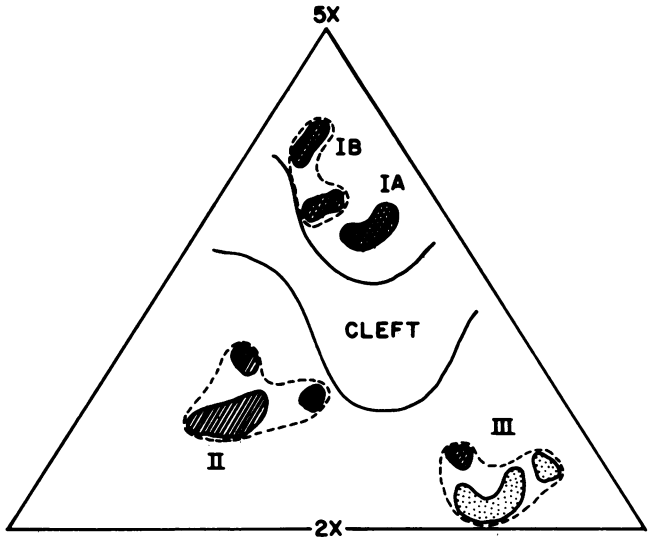

FIG. 8. Proposed immunogen locations on virus surface. The axes of symmetry for the virus are indicated on this diagram of the crystallographic structure unit (icosahedral asymmetry unit [29a]). Most of VP1 is located in the top half of the structure unit, including the cleft. Most of VP2 occupies the bottom left, and most of VP3 occupies the bottom right. The noncontiguous antigens are circumscribed (dotted lines), with the coat protein locations represented by cross-hatched lines (VP1), hatched lines (VP2), or stipples (VP3). Amino acid substitutions observed in HRV-14 are as follows: for NIm-IA, VP1 91 and 95; NIm-IB, VP1 83 and 85 (top) and VP1 138 and 139 (bottom); NIm-II, VP2 136 (top), VP2 158, 159, 161, and 162, and VP1 210; NIm-III, VP3 72, 75, and 78 (bottom), VP3 203 (right), and VP1 287.

posed of noncontiguous sequences located on the same coat protein as the mutation cluster, or on a different one (Fig. 8). NIm-IA and NIm-IB lie very close to each other, raising the question of whether they are actually part of a single immunogen. We have no evidence for their linkage, in that no mutants selected with antibodies recognizing one immunogen were resistant to antibodies recognizing the other. However, selection of additional mutants could possibly reveal such a linkage. There has been no published evidence for a NIm-IB equivalent in poliovirus. However, the dominant immunogen of that virus may actually span both the NIm-IA region and the amino half of the NIm-IB region. Minor et al. cross-tested monoclonal antibodies with resistant poliovirus type 3 mutants to define a single immunogen (28), and the substitutions in these mutants fell at VP1 residues 93 through 100 (15). These poliovirus residues may correspond to HRV-14 residues 83 through 94, encompassing the amino half of NIm-IB and part of NIm-IA. A possible linkage between the two sites in poliovirus would not have been evident in many of the studies with synthetic peptides (5, 10), as most of these peptides spanned both sites. A similar situation may exist in the hemagglutinin of influenza virus: extensive characterization has revealed completely independent antigens in influenza A, while two of the antigens may be linked in influenza B (2, 19). Thus, slight differences in conformation between two different virus serotypes may result in the fusion of two formerly separate antigens or the separation of a single antigen into two. It is possible that such a process occurs also in picornaviruses and represents a mechanism for the evolution of serotypes.

NIm-II is composed mainly of VP2 sequences, but a role for VP1 became evident with the discovery of HRV-14 mutant 28.3C, which encodes a substitution at VP1 residue 210 (Fig. 7). The foot-and-mouth disease virus dominant neutralization antigen, encoded in the region of VP1 amino acid residues 141 through 160 (3), evidently lies at a site analogous to HRV-14 NIm-II (29a). A poliovirus synthetic peptide of the VP2 sequence corresponding to HRV-14 NIm-II induces a low level neutralizing response (12); however, this site on the poliovirion is poorly immunogenic (11). Thus, the dominance of this site in the neutralization of HRV-14 and foot-and-mouth disease virus may contrast markedly with its role in poliovirus neutralization.

NIm-III is composed mainly of VP3 sequences, but participation of VP1 is evident in the case of mutant 9.6, which encodes a substitution at VP1 residue 287. The minor neutralization antigen of foot-and-mouth disease virus, encoded at the carboxy terminus of VP1 (3), may be in a position analogous to the HRV-14 NIm-III or may lie closer to a neighboring VP3 protrusion (29a). This site may also be used in poliovirus neutralization, as a neutralizing antiserum raised against a mixture of poliovirus VP3 and VP4 binds a poliovirus synthetic peptide corresponding to HRV-14 NIm-III (11).

Our strategy for locating the antibody resistance mutations was to sequence regions nonconserved between the three poliovirus serotypes and between poliovirus and HRV-14. Indeed, three of the four HRV-14 immunogens fell at highly nonconserved regions. Recent publication of the HRV-2 sequence (35) has allowed comparison of two different rhinovirus serotypes, and, not surprisingly, the mutation clusters defining the HRV-14 immunogens were notably nonconserved. In light of the solved virus structure, however, it is now clear that antigens which lie in similar locations on the virion (e.g., HRV-14 NIm-II and the foot-and-mouth disease virus major antigen [29a]) may be encoded in entirely different locations on the genome. Thus, while potential neutralization sites can be identified by comparing coat protein primary sequences between viruses, determination of the association of these sites with specific antigens on the virus surface requires knowledge of the three-dimensional structure.

Frequency of antibody resistance mutations and random second mutations. The frequency of nucleotide mutations in RNA viruses is estimated to be on the order of 10^{-4} (18). HRV-14 antibody-resistant mutants generally occur at a frequency of 10^{-4} to 10^{-5}, although frequencies as low as 2×10^{-6} have been observed (34). The frequency with which a given antibody could select resistant mutants was at least in part, determined by the way in which it interacted with the antigen; that is, for each of 10 antibodies examined, there was a high correlation between the frequency with which resistant mutants could be selected by an antibody and the number of different amino acid substitutions which could be tolerated by the antibody without loss of neutralizing ability (data not shown). A 10^{-4} frequency was approached only by the most discriminating antibodies, such as those recognizing the aspartate residue at position 91 in VP1, for which any substitution resulted in the loss of neutralizing activity (Fig. 5). Lower frequencies (as low as 10^{-6}) were observed with less discriminating antibodies which tolerated replacement by certain amino acids.

The fraction of mutants carrying a second fortuitous amino acid substitution, on the order of 1/20 (34), is also consistent with a random mutation frequency of 10^{-4}. The coat protein-encoding region of HRV-14 is approximately 2,500 bases. Thus, if the mutation frequency were 10^{-4} in picornaviruses, then one in four particles would have a

fortuitous mutation in the coat region. Assuming that only two-thirds of all nucleotide mutations result in an amino acid substitution (due to wobble at the third position in the codon), then only one-sixth would have an amino acid substitution in this region. However, only one-third of the amino acid substitutions due to a single-nucleotide mutation will result in a charge shift detectable on an isoelectric focusing gel (see Table 2 in reference 34). Therefore, only 1/18 of the viruses will have a fortuitous, charge-altered amino acid substitution in the coat protein-encoding region. In fact, we have observed 3 cases in 59 (Fig. 7), or, approximately 1/20 of the cases, consistent with the frequency predicted by a 10^{-4} mutation frequency.

The sequence of the coat protein-encoding region of our wild-type HRV-14 was compared with those of the two published HRV-14 sequences (4, 36). Our nucleotide sequence appeared to be identical to that of Stanway et al. (Fig. 4), consistent with the limited passaging of these two virus stocks after separation from a common source. In contrast, the sequence of Callahan et al. (4) differed from ours in 11 positions, resulting in five amino acid substitutions and a six-amino acid frameshift (four of the nucleotide differences were silent). These differences may reflect different passage histories.

Molecular basis of serotypes. Serotype is determined by the number and relative immunogenicities of surface neutralization antigens. The four NIms of HRV-14 provide a foundation for defining the molecular basis of picornavirus serotypes. Additional work with many viruses will be required to investigate whether these four antigens exhaust the immunogenic repertoire. The frequency of NIm-specific murine monoclonal antibodies, however, does not necessarily measure relative immunogenicity for two reasons. First, the distribution of antibodies stimulated by laboratory methods of immunization may not reflect that stimulated by natural infection. Secondly, it is well known that serologic specificity varies with species. Therefore, it is unclear whether immunodominance in the natural host is accurately reflected by the frequency of NIm-specific monoclonal antibodies isolated from the mouse.

The definition of serotype is somewhat arbitrary; cross-reaction of neutralizing antisera is commonly observed at levels of a few percent. This is particularly evident with human rhinoviruses. For example, HRV-3 and HRV-6 cross-react with HRV-14 (7). Monoclonal antibodies with defined specificity provide important new tools for investigating which antigens are involved in these cross-reactions and which are not.

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ADDENDUM IN PROOF

Recent analyses of type 1 (D. C. Diamond, B. A. Jameson, J. Bonin, M. Kohara, S. A. Abel, H. Itoh, T. Komatsu, M. Arita, S. Kuge, A. Nomoto, A. D. M. E. Osterhaus, R. Crainic, and E. Wimmer, 1985, Science **229**:1090–1093) and type 3 (P. D. Minor, M. M. A. Evans, M. Ferguson, G. C. Schild, G. J. Westrop, and J. W. Almond, 1985, J. Gen. Virol. **66**:1159–1165) poliovirus mutants suggest a role for NIm-II and NIm-III in poliovirus neutralization (J. M. Hogle, M. Chow, and D. J. Filman, 1985, Science **229**:1358–1365).

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