JOURNAL OF VIROLOGY, Jan. 1989, p. 36–42
0022-538X/89/010036-07$02.00/0

Vol. 63, No. 1

Inhibition of Rhinovirus Attachment by Neutralizing Monoclonal Antibodies and Their Fab Fragments

RICHARD J. COLONNO,[1]* PIA L. CALLAHAN,[1] DONNA M. LEIPPE,[2] ROLAND R. RUECKERT,[2] AND JOANNE E. TOMASSINI[1]

Merck Sharp and Dohme Research Laboratories, West Point, Pennsylvania 19486,[1] and Institute for Molecular Virology and Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706[2]

Received 20 May 1988/Accepted 14 September 1988

Previous molecular and immunological studies have mapped four neutralization sites on human rhinovirus type 14 (B. Sherry, A. G. Mosser, R. J. Colonno, and R. R. Rueckert, J. Virol. 57:246–257, 1986). Eight monoclonal antibodies, one pair for each of the four target sites and all belonging to a single isotype, immunoglobulin G2a, were studied under conditions which resulted in 95% neutralization of infectious viral particles. All eight antibodies shifted the isoelectric point of virions from 6.7 to much more acidic forms, ranging from pI 1.8 to 3.2. In addition, antibodies targeted against three of the four neutralization sites caused significant aggregation of virions under the neutralization conditions employed. Aggregation could be reversed by digesting virus-antibody complexes with papain. Following papain digestion, the acidic pIs of three of the neutralized virus preparations returned to neutral and infectivity was restored. Membrane-binding assays with virus neutralized with a nonaggregating antibody showed a dose-related inhibition of virus attachment to cellular receptors. Purified Fab fragments at a 13- to 61-fold-higher concentration than intact antibodies caused a comparable isoelectric shift, neutralized virions in the absence of aggregation, and interfered with attachment of virions to host cell receptors in a membrane-binding assay. These findings suggest that neutralizing antibodies interfere with the attachment of rhinoviruses to cellular receptors and that bivalent attachment of antibody is not a prerequisite for neutralization.

While neutralization of picornaviruses has been studied extensively (11), the precise mechanism(s) of antibody-mediated inactivation remains controversial. Early studies by Mandel (20) suggested a single-hit model for neutralization of poliovirus based on first-order kinetics. According to this model, a single antibody binding at any one of a number of critical sites on the virus surface is sufficient to inactivate a virus particle. He proposed a novel mechanism by which inactivation of poliovirus was associated with a change in the isoelectric point of the virus. After reviewing the published data, Daniels (10) concluded that the available evidence does not support a single-hit model but instead is compatible with a multihit mechanism of neutralization. A major difficulty with a number of the early studies has been the use of hyperimmune antiserum that contains a mixture of antibodies, not all of which neutralize, and which are directed against different epitopes on intact virions. More recently, aggregation or polymerization of virions has been proposed (3, 27, 28) as the major mechanism of neutralization. Supportive of this mechanism is the finding that papain treatment of poliovirus-antibody complexes disrupts the aggregates and restores virus infectivity (13, 16, 28).

The step(s) in viral infection that is affected during antibody-mediated neutralization is unclear. Early reports suggested that neutralizing antibodies had little effect on poliovirus attachment (12) and failed to completely inhibit penetration once the virus bound to the cell. However, these antibodies had an effect early in the infectious cycle, since neutralized virus failed to initiate viral RNA synthesis and did not shut off host cell macromolecular synthesis. Furthermore, it is unclear how many antibody molecules are needed to cause neutralization of a picornavirus. Icenogle et al. (16) reported that an average of four bound monoclonal antibodies were required per poliovirus virion to reduce infectivity, while Wetz and co-workers (30) found that a single polyclonal immunoglobulin G (IgG) molecule was sufficient for neutralization.

The human rhinoviruses (HRVs) represent a subgroup of picornaviruses and contain 100 antigenically distinct serotypes (15). Because they are the predominant cause of the common cold in humans, the mechanism by which they are neutralized is of particular medical importance. The sites involved in the neutralization of HRV-14 have been studied in some detail. The binding sites of 35 murine neutralizing monoclonal antibodies were mapped to specific regions on the viral capsid by comparing the genome sequence encoding the structural proteins of escape mutants with that of wild-type virus (23). These results revealed the existence of four neutralizing immunogen sites (NIms) within each viral protomer. The first (NImIA) and second (NImIB) map to amino acids 91 to 95 and to 83 to 85 and 138 to 139 of VP1, respectively. A third site (NImII) was mapped to amino acids 136 and 158 to 162 of VP2 and amino acid 210 of VP1. The last site (NImIII) mapped to amino acids 72 to 78 and 203 of VP3 and amino acid 287 of VP1. From the predicted amino acid sequence (5, 25) and the available atomic structure of HRV-14 (22), it was determined that the neutralization sites consist of surface amino acids and can be formed by the interaction of two different capsid proteins. The amino acid sequences encoding these surface regions represent hypervariable areas that can be detected by comparing the nucleotide sequences of many picornaviruses.

The present study was undertaken to discern the mecha-

* Corresponding author.

nism(s) by which antibody binding to each of the four neutralizing sites results in inactivation of HRV-14.

MATERIALS AND METHODS

Virus, cells, and antibodies. The growth and preparation of radiolabeled HRV-14 has been described previously (1, 23). Virus titers were determined by the standard plaque assay (1), and stock preparations were divided into equal portions and stored at −70°C. Particle and radioactivity concentrations were determined by electron microscopy and scintillation counting, respectively.

Neutralizing monoclonal antibodies were prepared from ascites fluid by DEAE, protein A, and MONO-Q column chromatographies as described previously (8). Concentrations were determined by the assay of Lowry et al. (19a) and by optical density by the procedure of Kirschenbaum (19).

Determination of neutralizing titers. Antibody titers were determined by incubating [^{35}S]methionine-labeled HRV-14 (final concentration, 7.4×10^{10} particles per ml, 1.23×10^{-10} M, 5.8×10^8 PFU/ml, 6×10^5 dpm/ml) with serial dilutions of antibody in McCoy 5A medium for 1 h at 25°C in a 10-μl volume. The reaction mixes were diluted to 500 PFU/ml and assayed for plaque formation as described before (1, 23), except that free antibody was removed from the monolayer by washing after a 1-h absorption period.

Preparation of Fab fragments. Following buffer exchange via a PD-10 gel filtration column (Pharmacia, Uppsala, Sweden), purified monoclonal antibodies were added to prewashed, immobilized papain (Pierce Chemical Co., Rockford, Ill.) in 20 mM NaH_2PO_4–20 mM cysteine hydrochloride–10 mM EDTA-Na_4, pH 7.2, according to the directions supplied by Pierce. The digestion mixture was incubated for 18 h at 37°C with rocking, and immobilized papain was then removed by low-speed centrifugation. Fc antibody fragments and any residual intact antibody were removed by affinity chromatography on an Affi-Gel protein A column (Bio-Rad Laboratories, Richmond, Calif.) with the MAPS buffer system and directions supplied by Bio-Rad. Fab fragments were collected as they passed through the column, and their purity was demonstrated by analysis on silver-stained polyacrylamide gels. In all cases, no intact antibody could be detected on overloaded gels, indicating that >98% purification was achieved. The concentration of Fab fragments was determined by standard protein determinations (19a).

Isoelectric focusing. Isoelectric focusing was performed in a 110-ml LKB isoelectric focusing column (21). The column contained a 100-ml preformed 0 to 50% (wt/vol) linear sucrose gradient containing 1% ampholytes (Pharmalyte pH 3 to 10; Pharmacia, Uppsala, Sweden) and 0.1% Triton X-100. Samples (about 30,000 cpm of ^{35}S-labeled virus) were added to the gradient former so as to distribute the sample over the top half of the gradient. The top cathode buffer consisted of 1.6% ethanolamine, and the bottom anode buffer was 60% (wt/vol) sucrose containing 1.5% H_2SO_4. The column was chilled to 10°C, and the LKB power supply limits were set to 1,800 V, 15 W, and 15 mA. After 16 h, the column was fractionated from the bottom into 1.5-ml fractions. The pH of every fifth fraction was determined, and 0.5-ml samples of each fraction were assayed for radioactivity. Except when indicated, pI values given in the tables are an average of at least two determinations and represent the position at which >90% of the input radioactivity was recovered.

Assays for viral aggregation. The formation of viral aggregates was determined by sucrose gradient analysis of neutralized [^{35}S]methionine-labeled virus (see above). Samples (0.2 ml) of antibody-treated virus were layered on a 4.8-ml 5 to 20% preformed sucrose gradient and centrifuged for 25 min at 44,000 rpm in a Beckman SW 50.1 rotor at 10°C. The gradients were fractionated (50 μl) from the bottom, and the radioactivity in each fraction was determined by scintillation counting. The pellet, which contained aggregates of five or more virions, was suspended in 0.6 ml of phosphate-buffered saline (PBS) containing 0.1% sodium dodecyl sulfate (SDS), and radioactivity was counted to determine the percent precipitation.

Alternatively, radiolabeled virus was incubated with antibody or Fab fragments for 1 h at 25°C and diluted to 0.1 ml in PBS prior to centrifugation in an Eppendorf tube at 12,000 × *g* for 2 min. Aggregation was determined by assaying the radioactivity in the supernatant and pellet (after suspension in 0.1 ml of 1% SDS). Untreated virus did not form pellets in control experiments.

Papain treatment of neutralized virus. [^{35}S]methionine-labeled HRV-14 was incubated with individual neutralizing antibodies or PBS in 25 μl under the conditions described above. A 2-μl sample was removed and assayed for aggregation by the microfugation method detailed above. The remaining sample was digested in 20 mM cysteine with 50 μg of papain (Sigma) per ml in a final volume of 34 μl for 15 min at 37°C. A portion (2 μl) was again assayed for aggregation by microfugation. The remainder of the sample was analyzed on a pI column as described above. To follow infectivity, parallel reaction mixes (25 μl) were incubated with PBS or antibody, and then one half was treated with papain. Untreated samples were incubated in the presence of 20 mM cysteine. Aggregation assays were again performed on both treated and untreated samples to monitor the effect of digestion on aggregation. The remainder of both treated and untreated samples was then assayed for infectious virus by the plaque assay (1).

Membrane-binding assay. Preparation of HeLa cell membranes has been previously described (8). Solutions (10 μl) containing intact antibody or Fab fragments were incubated with 10 μl of [^{35}S]methionine-labeled HRV-14 (10^4 cpm) in PBS containing 10 mM $MgCl_2$ for 1 h at 25°C in 1.5-ml Eppendorf tubes before the addition of 20 μl of HeLa cell membranes. After an additional 1 h of incubation at 25°C, the membranes were pelleted at 12,000 × *g* for 2 min, and the radioactivity in the supernatants was counted. Pellets were suspended in 0.2 ml of PBS and also assayed for radioactivity to determine percent binding.

RESULTS

Purification and titration of neutralizing antibodies. Two different monoclonal antibodies from each of the four HRV-14 neutralizing groups (NImIA, -IB, -II, and -III) were selected for study; all were of the IgG2a isotype. Antibodies were purified from ascites fluid as described previously (8), and their purity was confirmed by SDS-polyacrylamide gel electrophoresis (PAGE) analysis (Fig. 1). The HRV-14 preparations used in these studies were purified on sucrose gradients, titered by plaque assay, and determined by electron microscopy to have particle/PFU ratios ranging from 20 to 127. Preparations of each monoclonal antibody were titrated in a plaque reduction assay to determine the concentration necessary to achieve an average 95% neutralization (ND_{95}) of a standard concentration of virus. The results (Table 1) showed that the ND_{95} concentrations varied little between antibody groups and less than fivefold overall, from

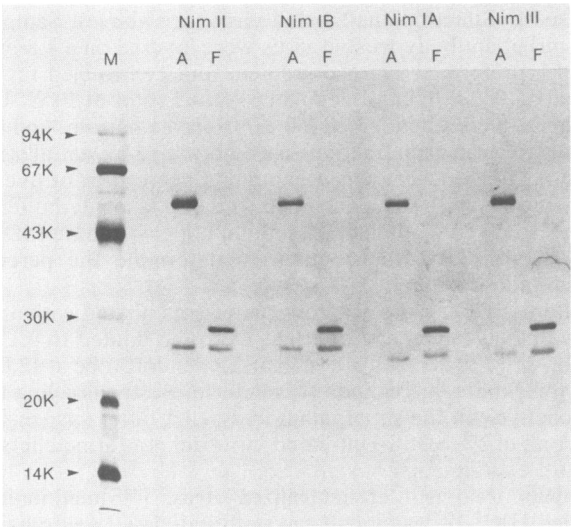

FIG. 1. SDS-PAGE analysis of antibodies and Fab fragments. Samples (0.8 μg) of NImII-28, NImIB-29, NImIA-34, and NImIII-33 antibodies (A) and each of their derived Fab fragments (F) were analyzed by 10% polyacrylamide SDS-PAGE under reducing conditions and silver stained as described previously (29). Standard protein markers are shown in lane M, and their molecular masses are indicated (in kilodaltons).

2.3×10^{-8} M (35 ng/10 μl) to 10.7×10^{-8} M (161 ng/10 μl) between individual antibodies. Despite the small number of antibodies examined in this study, the data suggest that neutralization at each of the four sites is comparable, with no obvious indication that antibody binding to one site was more efficacious than to the others.

Preliminary determinations of IgG/virion ratios of isolated aggregates suggested that less than 20 antibody molecules were present per virion under conditions that gave 95% neutralization (unpublished data). While these experiments have little bearing on the number of antibodies required for neutralization, they indicate that the neutralization effects observed below were not due to antibody saturation of virus particles.

Isoelectric focusing of neutralized virus. Earlier studies on poliovirus showed that neutralization coincides with a shift in the isoelectric focusing point (pI) of the virus from neutral to acidic (12, 20). To determine whether HRV-14 undergoes a shift in pI following antibody attachment, ^{35}S-labeled HRV-14 was incubated with each of the eight monoclonal antibodies under ND_{95} conditions before the virion pI was determined as described in Materials and Methods. Representative chromatographs are shown in Fig. 2. All of the neutralizing antibodies were capable of shifting the pI of native HRV-14 from 6.7 to between 1.8 and 3.6 (Table 1). The shift in pI appeared to be an all-or-none phenomenon, since treatment with lower concentrations of antibody yielded only two peaks, one at 6.7 and one at 1.8 to 3.6, with no intermediate peaks (data not shown). To show that neutralizing antibodies were still attached to the acidic particles after isoelectric focusing, radiolabeled NImII antibody 35 (NImII-35) was incubated with unlabeled HRV-14 and chromatographed on the pI column. The results (data not shown) demonstrated that the radioactivity migrated to the same acidic pI location at which the neutralized virus had focused. The observed pI shift was not merely due to the presence of the IgG antibody, since radiolabeled NImII-35 antibody chromatographed in the absence of virus focused with a pI of 6.6 (data not shown).

TABLE 1. Titration of HRV-14 neutralizing antibodies

Neutralizing antibody	Concn[a] (10^{-8}M)	pI	% Virus aggregation
None		6.7	1
NImIA-17	10.7	2.2	18
NImIA-34	3.7	2.5	14
NImIB-13	4.9	3.2	97
NImIB-29	3.3	2.3	96
NImII-28	7.9	1.8	96
NImII-35	4.8	3.6	97
NImIII-21	4.9	3.2	96
NImIII-33	2.3	3.1	94

[a] ND_{95} values (see text).

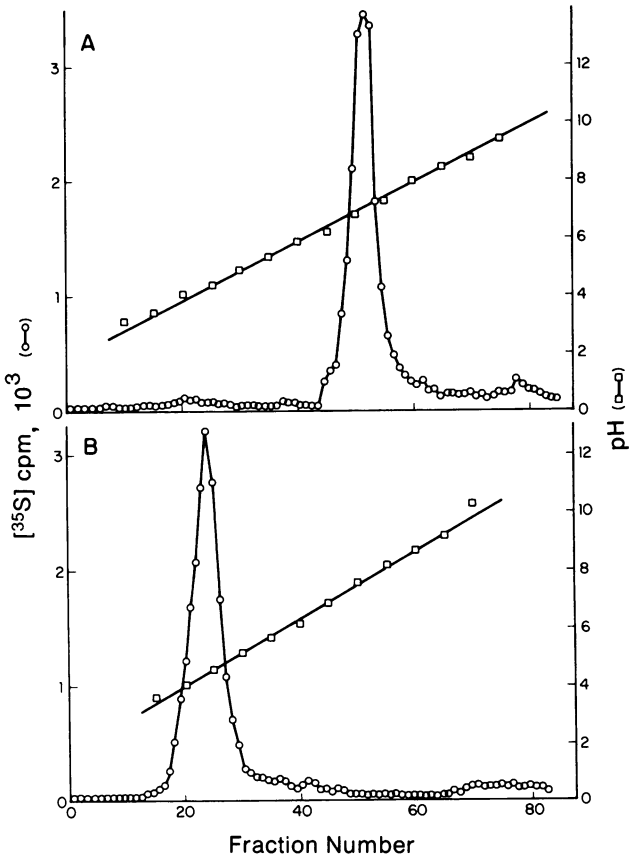

FIG. 2. Isoelectric focusing of neutralized HRV-14. Purified HRV-14 which had been metabolically labeled with [^{35}S]methionine was incubated with (B) or without (A) 0.8 μg of NImIA-17 antibody in 50 μl for 1 h at 25°C. Samples were then introduced into a 110-ml isoelectric focusing column and allowed to reach equilibrium for 16 h as described in Materials and Methods. Following fractionation (1.5 ml), 0.5-ml portions were assayed for radioactivity (○), and the pH (□) of every fifth fraction was determined.

Aggregation of virus particles. Previous neutralization studies (3, 16, 26, 27) on poliovirus have demonstrated that virions aggregate as a result of antibody neutralization. To determine whether any of the HRV-14 neutralizing antibodies caused a similar aggregation of HRV virions under ND_{95}

TABLE 2. Papain treatment of neutralized HRV-14

Antibody	Papain treatment	% of virus in pellet	pI[a]	% of control titer[b]
None (control)	−	4	6.7	
	+	2	6.7	98
NImIA-34	−	13	2.5	11
	+	3	6.6	98
NImIB-13	−	86	3.2	3
	+	3	6.5	88
NImII-35	−	89	2.6	2
	+	3	3.4 (5.5)[c]	39
NImIII-33	−	86	3.1	4
	+	3	6.7	89

[a] The pI values for neutralized virus samples that were not treated with papain are derived from Table 1.
[b] Average of three experiments.
[c] Number in parentheses represents 30% of recovered radioactivity.

conditions, radiolabeled HRV-14 was incubated with each of the eight monoclonal antibodies and then analyzed by centrifugation in sucrose gradients. Monoclonal antibodies mapping to the NImIB, NImII, and NImIII sites caused greater than 94% of the neutralized virions to pellet at the bottom of the gradient (Table 1). However, treatment of HRV-14 with either of the two NImIA antibodies resulted in substantially less aggregation of the particles, between 14 and 18%. The unprecipitated material in immunoprecipitates from NImIA antibodies 17 and 34 sedimented in soluble complexes (virus monomers, dimers, trimers, etc.) not observed with antibodies against the other target sites at this degree of neutralization. These results suggest that intraparticle binding is favored in the case of NImIA antibodies at the ND_{95} dose levels used.

Papain treatment of neutralized virus. If bivalent antibody attachment is crucial for viral neutralization, then this process might be reversible by cleavage of the bivalent structure of the antibody to form monovalent arms. This approach was reported to work successfully with poliovirus, since neutralized virions treated with papain to disrupt antibody binding became infectious and displayed a neutral pI (12, 16). Radiolabeled HRV-14 was incubated with ND_{95} quantities of each of four neutralizing antibodies, and one-half was treated with papain prior to further analysis. Papain treatment completely disrupted the viral aggregates (Table 2). Subsequent determination of virion pIs revealed that, with the exception of virus neutralized with antibody NImII-35, virion pIs returned to neutral values between 6.5 and 6.7 (Table 2). In contrast, the majority (70%) of the NImII-35-neutralized virions continued to display an acidic pI of 3.4, while 30% of the virions displayed an elevated pI of 5.5. The consequences of aggregate disruption and restoration of virion pIs were further evaluated by plaque assay. From 88 to 94% of the starting inocula were recovered from virus samples neutralized with antibodies NImIA-34, NImIB-13, and NImIII-33 (Table 2). However, the infectivity of virions neutralized with antibody NImII-35 was only partially (39%) revived. These results indicated that the effects of neutralization are reversible and suggest that the observed pI shift may not be the result of aggregation.

Activity of Fab antibody fragments. To explore further the relationship between aggregation and the acidic shift in the virion pI, Fab fragments were prepared from antibodies representing each of the four neutralizing groups and purified as described in Materials and Methods. Purity of the Fab fragments was again demonstrated by PAGE analysis (Fig. 1). Examination of all four Fab preparations showed the absence of full-length heavy-chain (50 kilodalton [kDa]) and the appearance of the expected truncated heavy-chain species at 28 kDa. Titration of the Fab fragments against HRV-14, under conditions identical to those used with intact antibody, demonstrated that the Fab fragments alone were capable of neutralizing virus (Table 3). Direct comparison of the bivalent antibody molecule and monovalent Fab fragment concentrations required for ND_{95} neutralization indicated that a 13- to 61-fold-greater concentration (0.8×10^{-6} to 2.0×10^{-6} M, 0.4 to 1.0 μg/10 μl) of Fab fragments was required to obtain equivalent neutralization (Table 3).

TABLE 3. Neutralization by Fab fragments

Antibody	ND_{95} concn[a]		pI
	10^{-6} M	Fab/intact	
NImIA-34	0.8	22	2.3
NImIB-29	2.0	61	3.6
NImII-28	1.0	13	2.0
NImIII-33	1.0	44	2.0

[a] Molar concentration of purified Fab fragments required to achieve 95% neutralization of [^{35}S]methionine-labeled HRV-14 at a final concentration of 7.4×10^{10} particles per ml after a 1-h incubation at 25°C. Fab/intact, Ratio of intact antibody concentrations (Table 1) to Fab concentrations needed to achieve equivalent neutralization (ND_{95}).

Since Fab fragments are only capable of monovalent attachment, viral aggregation should not occur. Analysis of Fab-neutralized virus by the microfuge procedure confirmed both that viral aggregates were absent and that the neutralizing activity observed was not the result of contaminating intact antibody (data not shown). Addition of anti-mouse IgG antiserum to the Fab-neutralized virions reestablished the interparticle networks and resulted in aggregation of the virus (data not shown).

The ability of Fab fragments to neutralize virus in the absence of aggregation enabled further study into the cause of the isoelectric point shift observed above. Determination of virion pIs after neutralization with Fab fragments revealed that the Fab fragments alone were also capable of causing an acidic shift in the virion pI which was comparable to that observed with whole antibody (Table 3). These results imply that the interaction of antibody with the virion capsid proteins alone is sufficient to cause the observed pI change and that cross-linking or aggregation of virions is not required to effect this change.

Effect on viral attachment. Earlier studies have suggested that neutralizing monoclonal antibodies do not interfere with the attachment of poliovirus to cellular receptors (12). This seems surprising for several reasons. A virion can bind at least 30 and perhaps as many as 60 molecules of a given antibody on its surface; immune complexes almost invariably form aggregates; and the acidic pI shift is suggestive of substantial conformational changes within the virion. To determine whether neutralized HRV-14 could still attach to cellular receptors, radiolabeled virions were treated with neutralizing antibodies under ND_{95} conditions and assayed in a membrane-binding assay. As shown in Fig. 3, each of the neutralizing antibodies had a profound effect on viral attachment. The antibody to the NImIA site caused a dose-dependent inhibition of HRV-14 binding, with 95% inhibition of virus binding obtained at 1.9×10^{-8} M. This value is very close to the 3.7×10^{-8} M (Table 1) concentration required to achieve an ND_{95} by plaque assay. Antibodies to the other three sites appeared to increase the amount of virus bound. However, controls showed that the increase in virus binding was due to the aggregation of virus particles,

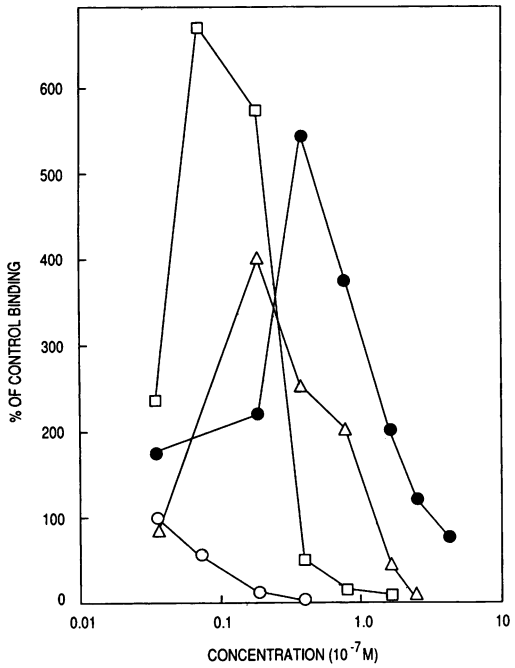

FIG. 3. Effect of antibody neutralization on HRV-14 attachment to membranes. Duplicate samples of ^{35}S-labeled HRV-14 were treated with the indicated concentrations of NImIA-34 (○), NImIB-29 (●), NImII-28 (□), and NImIII-33 (△) antibodies in a total volume of 10 μl for 1 h at 25°C. HeLa cell membranes (20 μl) were added to each sample, and the ability of the virus to attach to cellular receptors was measured as described in Materials and Methods. Virus binding is expressed as a percentage of the specific binding observed with untreated virus.

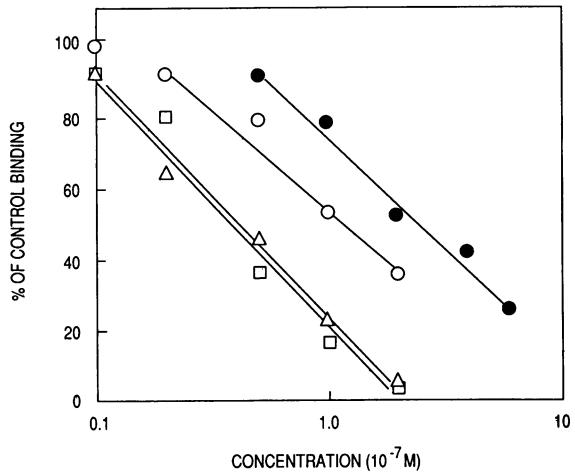

FIG. 4. Effect of Fab fragments on HRV-14 attachment. Fab fragments were prepared and purified as described in Materials and Methods. Duplicate samples of ^{35}S-labeled HRV-14 were treated with the indicated concentrations of NImIA-34 (○), NImIB-29 (●), NImII-28 (□), and NImIII-33 (△) Fab fragments in 10 μl for 1 h at 25°C. HeLa cell membranes (20 μl) were added to each sample, and incubation was continued for an additional hour at 25°C. Membranes were pelleted, and the amount of radioactivity present in the pellets was determined. Virus binding is expressed as a percentage of the specific binding observed with untreated virus.

since the majority of antibody-treated virus could also be pelleted in the absence of membranes.

The formation of aggregates made it impossible to measure the effect of these three antibodies on viral attachment but indicated that viral attachment was indeed affected. Fab fragments were then used to overcome this problem. Titration of Fab fragments in the membrane-binding assay showed dose-dependent response curves and inhibition of HRV-14 attachment by each of the four Fab fragments (Fig. 4). The concentration of NImII-28 and NImIII-33 Fab fragments needed to show a 95% inhibition of control binding was 1.6×10^{-7} M, while the projected concentrations of NImIA-34 and NImIB-29 Fab fragments required for comparable binding inhibition were 6.3×10^{-7} and 1.1×10^{-6} M, respectively. The inhibition was shown to be specific, since parallel assays involving a different HRV serotype, HRV-15, showed no binding inhibition (data not shown). The concentrations of Fab fragments required for 95% inhibition of virus attachment versus 95% neutralization were nearly equivalent for NImIA-34 and NImIB-29 Fab fragments and sixfold less for Fab fragments derived from antibodies NImII-28 and NImIII-33.

DISCUSSION

These studies support the contention that neutralization of picornaviruses occurs by several mechanisms. Rhinoviruses, like other picornaviruses, have four sites on the virion capsid to which neutralizing antibodies can bind and inactivate the virus particle. The precise locations and structures of these four sites have been determined by sequencing the RNA genome of neutralization escape mutants and subsequently visualized from the atomic structure determined for HRV-14 (22, 23). All four of these sites reside on the surface of the virion and represent hypervariable regions located in the structural proteins VP1, VP2, and VP3. Of the 35 HRV-14-neutralizing antibodies isolated, 15 recognize the NImIA site, 5 the NImIB site, 4 the NImII site, and 11 the NImIII site (23).

In order to generalize interpretation of our results, we selected two monoclonal antibodies from each group that had the same IgG2a isotype, and all were produced and purified by identical protocols. The amount of antibody required to achieve an ND_{95} differed only slightly between neutralizing sites, and this fact suggests that antibody binding to any one site was not markedly different from binding to any of the other sites.

The two major consequences of neutralization which have been reported for other picornaviruses include an acidic shift in the pI of virions and the formation of viral aggregates (3, 12, 16, 20, 27, 28). Analysis of neutralized HRV-14 particles also demonstrated the involvement of these effects in the neutralization of rhinoviruses. The importance of studying several neutralizing monoclonal antibodies became apparent when we found that antibody binding to NImIB, NImII, and NImIII sites resulted in the polymerization of 86 to 97% of treated virions into large aggregates, while under the same conditions only 13 to 18% of the virions containing bound NImIA antibodies became aggregated (Tables 1 and 2). Such differences have also been obtained in the case of poliovirus by immunoelectron microscopy (26). The decreased amount of aggregation found when antibodies bound to NImIA sites probably reflects an optimal distance between adjacent NImIA sites that favors intraparticle, bivalent attachment of an IgG molecule. The optimal distance which favors bivalent attachment of two antibody arms has been reported to be between 9 and 15 nm (16). Examination of the HRV-14 atomic structure (22) indicates that bivalent antibody attach-

ment spanning two Asp-91 amino acid sites in VP1 across a two-fold axis would bridge an ideal distance of 10.7 nm. However, similar examination of each of the NIm sites indicates that an optimal distance can be found between two NIm sites if all of the possible orientations are considered. Therefore, other criteria such as the spatial orientation of the NIm sites on the virus particle need to be considered in explaining interparticle versus intraparticle binding.

Treatment of viral aggregates with papain destroyed these complexes and confirmed the antibody-induced interparticle nature of these structures (Table 2). With the exception of antibody NImII-35, the nonaggregated virions displayed neutral pI values and restored infectivity (Table 2). These data are similar to data obtained in earlier studies performed on poliovirus (13, 16, 28). Virions characterized after neutralization with NImII-35 and subsequent papain treatment differed significantly from virions neutralized with the other antibodies tested. While aggregation was disrupted in a manner similar to that caused by the other antibodies, these virions showed only a partial shift in their pI values toward neutrality and a similar partial (39%) restoration of infectivity. This result remains an anomaly since it is the only result obtained which shows virus infectivity in the presence of an acidic pI value. The observed result may reflect partial neutralization by the Fab fragments left associated with the virus. It is worth noting that the Fab fragments of antibody NImII-28 were the most effective, relative to intact antibody concentrations (Table 3), at neutralizing HRV-14.

The data presented indicate that an ND_{95} can be achieved in the absence of bivalent antibody attachment to virions. Fab fragments derived from each of four neutralizing antibodies were capable of neutralizing HRV-14, although 13- to 61-fold-higher concentrations were required for equivalent neutralization (Tables 1 and 3). The higher Fab concentrations needed may reflect decreased binding affinity or inactivation following digestion and repurification or indicate that aggregation is a more efficient means of blocking viral attachment. Keller (17, 18) also reported that monovalent Fab fragments obtained by papain digestion were effective in neutralizing poliovirus to a lesser extent than intact, bivalent antibodies, while Mandel (20) reported complete neutralization with Fab fragments obtained from polyclonal antiserum. In contrast, Thomas et al. (27) could not prepare Fab fragments that were capable of binding to poliovirus.

The second major consequence of neutralization frequently found is an acidic shift in the normally neutral pI of virions. Our results with HRV-14 also show a strong correlation between neutralization and shift in the virion pI. The studies (Table 3) with Fab fragments indicate that a shift of neutralized virions to an acidic pI is not a consequence of aggregation, since Fab fragments were also effective at causing this change. One interpretation of the pI change is that it is the result of a cooperative conformational transition or allosteric change of capsid subunits (20). Such a conformational shift has been observed when drugs are bound to crystals of HRV-14 (2). The present studies suggest that the antibody-induced pI shift observed is most likely the result of antibody interaction with viral capsid proteins. The precise molecular mechanism remains unclear, but the isolation of antibodies which can bind to poliovirus (12) without neutralizing them indicates that there is more to neutralization than antibody interaction with virion capsid proteins. It may be due to alterations in charged groups exposed on the surface of the virus. A similar conclusion was reached by Carthew (6), who found that when bovine enterovirus was iodinated before and after neutralization, a different pattern of labeled polypeptides was obtained. These data differ from those of Brioen et al. (4), who, using both monoclonals and polyclonals, reported that the pI shift was coincidental and that there was no strict correlation between virus neutralization and change in isoelectric points.

Whether through aggregation or pI change, it is clear that attachment of neutralizing antibodies interferes with attachment of HRV-14 to cellular receptors. In no instance was normal binding to receptors observed when ND_{95} quantities of antibody or Fab fragments were present. This result is not surprising considering the amount of surface area covered by the arm of an antibody molecule, the high probability of steric hindrance, and possible conformational changes resulting from a drastic shift in the pI of the virion. However, these results do not agree with earlier studies on poliovirus, which suggest that six of seven neutralizing monoclonal antibodies tested failed to block attachment or penetration of treated poliovirus virions (12). The reasons for these differences are unclear but may be related to the specificity and sensitivity of the binding assays employed. Under the conditions used in the poliovirus study, a good deal of nonspecific binding to cells lacking poliovirus receptors (L cells) was observed, which may have decreased the sensitivity in determining specific binding.

Recent studies have determined that the viral attachment site resides deep within a crevice located on the surface of HRV-14 (7, 9). A 90-kDa cell surface protein apparently interacts with this region of the virus by entering the canyon (29). It is quite conceivable that attachment of antibody or Fab fragments could restrict entrance of the receptor protein into the opening of the canyon. The ability of intact antibodies to span this opening may account for their greater efficacy in blocking viral attachment. It is doubtful that any of the neutralizing antibodies interact directly at the viral attachment site, since the amino acid sequences composing the four NIm sites are unique to HRV-14. Attempts to neutralize other HRV serotypes belonging to the same receptor family have been unsuccessful (unpublished data).

Clearly the mechanisms by which the host immune system combats viral challenge are both complicated and customized to individual viruses. The use of X-ray crystallography, molecular biology, and immunological techniques will continue to refine our understanding of this process.

ACKNOWLEDGMENTS

We thank Eddy Arnold (New Jersey Center for Advanced Biotechnology and Medicine) for helpful discussions regarding the spacial orientation of neutralization sites on HRV-14.

This work was supported in part by Public Health Service grant AI24939 from the National Institutes of Health to Roland Rueckert.

LITERATURE CITED

1. **Abraham, G., and R. J. Colonno.** 1984. Many rhinovirus serotypes share the same cellular receptor. J. Virol. **51**:340–345.
2. **Badger, J., I. Minor, M. J. Kremer, M. A. Oliveira, T. J. Smith, J. P. Griffith, D. M. Guerin, S. Krishnaswamy, M. Luo, M. G. Rossmann, M. A. McKinlay, G. D. Diana, F. J. Dutko, M. Fancher, R. R. Rueckert, and B. A. Heinz.** 1988. Structural analysis of a series of antiviral agents complexed with human rhinovirus 14. Proc. Natl. Acad. Sci. USA **85**:3304–3308.
3. **Brioen, P., D. Dekegel, and A. Boeye.** 1983. Neutralization of poliovirus by antibody-mediated polymerization. Virology **127**: 463–468.
4. **Brioen, P., B. Rombaut, and A. Boeye.**1985. Hit-and-run neutralization of poliovirus. J. Gen. Virol. **66**:2495–2499.
5. **Callahan, P. L., S. Mizutani, and R. J. Colonno.** 1985. Molecular cloning and complete sequence determination of the RNA

genome of human rhinovirus type 14. Proc. Natl. Acad. Sci. USA **82**:732–736.
6. **Carthew, P.** 1976. The surface nature of proteins of a bovine enterovirus, before and after neutralization. J. Gen. Virol. **32**: 17–23.
7. **Colonno, R. J.** 1987. Cell surface receptors for picornaviruses. BioEssays **5**:270–274.
8. **Colonno, R. J., P. L. Callahan, and W. J. Long.** 1986. Isolation of a monoclonal antibody that blocks attachment of the major group of human rhinoviruses. J. Virol. **57**:7–12.
9. **Colonno, R. J., J. H. Condra, S. Mizutani, P. L. Callahan, M. E. Davies, and M. A. Murcko.** 1988. Evidence for the direct involvement of the rhinovirus canyon in receptor binding. Proc. Natl. Acad. Sci. USA **85**:5449–5453.
10. **Daniels, C. A.** 1975. Mechanisms of viral neutralization, p. 79–97. *In* A. L. Notkins (ed.), Viral immunology and immunopathology. Academic Press, New York.
11. **Dimmock, N. J.** 1984. Mechanisms of neutralization of animal viruses. J. Gen. Virol. **65**:1015–1022.
12. **Emini, E. A., S. 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.
13. **Emini, E. A., P. Ostapchuk, and E. Wimmer.** 1983. Bivalent attachment of antibody onto poliovirus leads to conformational alteration and neutralization. J. Virol. **48**:547–550.
14. **Greenwood, F. C., W. M. Hunter, and J. S. Glover.** 1963. The preparation of ^{131}I-labeled human growth hormone of high specific activity. Biochem. J. **89**:114–123.
15. **Hamparian, V. V., R. J. Colonno, M. K. Cooney, E. C. Dick, J. M. Gwaltney, Jr., J. H. Hughes, W. S. Jordan, Jr., A. Z. Kapikian, W. J. Mogabgab, A. Monto, C. A. Phillips, R. R. Rueckert, J. H. Schieble, E. J. Stott, and D. A. J. Tyrrell.** 1987. A collaborative report: rhinoviruses—extension of the numbering system from 89 to 100. Virology **159**:191–192.
16. **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.
17. **Keller, R.** 1966. The stability of neutralization of poliovirus by native antibody and enzymatically derived fragments. J. Immunol. **96**:96–106.
18. **Keller, R.** 1968. Studies on the mechanism of enzymatic reactivation of antibody-neutralized poliovirus. J. Immunol. **100**: 1071–1079.
19. **Kirschenbaum, D. M.** 1973. Molar absorbtivity and A_{ICM} values for proteins at selected wavelengths of the ultraviolet and visible regions. Anal. Biochem. **56**:237–263.
19a. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
20. **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.
21. **Righetti, P. G.** 1983. *In* T. S. Work and R. H. Burdon (ed.), Laboratory techniques in biochemistry and molecular biology—isoelectric focusing: theory, methodology and applications, p. 88–98, 298–299. Elsevier Biomedical Press, Amsterdam.
22. **Rossmann, M. G., 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.** 1985. Structure of a human common cold virus and functional relationship to other picornaviruses. Nature (London) **317**:145–153.
23. **Sherry, B., A. G. Mosser, R. J. Colonno, and R. R. Rueckert.** 1986. Use of monoclonal antibodies to identify four neutralization immunogens on a common cold picornavirus, human rhinovirus 14. J. Virol. **57**:246–257.
24. **Sherry, B., and R. R. Rueckert.** 1985. Evidence for at least two immunodominant neutralization antigens on human rhinovirus 14. J. Virol. **53**:137–143.
25. **Stanway, G., P. J. Hughes, R. C. Mountford, P. D. Minor, and J. W. Almond.** 1984. The complete nucleotide sequence of a common cold virus: human rhinovirus 14. Nucleic Acids Res. **12**:7859–7875.
26. **Taniguchi, K., and S. Urasawa.** 1987. Different virus-precipitating activities of neutralizating monoclonal antibodies that recognize distinct sites of poliovirus particles. Arch. Virol. **92**: 27–40.
27. **Thomas, A. A. M., P. Brioen, and A. Boeye.** 1985. A monoclonal antibody that neutralizes poliovirus by cross-linking virions. J. Virol. **54**:7–13.
28. **Thomas, A. A., M. R. Vrijsen, and A. Boeye.** 1986. Relationship between poliovirus neutralization and aggregation. J. Virol. **59**: 479–485.
29. **Tomassini, J. E., and R. J. Colonno.** 1986. Isolation of a receptor protein involved in attachment of human rhinoviruses. J. Virol. **58**:290–295.
30. **Wetz, K., P. Willingmann, H. Zeichhardt, and K.-O. Habermehl.** 1986. Neutralization of poliovirus by polyclonal antibodies requires binding of a single IgG molecule per virion. Arch. Virol. **91**:207–220.