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Structural studies on the mechanisms of antibody-mediated neutralization of human rhinovirus

Thomas J. Smith, Anne G. Mosser and Timothy S. Baker*

Antibodies represent a major component of the mammalian immunological defense against picornavirus infection. The work reviewed here examines structural details of antibody-mediated neutralization of human rhinovirus 14 (HRV14) using a combination of crystallography, molecular biology and electron microscopy. The atomic structures of the Fab fragment from a neutralizing monoclonal antibody (Fab17-IA) and HRV14 were used to interpret the ~25Å resolution cryo-electron microscopy structure of the Fab17-IA/HRV14 complex. While there were not any observable antibody-induced conformational changes in the HRV14 upon antibody binding, there was evidence that charge interactions dominate the paratope-epitope interface and that the intact antibody might bind bivalently across icosahedral two-fold axes. Site-directed mutagenesis results confirmed that charge interactions dominate antibody binding and electron microscopy studies on the mAb17-IA/HRV14 complex confirmed that this neutralizing antibody binds bivalently across icosahedral two-fold axes.

Key words: antibodies / neutralization / rhinovirus

Structure of human rhinovirus 14 (HRV14)

Human rhinoviruses are a major cause of the common cold, and are members of the picornavirus family.[1] These non-enveloped viruses have a characteristically small capsid (~280Å diameter) that encases a single-stranded RNA (ssRNA) genome. Other members of this family include viruses such as poliovirus, Coxsackievirus, hepatitis A virus, and foot-and-mouth disease virus. The structures of several picornaviruses have been determined to atomic resolution.[2,3] The icosahedral shells of all of these viruses are composed of four viral proteins; VP1, VP2, VP3 and VP4. The structures of the first three capsid proteins share a similar eight-stranded, anti-parrell, β-barrel motif. The major differences between the β-barrel structures appear on the external loops that connect β-strands.

Studies of picornavirus neutralization-escape mutants, isolated in the presence of individual monoclonal antibodies, demonstrated that viruses typically had three or four antigenic patches (neutralizing immunogenic sites; NIm sites) on their surfaces.[4-6] These ssRNA viruses have a high error rate when replicating their genome, with every population of virus containing a significant number of mutants capable of escaping antibody neutralization.

The antigenic sites of human rhinovirus 14 have been mapped onto its three dimensional structure (Figure 1). Thirty-five neutralizing monoclonal antibodies against HRV14 were isolated[5] and characterized by the isolation of 62 neutralization-escape viral mutants which were cross-tested against the entire panel of antibodies. The isolates, and the antibodies that failed to neutralize them, naturally segregated into four groups that formed four NIm sites located on protuberance on the viral surfaces. Most of these surround large depressions on the capsid that encircle each five-fold axis (Figure 1).[7] Residues that line the canyon were found to be more conserved among the rhinoviruses than those that occur on the rim. In addition, the narrowness of the canyon sterically hinders antibodies from binding into its deeper recesses. Hence, the floor of the canyon was postulated to be involved in viral recognition of the cell receptor protein.[7] Virus is thus able to tolerate mutations about the rim of the canyon and thwart antibody binding, while maintaining an ability to recognize host cells.[7]

Several lines of evidence support the hypothesis that the canyon is the site of receptor binding. Site-directed mutagenesis of the canyon floor identified several residues critical for cell-receptor binding.[8] Also, deformations in the canyon floor caused by antiviral drug binding were shown to abrogate cell receptor attachment.[9] Finally, a recent cryoelectron-

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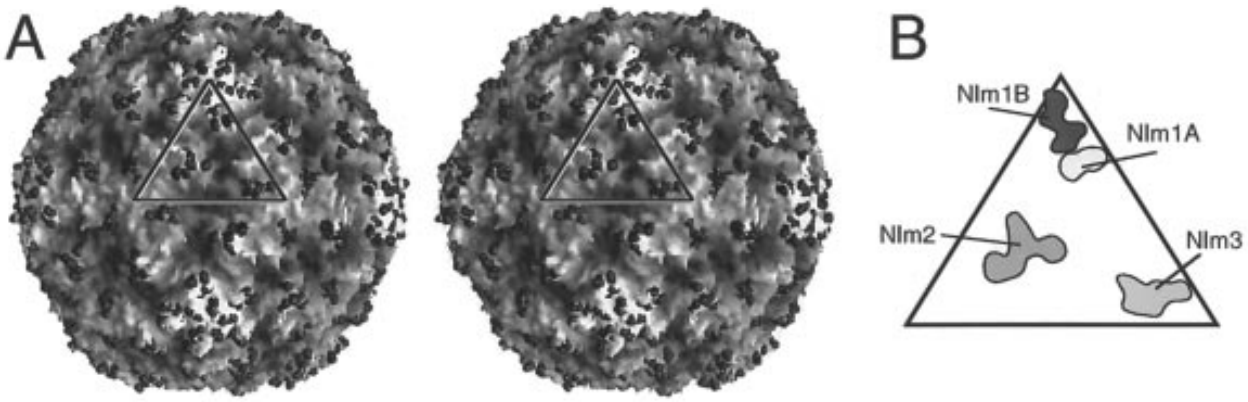

Figure 1. (A) Stereo view of HRV14 showing topography of viral surface with antibody-escape mutation sites shown in black. This figure was produced by J.-Y. Sgro using the program GRASP. (B) Schematic diagram of a single crystallographic subunit that corresponds to the triangles in Panel A, showing location of NIm sites.

microscopy study of HRV16 complexed with the two amino-terminal immunoglobulin-like domains of its receptor, ICAM-1, provided direct proof of the hypothesis.[10,11]

Proposed mechanisms of antibody-mediated neutralization of viruses

When HRV14 binds to its cellular receptor, ICAM-1 (Figure 2A), it recruits additional receptors, is taken up inside an endocytic vesicle, and the capsid undergoes a series of conformational changes that result in the transferral of the viral RNA to the cytoplasm. The details of this process are not known and may vary for different rhinoviruses. Theoretically, each step in this infectious process is vulnerable to antibody attack (Figure 2B).

Antibodies may neutralize viruses by aggregating them, by altering the capsid structure, or by interfering with some aspect of the replicative cycle. Almost all monoclonal antibodies aggregate virions to some extent. Some antibodies precipitate virions over a wide range of virus:antibody ratios while others cause very little aggregation. Aggregation reduces the number of independent infectious units and thereby provides the simplest conceptual mechanism of neutralization.

Antibody binding might also alter the conformation of the virus coat. This has long been proposed as the prime mechanism of neutralization, since early observations showed that antiserum neutralized virus with one-hit kinetics,[12] and antiserum-treated picornaviruses had a pI < 4.0, that is far below the normal pI of 7.0.[13] The popular model based upon these observations was that the binding of a single antibody molecule caused a concerted conformational change in the virus capsid that abolished infectivity and changed the surface charge. The two phenomena have been repeatedly confirmed for picornaviruses,[14,15] although the causal relationship between the pI shift and neutralization has been questioned.[16,17]

Studies have shown that Fab fragments and intact antibodies can block attachment,[14,15] prevent disappearance of virus from the cell surface,[14] and block the first stage in viral uncoating.[18] Antibodies might also trap the virus in a non-infectious form,[15] stabilize poliovirus capsids against the disrupting affects of hypotonic[19] and acidic buffers,[20] and prevent the appearance of intact viral RNA in cells.[21]

Antibody-mediated neutralization of HRV14

Although there is considerable evidence that antibodies might interrupt one or more steps in the infectious cycle, little is known about the relative importance of different mechanisms, or if recognition of different viral epitopes results in different neutralization mechanisms. To address these issues, the neutralizing[22,23] and aggregation[22] properties of thirty-two neutralizing anti-HRV14 monoclonal antibodies were further characterized.

When antibodies and virus were mixed at a variety of antibody:virion ratios, the antibodies differed in their tendency to aggregate virions.[22] Antibodies that produced large aggregates over a wide range of antibody:virus ratios were found at all NIm sites

(Table 1). Most of them were unable to reduce the surviving infectivity to less than 0.5% and were defined as weakly neutralizing antibodies. Frequently, an intermediate antibody:virus ratio gave stronger neutralization than at higher ratios. At high antibody concentrations, the virions probably became saturated with antibody and this prevented aggregation. These antibodies may neutralize virus simply by

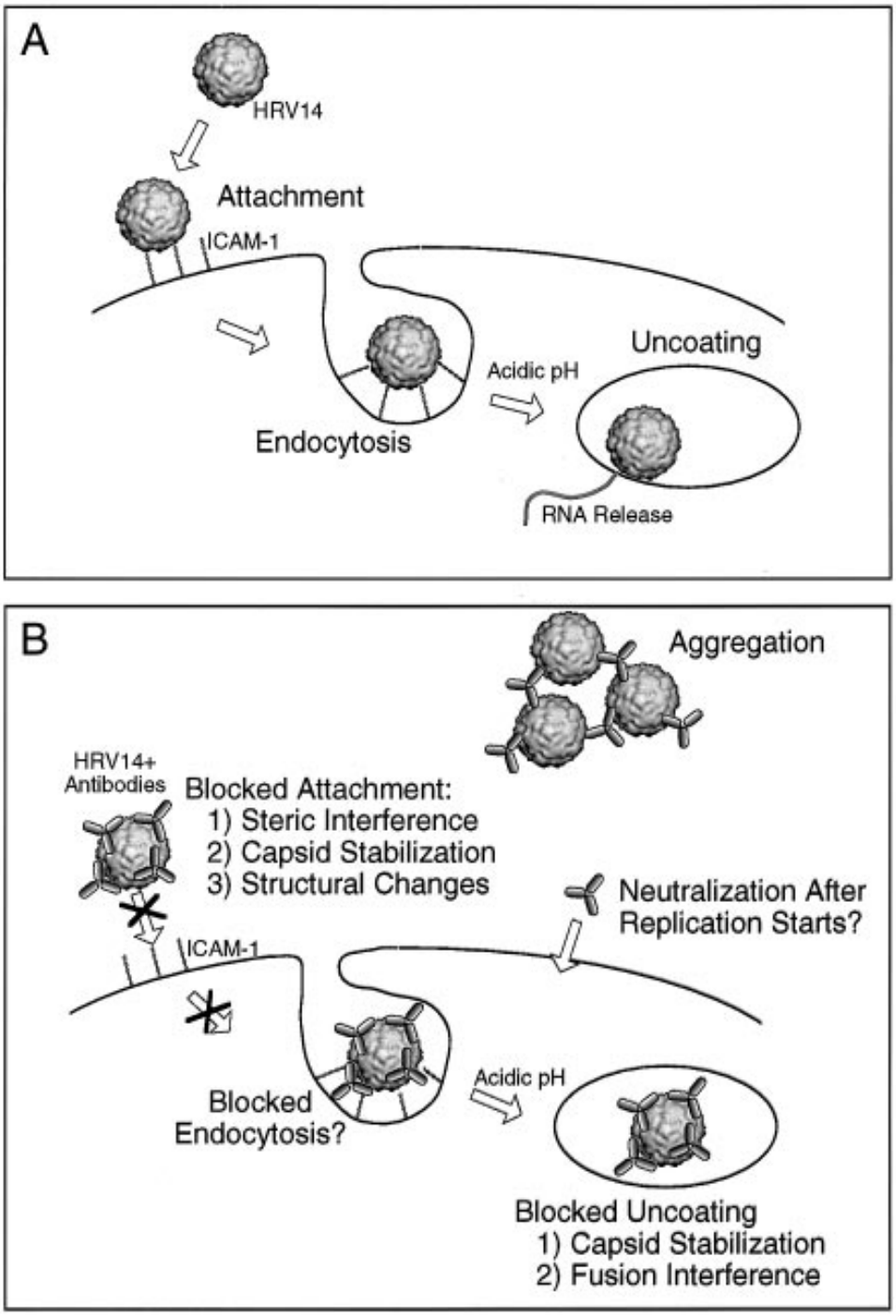

Figure 2. Schematic diagrams of HRV14 infection (A) and possible mechanisms of antibody-mediated neutralization (B).

Table 1. Charateristics of HRV14-neutralizing monoclonal antibodies

Site	Number of antibodies with virus aggregating ability: Weak	Strong
NIm1A	11 (10S, 1I)*	4 (2S, 2W)
NIm1B	0	3 (3W)
NIm2	0	4 (3W, 1I)
NIm3	3 (3I)	7 (6W, 1I)

*Numbers in parentheses give the classification by neutralization type. For instance, of the 11 weakly-aggregating NIm1A antibodies, 10 are strongly neutralizing and one neutralizes at intermediate strength.

aggregating them and thus reducing the concentration of soluble virus.

In contrast, 14 antibodies which aggregated virions poorly were only found at only two of the four NIm sites. Ten of these, all of which recognized the NIm-IA site, reduced viral infectivity to 0.01% or less of the original value and were therefore defined as strongly neutralizing antibodies.

These weakly-aggregating antibodies formed stable unaggregated immune complexes. When these antibody–virus complexes were isolated on sucrose gradients, the average number of antibodies bound per virion and the infectivity of the complexes could be determined.[22] Four NIm1A antibodies were shown to require 6 to 20 antibodies to neutralize a single virion. None were capable of neutralizing virus with a single antibody.

The number of antibodies bound to unaggregated virus-antibody complex at extremely high antibody-:virus ratios (the saturation number), for the weakly-precipitating antibodies ranged from 32 to 49.[22] This is consistent with the hypothesis that these antibodies bind with both arms to a single virion. It should be noted that the stoichiometry is consistently larger than the expected value of 30 antibodies per virion. Because the antibodies are added to the virus in such large excess, it is likely that some antibodies bind monovalently before others can bind bivalently to the virions. Strongly-precipitating antibodies, on the other hand, gave saturation numbers of 58-72, consistent with the model in which antibodies bind monovalently to each virion. These latter antibody–virus complexes were very unstable, suggesting weak binding constants.

Thus, these antibodies clearly segregated into at least two groups on the basis of their virus-aggregating and virus-neutralizing abilities. Strong neutralization appeared to be linked only to the NIm-IA site. One of these weakly-aggregating NIm1A antibodies, mAb17-IA, was chosen for further study and was shown to require 16 antibodies per virion to inhibit attachment by 90%.[20] In addition, 14 antibodies per virion were required to neutralize 90% of the input infectivity.[20] This suggests that, for this antibody, inhibition of attachment may be the primary mechanism of neutralization.

HRV14, like other rhinoviruses, is unstable at a pH below 6.0.[1] mAb17-1A and all other NIm-IA antibodies tested stabilized HRV14 at pH 5.0.[1] Four strongly-aggregating antibodies that recognized other NIm sites could not protect HRV14 against acid denatura-

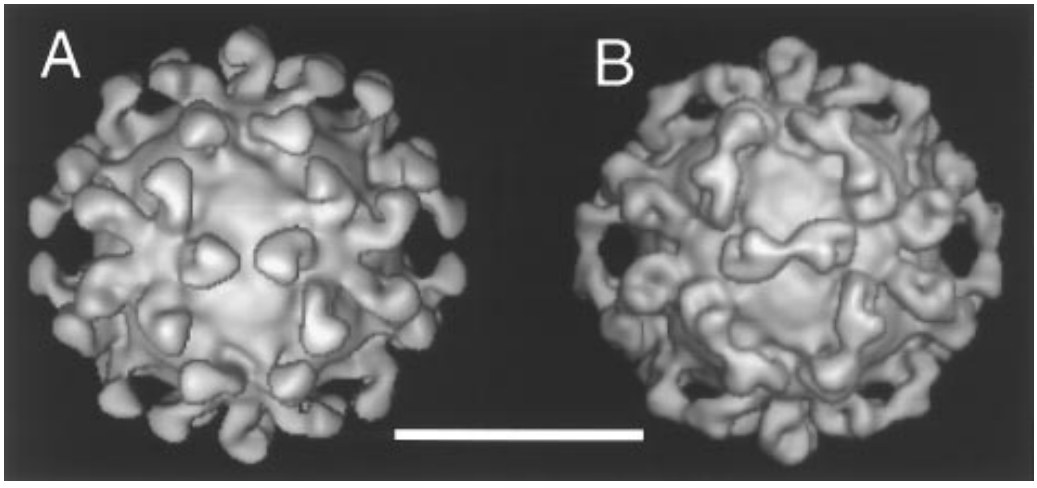

Figure 3. Cryo-electron microscopic image reconstructions of HRV14 complexed with Fab17-IA fragments (A) and the intact antibody mAb17-IA (B). White scale bar represents 200 Å.

tion. Fab fragments of mAb17-1A were not active even at a concentration of 200 Fab molecules per virion. This suggests that either bivalent binding of the antibody or high antibody affinity was necessary for pH stabilization. The latter alternative is favored by the fact that mAb1-IA, which is unlikely to bind bivalently,[22] was also able to protect the virions against low pH effects.[20]

Structural studies on antibody-mediated neutralization of HRV14

Monoclonal antibody mAb17-IA strongly neutralizes HRV14,[22] does not precipitate it, and is likely to bind bivalently to the capsid surface.[23] mAb17-IA was chosen for structural studies because it also induced a pI shift in the capsid.[15] The aim of these studies was to examine the binding contacts between antibody and virus and to see if the antibody induces conformational changes in the capsid.

Samples of HRV14 complexed with Fab17-IA fragments were purified, flash frozen, and the three-dimensional structure reconstructed from the resulting cryo-EM images. (Figure 3).[24] The image reconstruction of frozen-hydrated samples of Fab17-IA/HRV14 complexes exhibits several distinctive features. A dimple at the Fab elbow region clearly demarcates the constant and variable regions. The Fab molecules bind not radially to the virion surface, but rather at a tilted angle pointing from five-fold axes towards two-fold axes. The Fab's bind to the NIm-IA site on the rim of the canyon nearest the five-fold axes, bridge across the canyon making contact with the south rim, and meet an icosahedrally-related Fab at the nearest two-fold axis. The HRV14 capsid structure within the complex is nearly identical to the structure of the native (uncomplexed) virus. The lack of observable conformational changes suggests that, if Fab binding does cause conformational changes in the capsid, the magnitude of these changes is smaller than can be detected at the resolution of these studies.(~ 22Å). Therefore, the change in pI of the capsid upon antibody binding[13,14] is probably not due to large conformational changes in the virion.

The variable domain in each bound Fab17-IA makes extensive contact at the NIm-IA site and also contacts some of the NIm-IB site. Therefore, the position of Fab binding is as expected, but the area of contact is much larger than was implied simply by the natural mutations. One reason for this discrepancy may be that the initial studies only isolated those mutants with viability similar to the native virion rather than exhaustively examining all of the escape mutants.

The structures of both Fab17-IA[25] and HRV14[7] are known to atomic resolution and these were used to interpret the Fab/virus image reconstruction (Figure 4A). Although cryo-EM image reconstruction studies of spherical viruses do not yield atomic resolution structural details, they have provided remarkably accurate data. An atomic model of Fab17-IA can be docked quite easily and uniquely into the electron density envelope of the bound Fab protrusions (Figure 4A).[26] Translation of the Fab molecule by as little as 5Å over the virion surface or rotation of the Fab molecule by as little as 3–6° about its long axis produces significant disagreement between the observed image and the calculated model. Therefore, while it is not possible to assign exact conformations to side chains and peptide strands we can be certain of their general location.

On the basis of the model of the virus–Fab complex, the antibody-antigen interface was examined (Figure 4B).[25,27] The βB-βC loop of VP1, which contains the two residues that define the NIm-IA site (D1091 and E1095), fits well into the cleft between the heavy and light chain hypervariable regions (Figure 4B). This virus–Fab interaction was predicted in earlier modeling studies[27] because the acidic NIm-IA site is complemented by a basic cleft between the hypervariable regions of the heavy and light chains. In addition, these modeling studies suggested that the CDR2 loop of the Fab heavy chain might interact with a cluster of lysines on HRV14 (K1236, K1097 and K1085). To test this hypothesis, these residues on HRV14 were individually mutated to glutamine and glutamate.[20,26,27] As predicted, changes of all three residues had effects on antibody binding. Mutations of K1236 and K1085 decreased antibody binding by 2.4 and 3.9 fold, respectively, whereas the K1097 mutant was nearly equivalent to a natural escape mutant (36-fold decrease in affinity). When the atomic models of HRV14 and Fab17-IA were placed into the cryo-EM density map, residues D555 and D557 of the heavy chain CDR2 loop were juxtapositioned directly over K1097 (Figure 4B). K1085 and K1236, which seemed to be less important for antibody binding, were somewhat distal to this CDR2 loop. Their role in antibody binding may be to establish a positively charged potential in this region or to set up a water-lysine network to maintain the K1097 side chain position. From the extensive charge complementarity

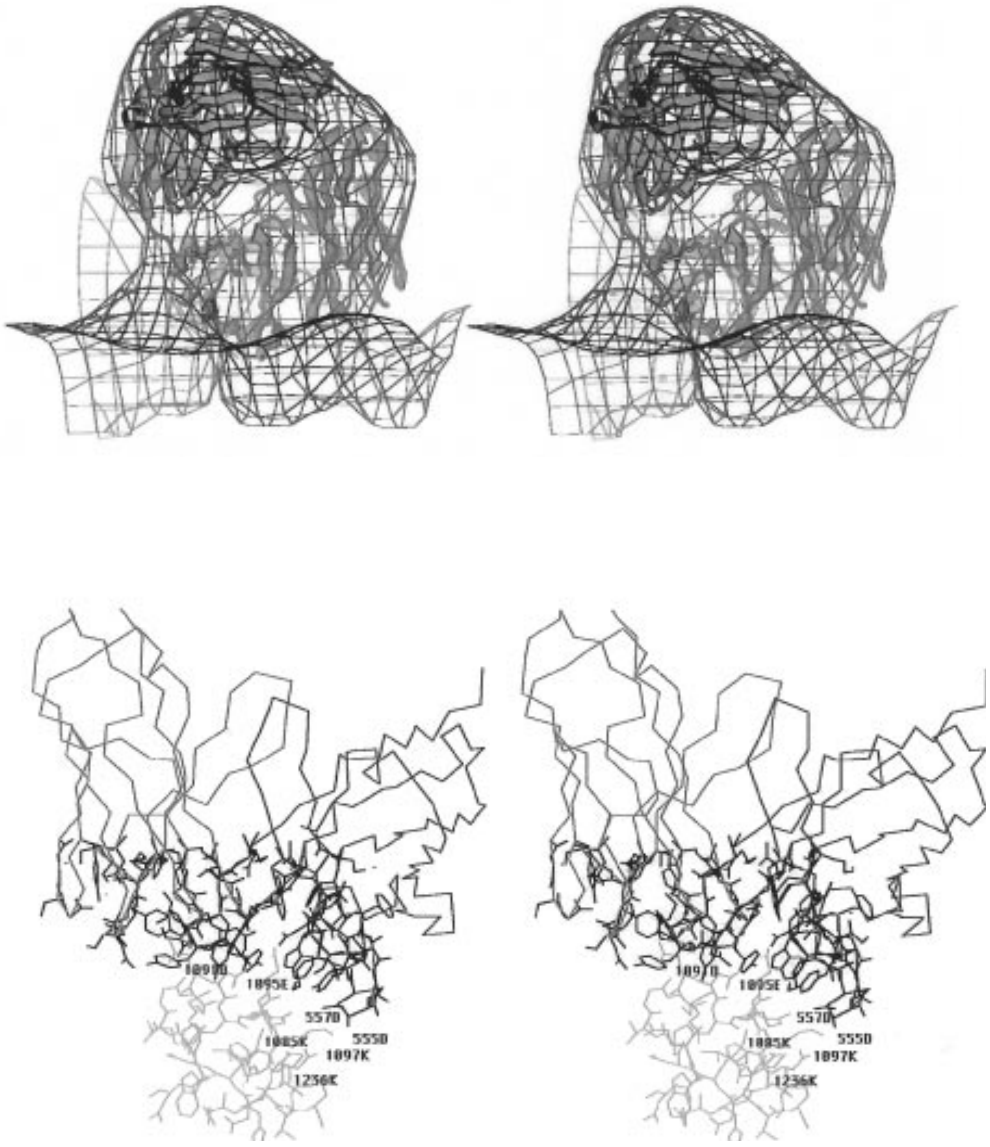

Figure 4. (A) Stereo pair of a ribbon diagram of the Fab17-IA structure fit into the envelope of the cryo-EM structure. (B) Details of the interactions between the bound Fab17-IA and HRV14. The variable domains of the heavy and light chains are represented by C-α traces, the HRV14 residues in contact with Fab17-IA are light grey, and the Fab17-IA contact residues are black. Key residues of HRV14 (1085K, 1091D, 1095E, 1097K and 1236K) and Fab17-IA (555D and 557D) are labeled.

of the two surfaces, it is clear that charge is indeed an important criteria for antigen recognition.

Bound Fab17-IA molecules nearly touched at icosahedral two-fold axes, suggesting that mAb17-IA might indeed bind bivalently to HRV14. A bivalently bound antibody could be easily modeled by simply rotating the constant domains of the bound Fab molecules towards the virion surface and leaving the variable domains unchanged.[27] The resulting interactions between the two-fold related, Fab constant domains were nearly identical to those observed in the structure of the intact antibody Kol.[28,29] The similarity between the constant domain interactions between the modeled mAb17-IA and the known Kol structures, suggested that the elbow region might 'flex' to accommodate bivalent binding to the antibody to the surface of the virion.

The binding of Fab17-IA did not cause large conformational changes in the HRV14 capsid, but its position on the capsid occludes the cell receptor binding site. The cryo-EM structure of the cell receptor (ICAM)/HRV16 complex has been deter-

mined.[11] The ICAM site lies immediately 'south' (towards the nearest icosahedral two-fold axis) of the Fab17-IA binding site and inside the canyon. The binding positions of the ICAM-1 and Fab17-IA molecules overlap to such an extent that it is impossible for both to bind simultaneously. This hypothesis was substantiated by studies showing abrogation of HRV14 binding to host cells by mAb17-IA.[27] Indeed, the correlation between the stoichiometries of mAb17-IA binding and neutralization strongly suggest that this antibody neutralizes by sterically blocking cell receptor attachment. Structural studies on other antibodies that recognize the same and different antigenic sites are necessary to ascertain whether these results are typical of all neutralizing antibodies.

The bivalent model was tested by performing image reconstruction studies on the IgG-virus complex (Figure 3B).[26] The Fab arms of mAb17-IA bound in a similar orientation as the Fab fragments. However, a strong connection at the icosahedral two-fold axes joined the two arms together. The Fc portion of the antibody was not visible in the image reconstruction even though SDS-PAGE analysis showed it to be present. Weak density was observed directly above the 'connected' Fab arms at the icosahedral two-fold axes. This suggests that the Fc region is quite flexible and therefore the corresponding density gets 'smeared' out in the reconstruction as a result of the averaging process involved in computing the density map. This concurs with previous crystallographic and electron microscopy studies that demonstrated the extremely dynamic nature of the hinge region.[29-31] As had been observed in the Fab-virus complexes, conformational changes were also not observed in the capsid upon bivalent binding of the antibody. Therefore, pI changes may result from alterations in surface accessibility upon antibody binding, or may require the antibody to be bound in the presence of the unusual conditions needed to measure pI's (e.g. low ionic strength and unusual buffers).

The conformation of the Fab17-IA structure, when bound to the virion surface in the Fab/virus complex, differs from that found in the mAb17-IA-virus complex. The variable domains of the Fab's in both complexes bind in nearly identical positions and orientations, but the constant domains differ by ~16-18° rotations about the elbow axis. In the mAb-virus complex, the light chain of the constant domain fills most of the density that 'connects' the two Fab arms. The cryo-EM studies provide direct evidence that the elbow region actively participates in immunoglobulin binding. Such motion is likely to occur not only when the antibodies bind to symmetrical viruses, but also when they bind to asymmetrical cell or bacterial surfaces. By allowing the antibody to bind bivalently, the elbow region theoretically helps increase the affinity (avidity) of the antibody for antigen by as much as a factor of 10.[3]

Future crystallographic[32] and mutational studies should be able to discern the importance of these findings. Currently, crystals of the Fab17-IA/HRV14 complex diffract to ~4-5Å resolution. At this resolution, potential main chain deviations in the two structures should be clearly visible. In addition, electron microscopy studies are underway to examine other antibodies that bind to the same and different antigenic sites to ascertain whether mAb17-IA is unusual in its *in*ability to produce large conformational changes in the capsid.

Conclusions

mAb17-IA blocks attachment and stabilizes the HRV14 capsid against acidic denaturation. Neither Fab or mAb binding causes discernable gross conformational changes in the capsid and antibody binding correlates strongly with abrogation of cell attachment. Therefore, the simplest mechanism for mAb17-IA neutralization is the blocking of cell attachment. The results described here do not rule out the possibility that neutralization is due to relatively small conformational changes in the capsid or stabilization of the capsid upon antibody binding. However, there is currently no evidence to require these effects to explain the results. The crystallographic structure of the Fab17-IA/HRV14 complex should address these remaining issues.

The experiments reviewed here suggest a few of the most likely *in-vitro* mechanisms of neutralization but do not address the most relevant *in-vivo* mechanisms. If a crucial element of antibody-mediated neutralization is antibody-mediated stabilization or destabilization of the capsid, then distal, compensatory mutations might be expected to arise when virus is grown in the presence of antibodies. Such mutations have been isolated only at the presence of capsid-stabilizing WIN compounds,[33] but not with antibodies. The WIN mutations were isolated in the presence of intermediate concentrations of WIN whereas antibody escape mutants have been mostly isolated at only high antibody concentrations. If distal, compensatory mutations can be isolated, perhaps in the presence of intermediate concentrations of antibody, then the

importance of putative capsid effects would be established. Also, if antibody stabilization or destabilization of the capsid is a major component of neutralization, then we might expect that more viruses would evolve large, flexible, immuno-dominant protuberances such as those found on foot and mouth disease virus[34] that would enable them to evade neutralization by isolating and limiting the effects of antibody binding. Perhaps, because of the remarkable interplay between immune system components, (Figure 5) the most biologically relevant antibody-mediated processes *in vivo* are those pertaining to only antibody binding

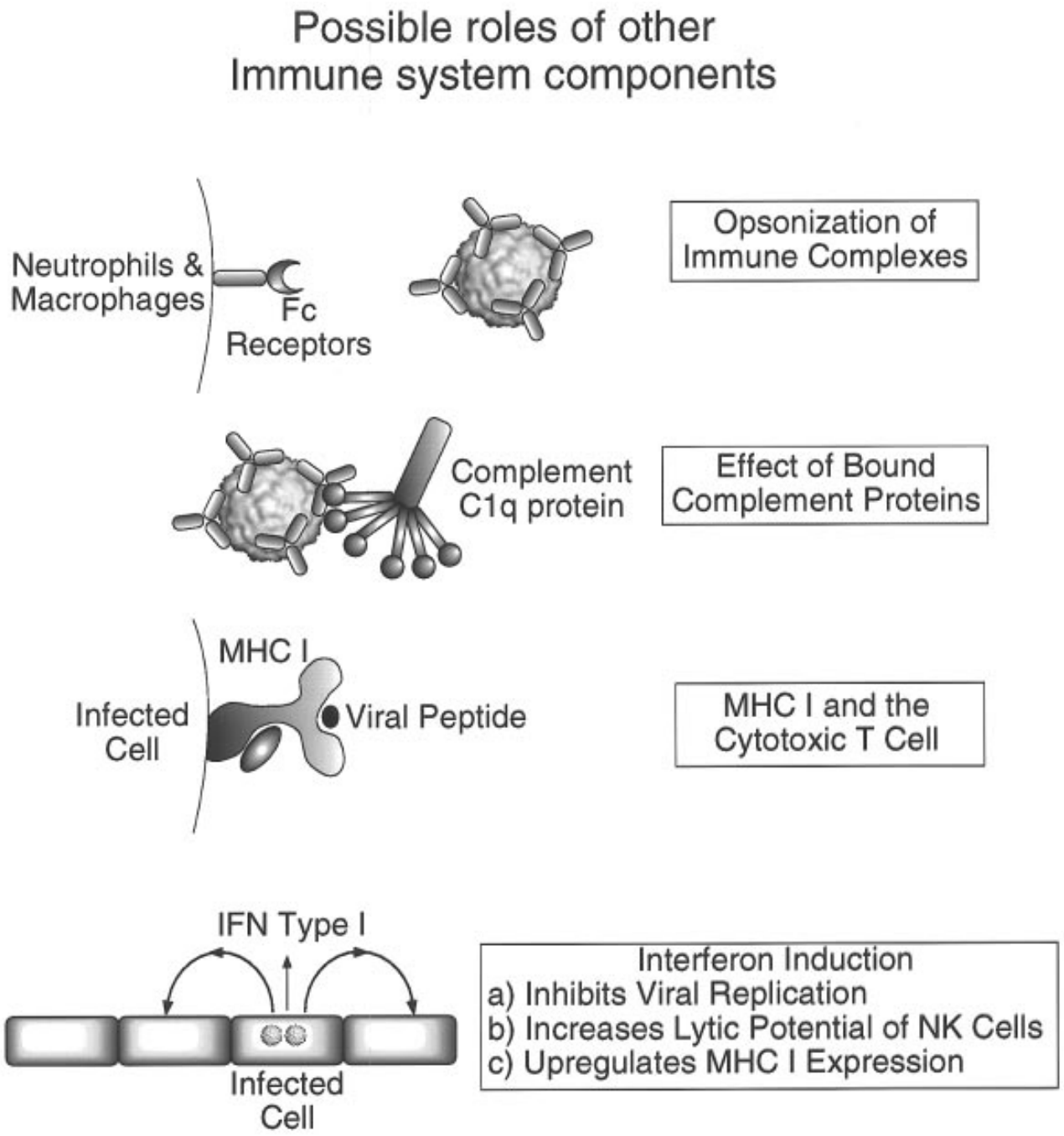

Figure 5. Other aspects of the immune system involved in the anti-viral response

such as inhibition of cell attachment, aggregation and opsonization.

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