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Efficient Neutralization and Disruption of Rhinovirus by Chimeric ICAM-1/Immunoglobulin Molecules

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The intercellular adhesion molecule 1 (ICAM-1) is used as a cellular receptor by 90% of human rhinoviruses (HRVs). Chimeric immunoadhesin molecules containing extracellular domains of ICAM-1 and constant regions of immunoglobulins (Igs) were designed in order to determine the effect of increased valency, Ig isotype, and number of ICAM-1 domains on neutralization and disruption of rhinovirus structure. These immunoadhesins include ICAM-1 amino-terminal domains 1 and 2 fused to the hinge and constant domains of the heavy chains of IgA1, IgM, and IgG1 (IC1-2D/IgA, -/IgM, and -/IgG). In addition, all five extracellular domains were fused to IgA1 (IC1-5D/IgA). Immunoadhesins were compared with soluble forms of ICAM-1 containing five and two domains (sICAM-1 and ICI-2D, respectively) in assays of HRV binding, infectivity, and conformation. In prevention of HRV plaque formation, IC1-5D/IgA was 200 times and IC1-2D/IgM and IC1-2D/IgA were 25 and 10 times more effective, respectively, than ICAM-1. The same chimeras were highly effective in inhibiting binding of rhinovirus to cells and disrupting the conformation of the virus capsid, as demonstrated by generation of ~65S particles. The results show that the number of ICAM-1 domains and a flexible Ig hinge are important factors contributing to the efficacy of neutralization. The higher efficiency of chimeras that bound bivalently in disrupting HRV was attributed to higher binding avidity. The IC1-5D/IgA immunoadhesin was effective at nanomolar concentrations, making it feasible therapy for rhinovirus infection.

Human rhinoviruses (HRV) cause 40 to 50% of all cases of common colds (34). These small, nonenveloped RNA viruses represent a subgroup of picornavirus (29). X-ray crystallography of rhinovirus identified a capsid 300 Å in diameter (1 Å = 0.1 nm) with icosahedral symmetry and constructed from 60 copies each of the viral coat proteins VP1, VP2, and VP3 (26). A surface depression or "canyon" on HRV was suggested as the receptor binding site (8, 26). The receptor for the major group of rhinoviruses was defined with a monoclonal antibody (MAb) (7) and subsequently shown to be ICAM-1 (18, 36); the binding site is located within N-terminal domains 1 and 2 (19, 35). A recombinant, soluble ICAM-1 (sICAM-1) has been shown to be effective in prevention of rhinovirus infection in vitro (19, 22).

Little is known about the mechanism by which picornavirus receptors induce the conformational change in the capsid that results in increased hydrophobicity of the capsid and release of viral RNA (16), except that it can be induced by soluble receptors (19, 20). This process is presumably closely related to the mechanism by which viral RNA gains entry into the cytoplasm and initiates infection.

We have constructed multivalent, chimeric ICAM-1/immunoglobulin (Ig) molecules (immunoadhesins). On cells, viral receptors are multimeric by virtue of their linkage to the cell surface; we wondered whether multivalent binding by multimeric soluble receptors might facilitate uncoating. Furthermore, multivalent binding might result in a higher effective affinity, termed avidity. Hence, immunoadhesins might be more effective than monovalent sICAM-1 in neutralizing HRV and thus would have increased therapeutic utility. We have compared immunoadhesins having heavy chains of IgM, IgA, and IgG because these classes differ in valence and because IgM and IgA, but not IgG, are secreted in the nasal mucosa, the site where infection with HRV occurs. With one heavy chain class, IgA, we examined the difference between constructs containing two and five Ig-like domains of ICAM-1. We find that valency, heavy chain class, and number of ICAM-1 Ig-like domains are all important factors for neutralization and conformational disruption of HRV.

MATERIALS AND METHODS

cDNA constructions. A human IgA1 cDNA was isolated from a λgt11 tonsil library by using a 22-mer (5′-CAAG GTCTTCCCGCTGAGCCTC) specific for the IgA1 subclass. It was subcloned in pCDM8 (2) to produce pCD/IgA5.3 and was verified by DNA sequencing to correspond to the IgA1 genomic sequence (15). A restriction site for the fusion of IgA to ICAM-1 was introduced by the polymerase chain reaction (31) with a 5′ primer (5′-ACTGTGCCCTGCCCG GTACCCTCAACTCCACCT-3′) containing a *Kpn*I site and the beginning of the IgA1 hinge region and a 3′ oligonucleotide (5′-GAATACAGCGGTACCTACACAAC-3′) including a *Kpn*I site in the vector sequence. A *Kpn*I site was incorporated into ICAM-1 between D2 and D3 in codon L-187 by mutagenesis directed by the oligonucleotide 5′-CCAGACCTTTGTGGTACCAGCGACTCCCC-3′ (35). The *Kpn*I fragment containing D3, D4, and D5 was removed and replaced with the IgA1 polymerase chain reaction product. The orientation and the complete coding sequence of pCDIC1-2D/IgA, encoding the immunoadhesin IC1-2D/IgA, were verified by sequencing.

For the construction of a plasmid (pCDIC1-5D/IgA) encoding a five-domain ICAM-1–IgA1 chimera (IC1-5D/IgA), the *Kpn*I site in the pCDM8 vector portion of pCDIC1-2D/IgA was eliminated by partial digestion, filling in recessed ends with T4 DNA polymerase I, and blunt-end ligation. The

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two-domain ICAM-1 fragment was then removed by *Kpn*I and *Hin*dIII restriction cuts and replaced by an ICAM-1 fragment containing all five extracellular Ig-like domains that was amplified with the primers 5′-CTTCTAGAGATCCCTC GACC-3′ and 5′-TTTGGTACCAGCACATTCACGGTCA CCTCGCGG-3′.

The IgM-ICAM-1 chimera (IC1-2D/IgM) was generated by replacing CD4 in the pCD4-Hu vector (39) with an ICAM-1 polymerase chain reaction product by using a pCDM8 5′ primer (5′-CTTCTAGAGATCCCTCGACC-3′) and a 3′ primer (5′-AAGTCGACAAGCTTGCGGCCGCTCTCACC AAAGGTCTGGAGCTGGTAGGGGGC-3′) that encodes 20 bases of ICAM-1, including the codon for F-185 at the end of domain 2, 8 additional bases to encode a 5′ donor splice site, and a multiple cloning site with *Not*I, *Hin*dIII, and *Sal*I restriction sites. The coding region of the resulting plasmid, pHμIIC1-2D/IgM, was again verified by sequencing.

The IgG ICAM-1 chimera (IC1-2D/IgG) was made with the human IgG1 hinge, C_H2 and C_H3 domains as described previously (37). sICAM-1, containing ICAM-1 domains 1 through 5 (22), and IC1-2D, containing ICAM-1 domains 1 and 2 (23), have been described previously.

Transfection. The expression of IC1-2D/IgA and IC1-5D/IgA constructs was initially verified by transfection in COS cells with DEAE-dextran (2). Subsequently, stable producing cell lines were obtained by transfection of CHO DG44 cells (40). CHO cells were grown in alpha minimal essential medium (MEM) supplemented with 0.1 mM hypoxanthine, 0.016 mM thymidine, and 10% fetal calf serum (FCS) and 50 μg of gentamicin per ml in 5% CO_2–air at 37°C. CHO cells (5×10^6) in 800 μl of HeBS (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.05], 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose) were electroporated (6) at 960 μF and 280 V with 20 μg of CDM8-IC1-2D/IgA or -5D/IgA and 5 μg of pDHIP plasmid, which contains the dihydrofolate reductase minigene (24). Cells were grown in 15-cm plastic culture dishes in supplemented alpha MEM medium for 48 h, and then were grown in medium with 5% dialyzed FCS and lacking thymidine. After the appearance of macroscopic colonies, selection was started with 0.005 μM methotrexate (21). Every week, the methotrexate concentration was increased until 2 μM was reached. Cell clones were picked with cloning cylinders and subcultured in a 24-well plate. Supernatants were tested by a capture enzyme-linked immunosorbent assay (ELISA) (27). The expressing clones were maintained in 0.5 μM methotrexate. Chimeric molecules were expressed between 0.5 and 2 μg/ml.

The IC1-2D/IgM construct (30 μg) was linearized by *Pvu*I and transfected into 5×10^6 J558L myeloma cells by electroporation as described above. Cells were grown in Dulbecco's MEM supplemented with 10% FCS–50 μg of gentamicin per ml and selected in a 96-well plate with 4 μM mycophenolic acid and 1.5 μM xanthine. IC1-2D/IgA plasmid linearized with *Sfi*I was cotransfected with the selectable marker pSV2gpt linearized with *Pvu*I (25) in J558L cells in the same way. Cells producing supernatants that were positive in the ELISA were cloned by limiting dilution and retested. Positive cell lines were maintained in 1 μM mycophenolic acid.

All cell lines were grown in roller bottles. Two-liter plastic bottles (Corning) were filled with 1 liter of cell culture (~5×10^5 cells per ml) and rolled at 37°C (1 rpm) until the pH became acidic.

Although the cell types in which chimeras were produced varied, previous comparisons between sICAM-1 and IC1-2D produced in CHO cells and insect cells, which differ significantly in glycosylation, showed no difference in antiviral activity (4a, 23).

Protein purification. The immunoadhesins were purified by immunoaffinity chromatography on ICAM-1 MAb R6.5-Sepharose. Chimeric molecules were eluted by high pH (22), and concentrations were calculated by measuring optical density at 280 nm. Adsorptions of 0.80 for sICAM-1, 0.81 for IC1-2D, 1.07 for IC1-2D/IgA, 0.97 for IC1-5D/IgA, 0.95 for IC1-2D/IgM, and 1.01 for IC1-2D/IgG were calculated for a concentration of 1.0 mg/ml by using the peptide structure program of the University of Wisconsin Genetics Computing Group (10). Molar concentrations are given for the monomer, in the case of multimeric molecules.

IC1-2D/IgM was expressed as dimeric and approximately decameric molecules. Separation of these forms was performed by sedimentation over a 5 to 15% sucrose gradient (40,000 rpm, 15 h). Fractions were tested in the capture ELISA, and two distinct immunoreactive peaks were obtained. Sucrose was removed by volume reduction by centrifugation in a Centricon 30 and dilution in 1% bovine serum albumin–phosphate-buffered saline (BSA-PBS). The concentrations of the dimeric and approximately decameric IgM were determined by capture ELISA with the unfractionated IgM preparation used as a standard.

Cell binding assay. Immunoadhesins were immobilized on plastic, and cell binding assays were performed as described earlier (11). Briefly, in order to obtain different amounts of binding sites per square micrometer, 25 μl of chimeric molecules with concentrations of 0.01 to 1.0 μM was spotted on 60-mm plastic culture dishes and incubated for 1 h at 37°C. The density of binding sites per square micrometer was determined by a saturation assay with the ^{125}I-R6.5 MAb as described in detail previously (13). Free binding sites were blocked with 1% BSA, and plates were incubated with 4×10^6 JY cells in cell binding buffer (PBS containing 1% BSA, 0.2% glucose, and 0.2 mM $MgCl_2$) for 30 min. After dishes were washed three times with cell binding buffer and two times with PBS, bound cells were counted under the microscope.

Rhinovirus binding assay. HRV3 was purified and labeled with [^{3}H]leucine to a specific activity of 10^4 to 10^5 cpm/μg of virus as described by others (30). Binding of viruses to HeLa cells (1) was examined after preincubation for 30 min at 24°C in PBS–5% FCS with different concentrations of recombinant ICAM-1/Ig chimeras. HeLa cells were seeded into wells of a 96-well plastic culture plate (1×10^5 cells per well) for 24 h at 37°C in 5% CO_2. ICAM-1-treated HRV3 (10^4 cpm in 25 μl) was added in triplicate to wells, and 1 h later wells were aspirated and washed twice with 50 μl of PBS–5% FCS. Cells and bound virus were detached with aliquots of 30 and 20 μl of 1% Triton X-100 and transferred to a scintillation vial for direct measurement of ^{3}H radioactivity. Binding of untreated HRV3 was 20% of input virus.

Plaque-forming assay. HeLa cells (3×10^5 cells) were seeded in 60-mm tissue culture dishes in 5 ml of Eagle's MEM with Earle's salts containing 10% FCS and nonessential amino acids at 37°C in 5% CO_2. After 24 h, the medium was removed and the cell monolayer was washed with PBS. After preincubation of 200 PFU of HRV3 with different ICAM-1 proteins in 200 μl of PBS containing 5% FCS at 24°C for 0 to 30 min as indicated, virus was allowed to attach to cells for 30 min at 24°C in triplicate. Untreated virus was included as a positive control in every experiment. An agarose overlay and medium were applied as previously described (32). After 48 h of incubation at 35°C in 5% CO_2,

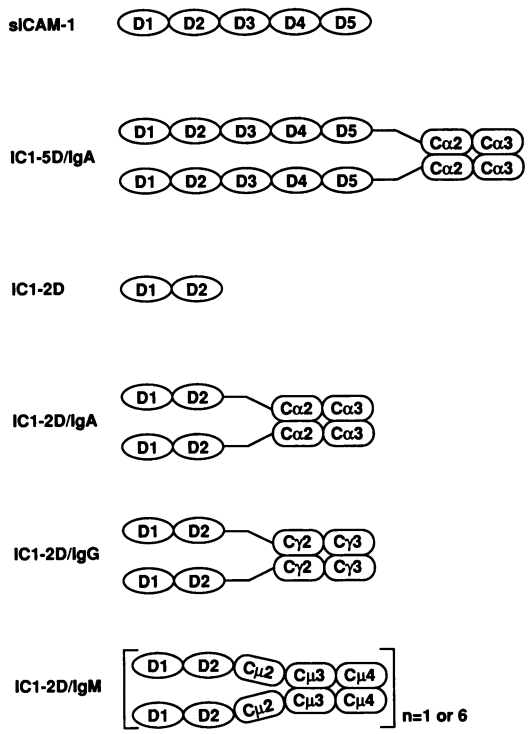

FIG. 1. ICAM-1/Ig chimeras, constructed by fusing extracellular domains of ICAM-1 (D1 plus D2, D1 through D5) to the hinge (⁀) and constant heavy chain (C_H) domains of IgA1 and IgM as shown. The IC1-2D/IgG1 chimera has previously been described (37).

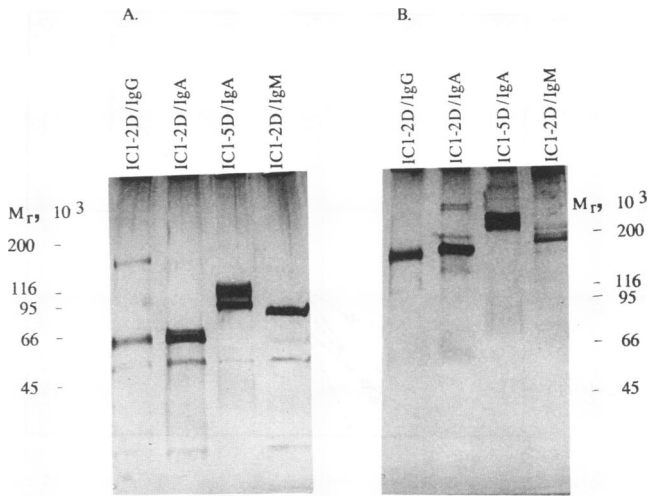

FIG. 2. SDS-PAGE of ICAM-1 immunoadhesins. The IC1-2D/IgA and IC1-2D/IgM immunoadhesins produced by myeloma J558L cells, the IC1-5D/IgA immunoadhesin produced by CHO cells, and the IC1-2D/IgG chimera produced by COS cells were immunoprecipitated from culture supernatants with ICAM-1 MAb R6.5-Sepharose CL-4B, eluted under nonreducing conditions, and subjected to reducing (A) or nonreducing (B) SDS-PAGE (4 to 15% polyacrylamide) and silver staining as described previously (12). The positions of relative molecular weight markers are shown.

cells were fixed with 4% formaldehyde, the overlay was removed, and cells were stained with 0.1% crystal violet. All plaques were counted, and the percentage of inhibition was calculated.

Assays of virus conformation. For sedimentation studies, HRV14 labeled with [^{3}H]leucine was preincubated with or without immunoadhesin or sICAM-1 preparations diluted in PBS containing 5% FCS for 0.5 h at 37°C. The sample (50 μl) was layered over a 5-ml 5 to 30% sucrose gradient in PBS with 0.1 mg of BSA per ml and was centrifuged for 1.5 h at 40,000 rpm at 4°C in a Beckman SW55 rotor. The gradient was collected from the bottom by inserting a microcapillary. Fractions were subjected to scintillation counting. For protease sensitivity, HRV14 labeled with [^{3}H]leucine was preincubated with or without immunoadhesin or ICAM-1 preparations for 15 min at 37°C in a total volume of 10 μl of RPMI 1640 containing 5% FCS. SV8 protease (1 U; Sigma) was added and incubated for 30 min at 37°C. After boiling with sodium dodecyl sulfate (SDS)-loading buffer containing 5% mercaptoethanol, samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (12% polyacrylamide) and fluorography.

RESULTS

Expression of immunoadhesins. ICAM-1/IgA1 chimeras were constructed by fusing ICAM-1 extracellular domains D1 and D2 or D1 through D5 to the hinge and C_H2 and C_H3 constant domains of IgA (Fig. 1). An ICAM-1/IgM chimera was made by fusing domains D1 and D2 of ICAM-1 to the C_H2, C_H3, and C_H4 domains of IgM. IC1-2D/IgA and IC1-5D/IgA were stably expressed in J558L myeloma cells or CHO cells, and IC1-2D/IgM was expressed in J558L myeloma cells. An IC1-2D/IgG1 chimera has previously been described (37). Immunoprecipitation with ICAM-1 MAb R6.5 followed by SDS-PAGE (Fig. 2) shows that IC1-2D/IgA is 65 kDa (reduced) and 130 kDa (nonreduced) and IC1-5D/IgA is 100 kDa (reduced) and 200 kDa (nonreduced). The two bands of IC1-5D/IgA resolved by both reducing and nonreducing SDS-PAGE may represent isoforms that differ in carbohydrate processing. IC1-2D/IgA expressed in J558L cells is similar in size in nonreducing SDS-PAGE to that expressed in CHO cells, suggesting that J chain expressed in J558L did not disulfide-link two or three dimers (data not shown). IC1-2D/IgM runs under reducing conditions as 80 kDa and runs under nonreducing conditions as a 160-kDa dimeric molecule and as a very-high-molecular-weight molecule at the top of the gel. The size of the latter band is consistent with five dimeric molecules linked by a J chain or six dimeric molecules as found for native IgM (9). All molecular weights in reducing SDS-PAGE were in the range expected for the fusion proteins.

For preparation of large quantities of chimeras, stably transfected cells were grown in roller bottles, and chimeric molecules were purified from culture media by immunoaffinity chromatography with ICAM-1 MAb R6.5 bound to Sepharose CL-4B. Quantities of 2 to 4 mg were purified and used in the assays described below. To allow comparisons between molecules of different valencies, concentrations given below are of the monomer units, i.e., of the ICAM-1 moiety.

LFA-1-dependent binding of lymphoblastoid cells to chimeras. ICAM-1/Ig chimeric molecules were immobilized on plastic, and binding of the LFA-1$^+$ B-lymphoblastoid cell line JY was examined (Fig. 3). JY cells bound similarly to a range of densities of ICAM-1 chimeric molecules and sICAM-1. Preincubation of cells with MAb against the LFA-1 α subunit (TS1/22) completely abrogated binding (not shown).

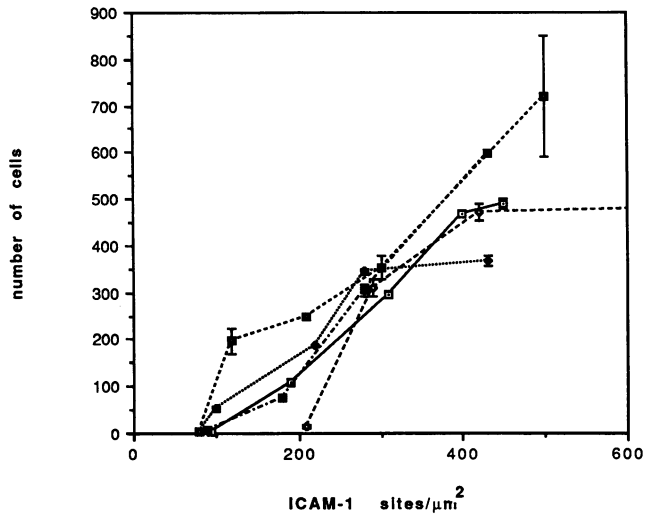

FIG. 3. Binding of LFA-1$^+$ cells (JY) to immobilized sICAM-1 (⊡) and ICAM-1 immunoadhesins (◆, IC1-2D/IgA; □, IC1-5D/IgA; ◇, IC1-2D/IgM; ■, IC1-2D/IgG). Plastic culture dishes coated with different concentrations of ICAM-1 proteins were incubated with JY cells and washed, and bound cells per square millimeter were counted under the microscope. ICAM-1 density was measured with the ^{125}I-R6.5 MAb.

Competitive inhibition of rhinovirus binding. ICAM-1/Ig immunoadhesins were examined for ability to inhibit binding of ^{3}H-labeled HRV3 to HeLa cells (Fig. 4). HRV3 binding was inhibited 50% with 6 nM IC1-5D/IgA, which was eight-fold more effective than sICAM-1. IC1-2D/IgM and IC1-2D/IgA were similar in activity to sICAM-1, whereas IC1-2D/IgG was 11-fold less effective than sICAM-1. ICAM-1 truncated after the second domain (IC1-2D) was less active than the five-domain truncated molecule (sICAM-1) as previously reported (19). The IC1-2D/IgG chimera was more effective than IC1-2D in inhibiting rhinovirus binding, whereas it was similar in efficacy to IC1-2D in other assays (see below).

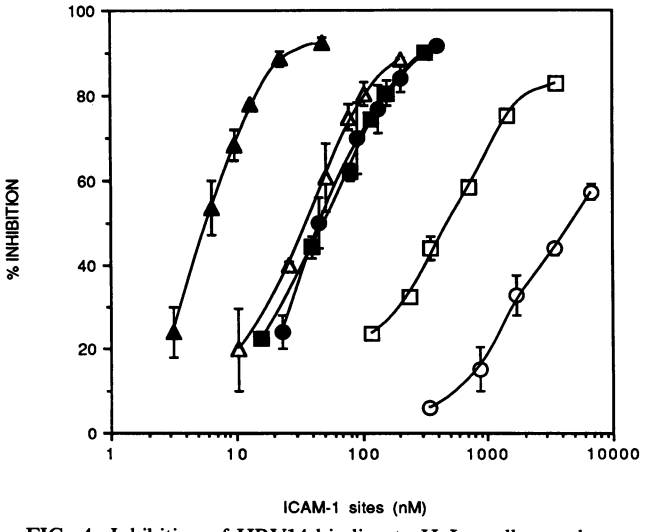

FIG. 4. Inhibition of HRV14 binding to HeLa cell monolayers. [^{3}H]HRV was mixed with sICAM-1 (●) or immunoadhesins (▲, IC1-5D/IgA; △, IC1-2D/IgA; ■, IC1-2D/IgM; □, IC1-2D/IgG; ○, IC1-2D) and added to HeLa cell monolayers. After 1 h of incubation, cells were washed with PBS–5% FCS and detached with 1% Triton X-100. The amount of bound HRV was measured by scintillation counting, and the percentage of inhibition was calculated. Error bars represent the standard deviations of two to four experiments.

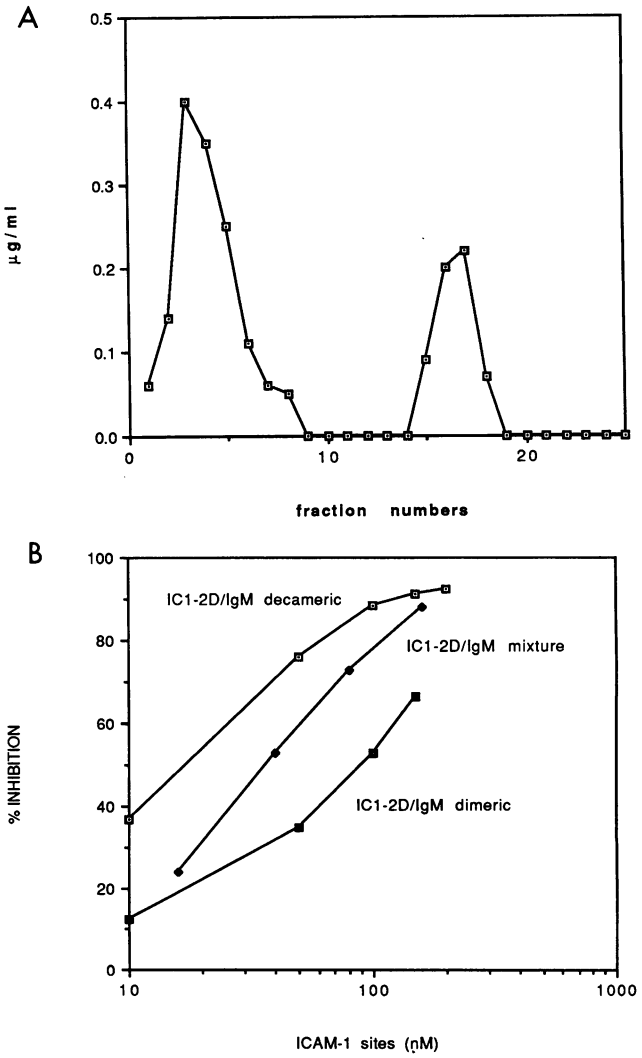

FIG. 5. Distinct species of IC1-2D/IgM. (A) Species of IC1-2D/IgM (approximately decameric and dimeric) separated by 5 to 20% sucrose gradient sedimentation. The bottom of the gradient is to the left. The concentration of the IC1-2D/IgM chimera was measured with a capture ELISA. (B) Inhibition of [^{3}H]HRV binding to HeLa cells, performed as in Fig. 4.

The IC1-2D/IgM preparation was subjected to sucrose gradient sedimentation because nonreducing SDS-PAGE (Fig. 2B) had suggested the presence of two species of IC1-2D/IgM differing in disulfide linkage, one migrating at 160 kDa and another at much higher molecular mass. Two peaks of IC1-2D/IgM were resolved on sedimentation, consistent with dimeric (~7S) and deca- or dodecameric (~19S) forms (Fig. 5A). Under nonreducing SDS-PAGE conditions, the ~19S fraction barely entered the gel whereas the ~7S form ran at 160 kDa (not shown). The approximately decameric chimera was five times more efficient than the dimeric chimera in inhibiting HRV3 binding to HeLa cells,

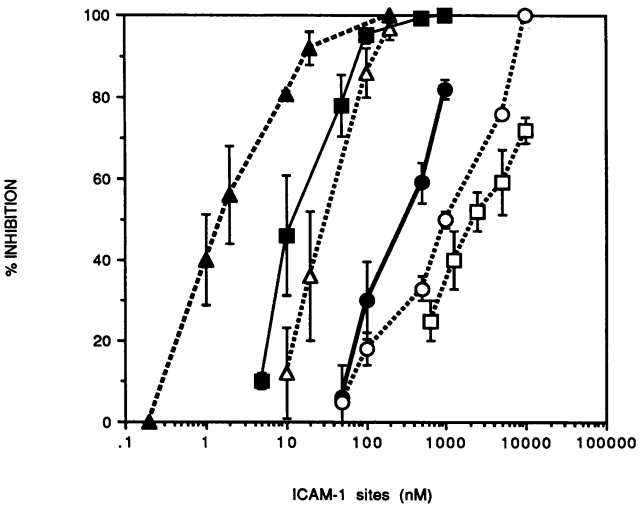

FIG. 6. Inhibition of HRV infectivity. HRV3 (200 PFU) was preincubated with various concentrations of sICAM-1 (●) or immunoadhesins (▲, IC1-5D/IgA; △, IC1-2D/IgA; ■, IC1-2D/IgM; □, IC1-2D/IgG; ○, IC1-2D), and then incubated on a HeLa cell monolayer for 30 min, followed by an agarose overlay. After 48 h, virus titer was determined by counting plaques after staining of the cell monolayer with 0.1% crystal violet. The percentage of inhibition was calculated relative to virus treated with buffer alone. The means ± standard deviations of two to four experiments are shown.

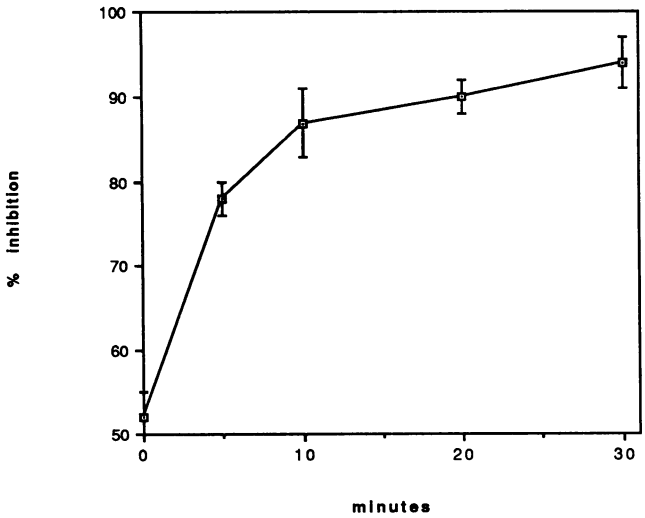

FIG. 7. Time course of HRV14 neutralization with IC1-2D/IgA. HRV (200 PFU) was preincubated with 200 nM IC1-2D/IgA for the indicated time and then was incubated on a HeLa cell monolayer for 30 min and virus titer was determined as described in Fig. 6. The means ± standard deviations of two experiments are shown.

and unfractionated IC1-2D/IgM was intermediate in inhibition (Fig. 5B).

Inhibition of plaque formation. The efficiency of different ICAM-1 immunoadhesins in inhibition of HRV3 infection of HeLa cells was determined in a plaque reduction assay (Fig. 6). To reduce infectivity of HRV3 by 50%, 300 nM sICAM-1 was required. ICAM-1 chimeras with IgA and IgM demonstrated much lower 50% infective concentrations (IC_{50}): 1.6 nM for IC1-5D/IgA, 12 nM for IC1-2D/IgM, and 30 nM for IC1-2D/IgA. Again, IC1-2D/IgG, with an IC_{50} of 2,200 nM, was less active than sICAM-1 and was similar in activity to IC1-2D (1,600 nM).

The kinetics of neutralization of infectivity was rapid (Fig. 7). Maximal inhibition by IC1-2D/IgA (at 400 nM) required only 10 min of preincubation with HRV.

Assays of virus conformation. Picornaviruses, including poliovirus (20) and HRV (19), have been shown to undergo a capsid conformational change and loss of viral RNA after incubation with purified receptor proteins. We examined protease sensitivity and the sedimentation coefficient as assays for conformational changes induced in HRV by ICAM-1/Ig chimeras. HRV capsid proteins VP1, VP2, and VP3 in the absence of ICAM-1 were not markedly sensitive to SV8 protease. Preincubation with sICAM or different ICAM-1 chimeras, however, resulted in increased SV8 protease sensitivity. A fragment migrating between VP1 and VP2 appeared, suggesting partial degradation of VP1 (data not shown).

Virus conformation was further examined by sucrose gradient sedimentation. Intact HRV virions sediment at 149S, whereas empty capsids lacking RNA sediment at 75S (28). Incubation of HRV with ICAM-1 immunoadhesins and sICAM-1 or IC1-2D resulted in a reduction of the 149S peak and the appearance of peaks at ~120 and ~65S (Fig. 8). The 149 and ~120S peaks were infectious, whereas the ~65S peak was not infectious (data not shown). The area under the 149 and ~120S peaks was integrated, and the area reduction was calculated as the percentage of conformational disruption (Fig. 9). If only the area under the 149S curve was integrated, similar results were obtained, but up to 95% abolition of the 149S curve was seen and the effective

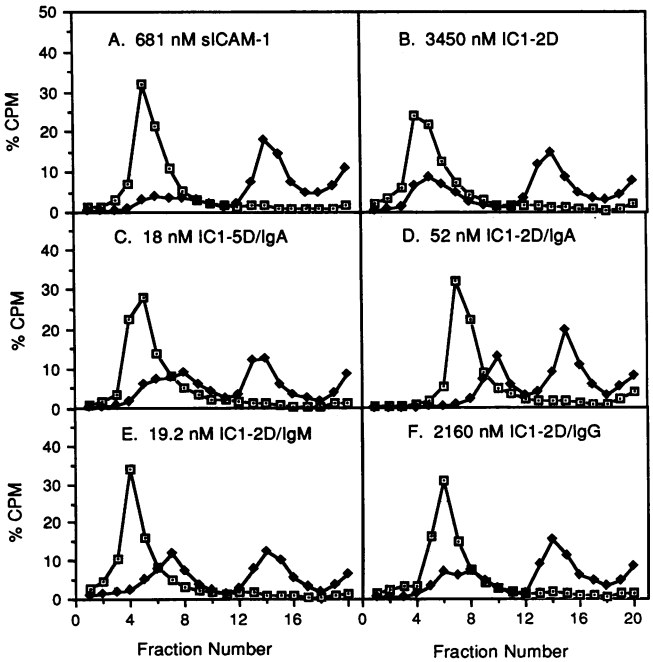

FIG. 8. Density gradient centrifugation of HRV14 particles. [^{3}H]HRV with (◆) or without (⊡) preincubation for 30 min with the indicated concentration and form of ICAM-1 was sedimented on 5 to 30% (wt/vol) sucrose gradients. The gradients were fractionated from the bottom into fractions of 200 μl, except for the last two fractions, which contained 600 μl, and total ^{3}H radioactivity was measured.

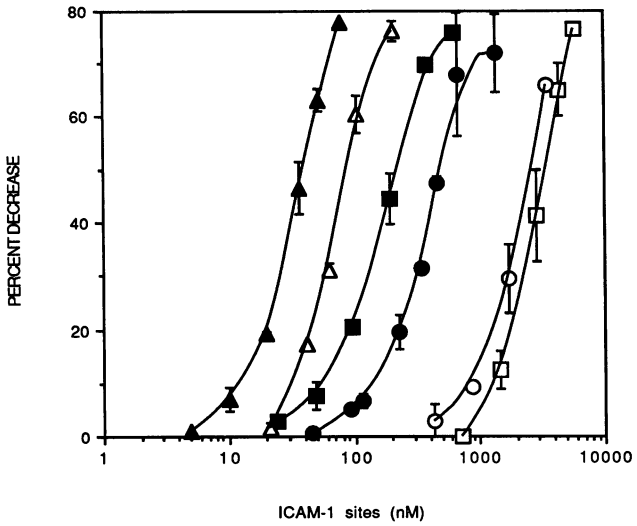

FIG. 9. Disruption of virus conformation. The area under the 149S plus ~120S peaks representing infectious virus was integrated. Reduction of 149S plus ~120S peaks induced by different ICAM-1 proteins (●, sICAM-1; ▲, IC1-5D/IgA; ■, IC1-2D/IgM; △, IC1-2D/IgA; □, IC1-2D/IgG; ○, IC1-2D) was calculated as percentage of disruption. The averages and standard deviations for two to three experiments are shown.

chimera concentration was ~1.1- to 2.4-fold lower. The order of induction of the shift to ~65S particles by ICAM-1 chimeras was IC1-5D/IgA>IC1-2D/IgA>IC1-2D/IgM> sICAM>IC1-2D/IgG = IC1-2D.

DISCUSSION

We have shown that fusing ICAM-1 to different Ig constant regions increased the efficiency of interactions with HRV. The valency of the immunoadhesins, the Ig heavy chain class to which ICAM-1 was fused, and the number of ICAM-1 Ig-like domains that were included were all important factors for neutralization capacity (Table 1). To fairly compare monomeric ICAM-1 molecules and multimeric ICAM-1 chimeras, concentrations were expressed as monomer molarity.

The effect of valency was assessed with chimeras containing both five and two domains of ICAM-1. The dimeric IC1-5D/IgA chimera was 200 times more effective than monomeric sICAM-1 in inhibition of HRV infectivity, whereas IC1-2D/IgM was 90-fold and IC1-2D/IgA was 36-fold more effective than IC1-2D. Multivalent chimeras also showed enhanced induction of capsid conformational change and inhibition of HeLa cell binding. Furthermore, the approximately decameric IC1-2D/IgM molecule was five times more potent than the dimeric IC1-2D/IgM molecule in inhibiting HRV binding to HeLa cells. These findings suggest that IgA and IgM chimeras can bind multivalently, resulting in a higher effective affinity (avidity).

TABLE 1. Concentrations of ICAM-1 analogs required for 50% inhibition of different assays of rhinovirus function

Analog	IC_{50} (nM) in terms of:		
	Binding to HeLa cells	149S virus particles	PFU
IC1-5D/IgA	6	38	1.6
IC1-2D/IgM	50	220	12
IC1-2D/IgA	35	90	30
sICAM-1	45	480	300
IC1-2D/IgG	500	3,400	2,200
IC1-2D	6,500	3,600	1,600

Comparison of bivalent chimeras showed that the class of Ig constant region chosen for construction of the chimeras had a major effect on efficacy. IC1-2D/IgA was dramatically more potent than IC1-2D/IgG in all assays. The findings discussed above suggest that IC1-2D/IgA bound bivalently to HRV. By contrast, the IC1-2D/IgG chimera was equivalent in IC_{50} to IC1-2D in inducing conformational change and neutralizing infectivity, implying that it bound monovalently in these assays. This interpretation is consistent with possession by IgA1 of a longer and hence more flexible hinge than IgG1 (14, 15). Segmental motion of Ig Fab regions, replaced in the immunoadhesins with ICAM-1, has been shown to correlate with the length of the upper hinge (38), which contains 20 amino acid residues in IgA1 and 5 residues in IgG1. IgA1 clearly has the longest upper hinge of any Ig; those of IgA2, IgG1, IgG2, IgG3, and IgG4 range from 3 to 12 amino acid residues in length. After the completion of the study reported here, we made an IgG1 immunoadhesin containing D1 through D5 of ICAM-1. IC1-5D/IgG is much more efficient than sICAM-1 and is almost as active as IC1-5D/IgA in inhibiting infectivity and inducing a shift in capsid conformation, suggesting that adding domains 3 through 5 of ICAM-1 enables bivalent binding.

For both monomeric soluble ICAM-1 constructs and ICAM-1/IgA chimeras, the five-domain proteins were more potent than the two-domain proteins. The soluble five-domain form of ICAM-1 has previously been reported to be 3-fold more effective in preventing HRV binding and 6- to 13-fold more effective in preventing infectivity than the two-domain form (19, 23). These findings are consistent with our earlier observation that binding of rhinovirus to cells transfected with full-length ICAM-1 is more efficient than binding with domains 3 through 5 deleted (35). By contrast to the results with ICAM-1, soluble CD4 or CD4 immunoadhesins containing Ig domains 1 and 2 are at least as active as those containing domains 1 through 4 (4, 17).

Studies on the effect of valency and Ig class of immunoadhesins to date have been limited, and our systematic comparisons may be useful in the design of more effective immunoadhesins containing other receptors, such as CD4. CD4/IgG1 immunoadhesins are identical to soluble CD4 in affinity for HIV gp120 and inhibition of infection in vitro by human immunodeficiency virus (3–5). Thus, there is no evidence to date that dimeric CD4 immunoadhesins bind with higher affinity than monomeric CD4. Our results with IgA1 immunoadhesins suggest that the use of other Ig subclasses might yield dimeric CD4 immunoadhesins with higher avidity for human immunodeficiency virus. A CD4 immunoadhesin made with murine IgM inhibits syncytium formation by human immunodeficiency virus 1,000-fold better than other CD4 constructs, confirming that multimerization can significantly increase the neutralization capacity of CD4 (39).

In the three different assays we examined, five domains of ICAM-1 were always superior to two domains, and chimeras with IgA1 or IgM constant domains were always better than monovalent ICAM-1; however, there were relative differences between the assays that may reflect differing mechanisms of neutralization (Table 1). For example, IC1-5D/IgA, IC1-2D/IgA, and IC1-2D/IgM were much more effective

relative to sICAM-1 in reducing plaques than in inhibiting binding to HeLa cells. This suggests that these immunoadhesins are improved relative to sICAM-1 in inhibiting post-binding events, such as endocytosis or entry into the cytoplasm. The IC1-2D/IgG immunoadhesin was more effective than IC1-2D in blocking binding to HeLa cells, perhaps because of its greater bulk, but was equivalent to IC1-2D in the other assays. Conversion of 149 to ~65S particles is a therapeutically attractive mechanism of neutralization by an antiviral agent, because it is accompanied by release of viral RNA and results in irreversible inactivation (19). The amount of capsid disruption can be increased by prolonging the incubation; however, within the time period employed here of 30 min, it was not the dominant mechanism of inactivation, because the IC_{50} for particle disruption was always higher than for plaque reduction.

ICAM-1 immunoadhesins could potentially be administered to prevent or ameliorate common colds caused by the major group of HRV serotypes. We have produced immunoadhesins that would be effective at much lower concentrations than soluble ICAM-1. Because IgA and IgM are specialized for secretion into the mucosa, chimeras of these Ig classes may be well suited for intranasal administration. The WIN series of drugs currently under development as antipicornaviral agents differ significantly from ICAM-1 analogs in mode of action (33). The WIN compounds bind reversibly in a hydrophobic pocket within viral capsid protein VP1 and stabilize the virus and prevent uncoating. By contrast, ICAM-1 analogs can both reversibly neutralize and irreversibly destabilize HRV. Virus mutants that escape neutralization have been demonstrated for both the WIN compounds (34) and a soluble poliovirus receptor (20). In the latter case, escaped mutants bound to the soluble receptor with lower affinity. Escape from multivalent receptors in the form of immunoadhesins would be expected to occur at lower frequency, because they should more closely resemble in avidity receptors that are multivalently displayed on the cell surface.

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