ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2003, p. 1503–1508 Vol. 47, No. 5
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.5.1593–1508.2003

Prevention of Human Rhinovirus Infection by Multivalent Fab Molecules Directed against ICAM-1

Catherine H. Charles, Guang X. Luo, Lori A. Kohlstaedt, Ianessa G. Morantte,† Elliott Gorfain,‡ Linguang Cao, Jimmy H. Williams, and Fang Fang*

Perlan Therapeutics, San Diego, California

Received 7 October 2002/Returned for modification 20 December 2002/Accepted 11 February 2003

We have developed a technology for improving avidity by making bivalent, trivalent, or tetravalent recombinant polypeptides. We designed tripartite proteins consisting of the Fab fragment of an antibody fused with a hinge derived from human immunoglobulin D that was further linked to polymerization domains derived from human coiled-coil proteins. We report here on the application of this method with a Fab domain directed against the major human rhinovirus receptor, intercellular adhesion molecule 1 (ICAM-1). Multivalent anti-ICAM-1 molecules were produced in bacteria and purified as soluble preassembled homogeneous proteins at high yield. These proteins successfully blocked rhinovirus infection in vitro, with the efficiency increasing from monomer to dimer, trimer, and tetramer. The diminished dissociation rate of these multivalent antibodies and their improved efficacy in preventing rhinovirus infection provide a foundation for producing prophylactic and therapeutic molecules against human rhinovirus, the causative agent of the majority of common colds.

Human rhinoviruses (HRVs) are the major cause of the common cold (23, 24) and are among the most frequently occurring human pathogens. These viruses infect cells of the nasal epithelium by binding to cell surface receptors. On the basis of their cellular receptor specificities, the more than 100 HRV serotypes can be divided into two groups. The major group contains about 90% of all serotypes and uses intercellular adhesion molecule 1 (ICAM-1) as its receptor (25). A receptor-blocking approach has shown that anti-ICAM-1 monoclonal antibody (MAb) 1A6 prevents HRV infection of cells in vitro (3). In human clinical trials, the antibody diminished cold symptoms but failed to prevent onset of the disease (8). The limited efficacy of MAb 1A6 is most likely due to its low functional affinity (or avidity) for ICAM-1 compared to that of the multivalent HRV particles. Consistent with this interpretation was a study in which several MAbs against ICAM-1 were shown to dissociate from ICAM-1 at the same rate as HRV itself (i.e., they had equal dissociation rate constants [k_{off}s]) (2).

For over a decade, science has tried to improve on nature by using multivalency to increase the avidities of antibodies. The majority of these proteins were engineered by fusing the antigen-binding scFv fragment of an antibody with a polymerization domain, such as streptavidin (10), the yeast protein GCN4 leucine zipper domain (7, 18), or the tetramerization domain of p53 (18). Fab fragments of antibodies, however, generally exhibit significantly higher binding affinities than their corresponding scFv fragments. Because of the greater complexity of assembling multichain proteins, little work has been done on making trimeric and tetrameric Fab molecules. Our method involves the generation of tripartite proteins consisting of a humanized Fab, a linker sequence derived from the human immunoglobulin D (IgD) hinge (13), and a multimerization domain derived from either of the human transcription factors, ATFα (15) or CREBPa (12). These molecules are produced and assembled in *Escherichia coli* at high levels. They are purified in soluble homogeneous form by a simple purification procedure. This method is suitable for broad application in making multivalent molecules.

To verify that this approach could be applied to a therapeutic target, we multimerized a humanized Fab based on MAb 1A6. Because the binding of bivalent MAb 1A6 was not strong enough to block HRV infection completely, we applied the premise that multivalency could enhance avidity by lowering k_{off} (4, 9). We designed trivalent and tetravalent antibodies with the goal of improving efficacy via increased avidity. The multivalent humanized proteins possess a dramatically improved ability to protect cells in culture from HRV infection.

MATERIALS AND METHODS

DNA constructions. The Fab19 protein was expressed from a bicistronic operon similar to that used by Carter et al. (1) for Fab molecules. DNAs encoding the variable domains are precisely fused on their 5′ ends to a gene segment encoding the enterotoxin II signal sequence (17). The intervening sequence in the bicistronic gene contains a ribosome entry site, while the 3′ end of the gene contains the bacteriophage λt_0 transcriptional terminator (20). The vector includes a mechanism for generating a variety of multimeric Fab molecules through insertion of polymerization domain sequences between a unique *Sac*I site just before the stop codon in the C_H domain and a unique *Eco*RI site just after the stop codon. The expression vector pTexK was derived from pBR322 and contains the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter and a kanamycin resistance gene.

The gene segment encoding the light chain was synthesized by PCR with two overlapping templates, the V_L fragment derived from two versions of humanized scFv MAb 1A6 (HscB for Fab19 and HscE for Fab48 [11]) and the C_L fragment derived from the human κ_1 light-chain constant region (14). The PCR product was cloned into the pCR 2.1 TOPO cloning vector (Invitrogen). The inserts of correct clones were sequenced in their entirety. A similar approach was used to synthesize the heavy chain and the terminator as an *Xba*I-*Hin*dIII fragment, with the C_H1 domain derived from human IgG1 (5). The expression plasmid for Fab

* Corresponding author. Mailing address: Perlan Therapeutics, 6310 Nancy Ridge Dr., Suite 102, San Diego, CA 92121 Phone: (858) 622-0400. Fax: (858) 622-0499. E-mail: ffang@perlan.com.
† Present address: The Salk Institute, San Diego, Calif.
‡ Present address: RNAture, Irvine, Calif.

TABLE 1. Amino acid sequences

Multimerization domain sequence		Composition of multimeric proteins				
Domain name	Amino acid sequence	Protein name	Fab portion	Hinge[a]	Multimerization domain	Form
ATFα wild type	VSSLEKKAEELTSQNIQLSNEVTLLRNEVAQ	CFY196	Fab19	ED	ATFα(1)-LI	Tetramer
ATFα(1)-LI	LSSIEKKLEEITSQLIQISNELTLIRNELAQS	CFY484	Fab48	D	ATFα(2)-LI	Tetramer
ATFα(2)-LI	LSSIEKKLEEITSQLIQIRNELTLIRNELAQS	CFY193B	Fab19	ED	ATFα(2)-LL	Trimer
ATFα(2)-LL	LSSLEKKLEELTSQLIQLRNELTLLRNELAQ	CFY195	Fab19	ED	ATFα(2)-II	Trimer
ATFα(2)-II	ISSIEKKIEEITSQIIQIRNEITLIRNEIAQ	CFY199	Fab19	ED	CREB-IL	Dimer
CREB-IL	IMSLEKKIEELTQTIMQLQNEISMLKNEIAQ	CFY202	Fab19	ED	GCN4-P1	Dimer
GCN4-P1	RMKQLEDKVEELLSKNYHLENEVARLKKLVGER					

[a] The hinge sequences were as follows: D hinge, ELKTPKAQASSVPTAQPQAEGSLAKATTAPATTRN; ED hinge, ELKTPKAQASSVPTAQPQAEGSLAKAT TAPATTRNTGRGGEEKKKEKEKEEQEERETKTPE.

was made by ligating the *Spe*I-*Xba*I light-chain fragment and the *Xba*I-*Hin*dIII heavy-chain fragment into *Spe*I-*Hin*dIII-digested pTexK to generate pTexK-Fab.

To produce multimeric Fab proteins, DNA cassettes were synthesized by PCR with overlapping oligonucleotides as templates. The amino acid sequences encoded by these domains are listed in Table 1. DNAs encoding each of the hinge and multimerization domains were ligated into the appropriate Fab vector as *Sac*I-*Eco*RI fragments.

Expression, purification, and column chromatography of anti-ICAM-1 proteins. To produce Fab and multimeric Fab proteins, cultures of *E. coli* strain JM83 (American Type Culture Collection [ATCC]) (26, 27) expressing plasmid pTexK-Fab19, CFY199, CFY193B, CFY195, CFY196, CFY202, or CFY484 were grown in selective TB (Terrific broth) medium to an optical density at 600 nm (OD_{600}) of at least 2.0. After induction by the addition of IPTG to a final concentration of 0.2 mM and incubation for 8 h at room temperature, the cells were harvested by centrifugation at 4,000 × *g* for 15 min at 4°C. The harvested cell pellets were washed once with 1/20 volume of TBS (50 mM Tris [pH 8.0], 200 mM NaCl) with 5 mM EDTA and frozen. The thawed cell pellets were resuspended in TBS containing 250 μM 1,10-phenanthroline (*ortho*-phenanthroline), 5 mM EDTA, and 200 μM phenylmethylsulfonyl fluoride and sonicated. The sonicated material was then clarified by centrifugation at 23,000 × *g* for 30 min. The supernatant was passed through a DE52 (Whatman) column equilibrated with TBS. The flowthrough of this column was adjusted to 1 M NaCl and loaded onto a protein A column (Amersham/Pharmacia). The column was washed extensively with 2 M NaCl–50 mM Tris (pH 8.0). Proteins were then eluted with 0.1 M glycine (pH 2.5) and neutralized with 1/10 volume of 1 M Tris (pH 9.0). For Fab19, protein-containing fractions were then pooled and dialyzed against TBS and stored at 4°C. For the other proteins, fractions from the protein A column were dialyzed against 200 mM KCl in 50 mM *N*-2-hydroxyethypiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 8.0) and then further purified over a hydroxyapatite column (Macroprep ceramic hydroxyapatite type II; Bio-Rad). After dialysis, buffer was loaded and the column was washed with 500 mM KCl–50 mM HEPES (pH 8.0). The multimeric Fab proteins were eluted with a phosphate gradient in 500 mM KCl–50 mM HEPES (pH 8.0). Positive fractions were pooled and dialyzed against TBS. The yield was 1 to 10 mg/liter of culture.

Size-exclusion chromatography was performed on a Superdex 200 column (Amersham/Pharmacia) equilibrated with TBS at a flow rate of 0.4 ml/min. The column was calibrated with standard proteins (carbonic anhydrase, 29 kDa; albumin, 60 kDa; alcohol dehydrogenase, 150 kDa; β-amylase, 200 kDa; blue dextran). The purified Fab and multivalent Fab proteins were analyzed at similar concentrations and resolved under the same running conditions.

Hydrodynamic characterization of anti-ICAM-1 proteins. Analytical ultracentrifugation and light scattering were performed by Alliance Protein Labs (Camarillo, Calif.) with purified proteins. All experiments were conducted in TBS at a concentration of test protein of approximately 0.5 mg/ml. Sedimentation velocity analysis was performed in a Beckman Optima XL-A analytical ultracentrifuge; data were analyzed with the programs SEDFIT (21) and SVEDBERG (16). Dynamic light-scattering data were collected with a Protein Solutions DynaPro MS/X instrument with 12-μl quartz cells and analyzed with the manufacturer's software. Samples were filtered through a Whatman Anotop 0.02-μm-pore-size filter prior to light-scattering analysis.

Surface plasmon resonance analysis. k_{off}s were determined by surface plasmon resonance for the Fab19 monomer, dimeric CFY199, and tetrameric CFY196 by Research Consulting Services, BIAcore, Inc., Piscataway, N.J. Analysis was done on a BIAcore TM 3000 instrument (Biosensor, Piscataway, N.J.) at 25°C. The response data were generated from a four-channel CM5 chip onto which soluble ICAM-1 (Bender MedSystems, Vienna, Austria) was immobilized at 50 and 1,650 response units. Proteins were injected onto the chip at 100 nM in a running buffer consisting of 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20. The data were fit with BIAcore software to generate the rate constant and the half-life for the dissociation of each protein from ICAM-1 (see Table 3).

Cell culture and viral production. HeLa cells (Ohio HeLa-I cells; a gift of Frederick G. Hayden, University of Virginia) were maintained in Dulbecco modified Eagle medium (Cellgro) with 10% fetal bovine serum (Gibco) for routine propagation. BEAS-2B cells (ATCC) (19) were grown in bronchial epithelial cell medium supplemented with growth factors (Clonetics, BioWhittaker Co.). They were maintained under subconfluent growth conditions during passage. HRV serotype 14, 15, 16, 39, and 1a stocks (ATCC) were amplified to high titer by successive infection of target HeLa cells. Viral infection was initiated in culture medium supplemented with 20 mM HEPES (pH 7.4)–10 mM $MgCl_2$. After 12 to 24 h of infection at 33°C, viruses were released from the cells by three freeze-thaw cycles at −80 and 33°C. The cell debris was discarded, while the supernatant containing amplified virus was aliquoted and frozen at −80°C.

Competitive ELISA. To prepare a tracer molecule that could compete with anti-ICAM-1 tetramers, we produced a horseradish peroxidase (HRP)-labeled protein. A cysteine was introduced into a tetrameric anti-ICAM-1 antibody by adding the sequence TGC to the carboxy terminus of CFY196 to generate a new tetrameric molecule, 196TGC. HRP was conjugated to 196TGC with the EZ-link Maleimide Activated Horseradish Peroxidase labeling kit (Pierce) to produce 196TGC-HRP.

To begin the enzyme-linked immunosorbent assay (ELISA), a 96-well enzyme immunoassay plate (Corning) was coated with 100 μl of soluble ICAM-1 (1 μg/ml in 0.1 M $NaHCO_3$; Bender MedSystems) per well. After the plate was washed with TBST (50 mM Tris [pH 8.0], 150 mM NaCl, 0.05% Tween 20), it was blocked with 3% nonfat milk in TBST at 37°C for 1 h. After the samples were washed with TBST, anti-ICAM-1 Fab samples (monomer or multimer) serially diluted in 1% nonfat milk–TBST solution were added and the mixture was incubated at room temperature for 1 h. The plate was incubated at room temperature for 2 h, and then the wells were thoroughly washed with TBST. Bound HRP activity was measured with 100 μl of 3,3′,5,5′-tetramethylbenzidine substrate solution (Kirkegaard & Perry Laboratories) per well. After 15 min, the color development was stopped by the addition of 100 μl of 0.12 N HCl per well and the absorbance was measured at 450 nm with a plate reader (ICN).

The percent inhibition of tracer antibody (196TGC-HRP) binding was calculated as $100 \times [(A_0 - A_s)/A_0]$, where A_0 is the OD_{450} of the reference well without samples (196TGC-HRP only) and A_s is the OD_{450} of the sample wells. The relative binding affinities of the anti-ICAM-1 antibodies were represented by the protein concentration that reduces tracer antibody binding by 50% (IC_{50}).

Cell protection assays. To measure protection against viral infection, the level of cell death due to HRV infection after pretreatment with various concentrations of our proteins was compared to that for uninfected cells. In a standard assay, 10^5 HeLa cells were plated on the day before an assay in each well of a 48-well dish. Triplicate wells of cells were then incubated for 1 h at 37°C with protein preparations in a volume of 100 μl of cell medium at the indicated concentrations. This mixture was removed, and virus at a multiplicity of infection of 1 was added in 100 μl of cell medium. No additional anti-ICAM-1 proteins were added in this viral inoculum or later in the experiment. The dishes were incubated for 2 h at 33°C. The monolayers in the wells were then washed once with medium to remove unbound material and incubated with fresh medium. The infection was then allowed to proceed for 48 h at 33°C. The remaining viable

cells were stained with 0.5% crystal violet in 20% methanol, washed extensively with water, extracted with methanol, and quantified by measuring the absorption at 570 nm. Under these conditions, the virus-infected, unprotected HeLa cells were all lysed by visual inspection; therefore, the absorbance of the triplicate virus samples represented the background crystal violet staining of the cell debris. The percent protection was calculated as 100 × [(absorbance of sample − absorbance of virus only)/(absorbance of uninfected samples − absorbance of virus only)].

Viral protection assays were performed in a similar manner with BEAS-2B cells, except these cells do not lyse upon rhinoviral infection. The progression of infection was measured by determining the titers of the progeny virus released into the medium. The BEAS-2B human respiratory epithelial cells were pretreated with 100 μl of 1 μg of monomeric Fab19 (21 nM), trimeric CFY195 (5.8 nM), tetrameric CFY196 (4.3 nM), or anti-ICAM-1 MAb 84H10 (6.7 nM; MAb 1379; Chemicon) per ml. These solutions were then removed, and the monolayers were infected with HRV serotype 15 at a multiplicity of infection of 30. After 2 h of viral attachment, the cells were washed with epithelial cell medium to remove any unbound virus and incubated in fresh medium at 33°C. After 48 h, the titer of the progeny virus released into the cell medium was measured by using HeLa cells as a target. The titers of virus from protected cells were compared and expressed as a percentage of the titers obtained from unprotected cells.

RESULTS

Design and production of multivalent anti-ICAM-1 Fab. We had three criteria for designing polymerization domains. Namely, they should be derived from human sequences; they should form tight, homogeneous multimers without significant dissociation; and they should be small to allow efficient expression and folding in *E. coli*. To this end, we modified the coiled-coil domain from human transcription factors ATFα and CREBPa. Changes of the residues in the a and d positions of the heptad repeats [$(abcdefg)_n$] resulted in dimers, trimers, and tetramers in a context-dependent manner (Table 1). These compact domains were attached to the Fab at the Fd carboxy terminus by linkers derived from the long, flexible human IgD hinge sequence. After translation, the light and composite heavy chains are secreted into the periplasmic space, where the complete assembly of multimeric Fab occurs.

Tetrameric, trimeric, dimeric, and monomeric Fab proteins were produced in *E. coli* and purified to a yield of 1 to 10 mg of homogeneous protein per liter of shaker flask culture. The tetrameric molecules have four flexible Fab arms available for binding. Cross-linking studies with a zero-length cross-linker revealed that the tetramers formed from ATFα coiled-coil domains are parallel in orientation (data not shown). A theoretical model of one of these tetrameric chimeric proteins, CFY484, is depicted in Fig. 1.

For the antigen-binding domain, we chose the Fab fragment because it has higher efficacy than the corresponding scFv fragment (data not shown). Fab19 and Fab48 are derived from two versions of humanized anti-ICAM-1 MAb 1A6 (R. J. Colonno, J. H. Condra, J. E. Tomassini, and V. V. Sardana, European patent application 0459577A2), called HscB and HscE, respectively (11). The expression vector produces protein from a bicistronic operon that directs the expression of both light-chain and composite heavy-chain polypeptides. The resultant composite heavy chain consists of three parts: the Fd fragment (V_H and C_H1), the D or ED hinge, and the polymerization domain (Table 1). Since we envision that multimeric anti-ICAM-1 might be used as a nasal spray to prevent rhinovirus binding in the nasal epithelium, we tested the stabilities of the molecules by incubating them in the presence of human nasal mucus. We evaluated the protein integrity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the multimeric status by size-exclusion chromatography after 48 h at 37°C. Under these conditions the multimeric polypeptides remained intact (data not shown).

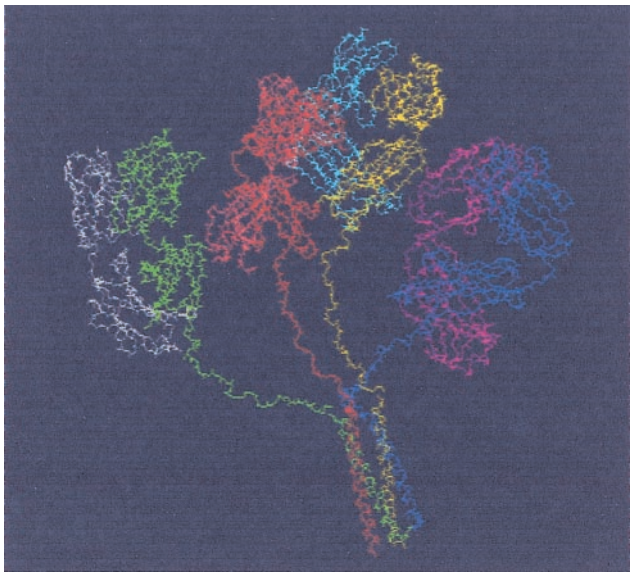

FIG. 1. Illustration of our conception of the tetrameric Fab anti-ICAM-1 molecule. A homology model of Fab19 was attached to a modeled representation of a hinge and multimerization domain to form a tetravalent Fab protein. Molecular models were constructed by using the programs Swiss Model and Swiss PDB Viewer (6). Each polypeptide chain is represented by a different color.

Characterization of oligomeric status. The oligomeric status of each fusion protein was characterized by size-exclusion chromatography, analytical ultracentrifugation, and dynamic light scattering. By gel filtration analysis with Superdex 200, monomeric Fab19 has a retention time of 24.83 min. Under the same chromatography conditions, the retention time of CFY199 is 18.35 min, that of CFY195 is 17.04 min, and that of CFY196 is 16.03 min. The mobility of standards in the same column suggests that Fab19 is a monomer, CFY199 is a dimer, and CFY195 and CFY196 are higher-order multimers. Sedimentation velocity experiments further characterize these multimers. Data from the analytical ultracentrifugation (22) as well as dynamic light scattering (28) experiments are summarized in Table 2. These analyses confirmed the results obtained by size-exclusion chromatography. They also demonstrated that CFY199 is a dimer, that CFY193B and CFY195 are trimers, and that CFY196 and CFY484 are tetramers. Therefore,

TABLE 2. Physical characteristics of purified multimeric proteins

Protein name	Sedimentation coefficient (S)	Hydrodynamic radius (nm)	Form
CFY196	6.6	8.98	Tetramer
CFY484	6.9	9.20	Tetramer
CFY193B	5.9	7.41	Trimer
CFY195	5.5	7.54	Trimer
CFY202	4.6	7.48	Dimer
CFY199	4.5	5.73	Dimer

TABLE 3. Dissociation kinetics of proteins

No. of response units and protein	k_{off} (s^{-1})	Half-life
50 response units		
Fab19	$3.00 \times 10^{-4} \pm 3.87\%$	38.56 min
CFY199	$3.78 \times 10^{-4} \pm 2.31\%$	30.93 min
CFY196	$7.35 \times 10^{-5} \pm 8.61\%$	2.62 h
1650 response units		
Fab19	$2.26 \times 10^{-4} \pm 0.20\%$	51.22 min
CFY199	$1.24 \times 10^{-4} \pm 0.32\%$	1.55 h
CFY196	$1.53 \times 10^{-6} \pm 42.70\%$	80.28 h

CREB-IL is a dimerization domain, ATFα-LL and ATFα-II are trimerization domains, and ATFα-LI is a tetramerization domain.

Analysis of k_{off}. Multivalency can increase functional affinity by decreasing k_{off}. Monomeric Fab19, bivalent CFY199, and tetravalent CFY196 were subjected to surface plasmon resonance analysis with ICAM-1 immobilized on the assay chip at a low level (50 response units) and a high level (1,650 response units). In contrast to the pattern of association and dissociation of MAb 1A6 scFv, which is very rapid (data not shown), Fab19 had a diminished k_{off}. Because of this slow k_{off}, the dissociation phase was measured for 6 min. The monomeric and dimeric proteins exhibited similar dissociation kinetics on the surface with a low level of substitution (Table 3). Tetrameric CFY196, in contrast, showed significantly lower k_{off} values. The effect of mulivalency on the dissociation kinetics was significantly enhanced on the surface with a high level of substitution.

While the surface with a high level of substitution exhibited a convolution of the effects of multivalency and mass action, the advantage of multivalent binding is apparent. If one compares the dissociation from the surfaces with low and high levels of substitution, the rate of dissociation of monomeric Fab19 is slowed 1.3-fold, dimeric CFY199 dissociates 3-fold more slowly, and tetrameric CFY196 dissociates 30-fold more slowly. These data demonstrate that multivalency has provided the characteristic that we sought: a tetravalent protein with a greatly diminished k_{off}.

Functional characterization of effectiveness of multivalent Fab fusion proteins against ICAM-1. We compared the effectiveness of multimeric Fab19 using three methods: a competitive ELISA measuring ICAM-1 binding and two viral protection assays.

Because conventional ELISA cannot distinguish between the high-affinity anti-ICAM-1 multimers, we designed a two-step competition ELISA using high-affinity tetrameric 196TGC linked to HRP (196TGC-HRP) as a tracer. The anti-ICAM-1 proteins to be assessed were bound to immobilized ICAM-1, and then 196TGC-HRP was added and allowed to compete with the tested protein for ICAM-1 binding. Monomeric Fab19 inhibited only 25% tracer antibody (196TGC-HRP) binding at the highest concentration tested; however, the trimer (CFY195) and the tetramer (CFY196) gave much higher percent inhibitions, with IC_{50}s equal to 0.54 and 0.069 μg/ml, respectively (Fig. 2).

We also tested the abilities of the Fab and multimer proteins to prevent HRV15 infection of cultured human cells. In this assay, we used HeLa cells as an HRV target. Since MAb 1A6 was not available, a commercially available anti-ICAM-1 MAb, MAb RR1/1 (MAb 2145; Chemicon), was also included for comparison. This viral infection protection assay (Fig. 3) illustrates that tetravalent CFY196 was the most effective, with a 50% effective concentration (EC_{50}) of 0.97 nM (0.22 μg/ml), which is over 200 times better than that of monovalent Fab19 (EC_{50}, >200 nM, or 100 μg/ml; data not shown). Trivalent CFY193B was less effective, with an EC_{50} of 1.35 nM (0.24 μg/ml), as was the bivalent CFY202, with an EC_{50} of 4.5 nM (0.52 μg/ml). However, all of the multivalent Fab fusion proteins functioned better than MAb RR1/1 (EC_{50}s, 12.5 nM, or 1.87 μg/ml) and a second commercial antibody, MAb 1379 (data not shown). Tetrameric CFY196 was 13 times as effective as MAb RR1/1. Since MAb RR1/1 was previously shown to be kinetically on par with the HRV particle in terms of the rate of dissociation from ICAM-1 (2), only a molecule with a higher avidity than MAb RR1/1 can effectively compete with HRV for receptor binding. Our tetrameric Fab has a true kinetic advantage over HRV and, therefore, is a promising HRV inhibitor. We know that the protective ability of our anti-ICAM-1 is specific to strains that bind to ICAM-1, because we observed

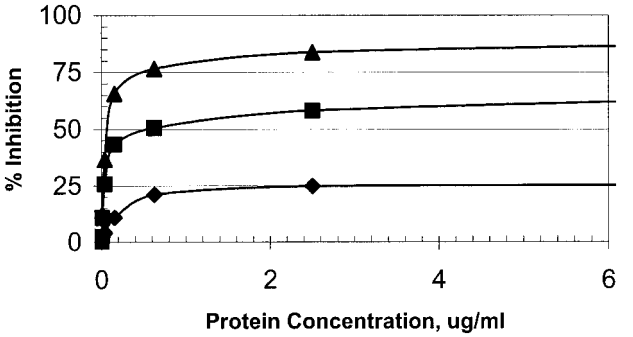

FIG. 2. Competitive ELISA results. The graph shows the percent inhibition of tracer antibody binding as a function of the amount of each protein added to wells coated with soluble human ICAM-1. Triangles, CFY196; squares, CFY195; diamonds, Fab19.

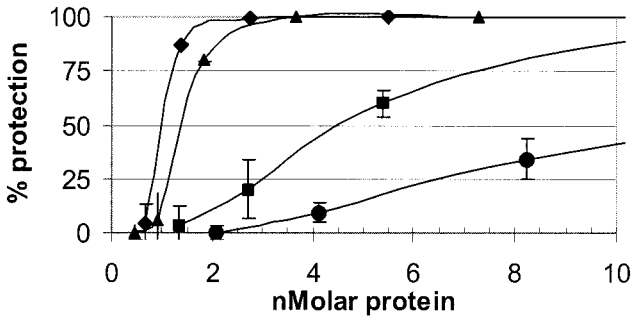

FIG. 3. Protection of HeLa cells from HRV infection by proteins that are multimers of anti-ICAM-1 Fab19. Percent protection was calculated as indicated in Materials and Methods, with 100% protection being equivalent to the staining of uninfected cells and 0% protection being equivalent to the staining of infected untreated cells. Circles, MAb RR1; triangles, CFY193B; squares, CFY202; diamonds, CFY196. The points represent mean values from one experiment in which the samples were tested in triplicate. The error bars represent standard deviations.

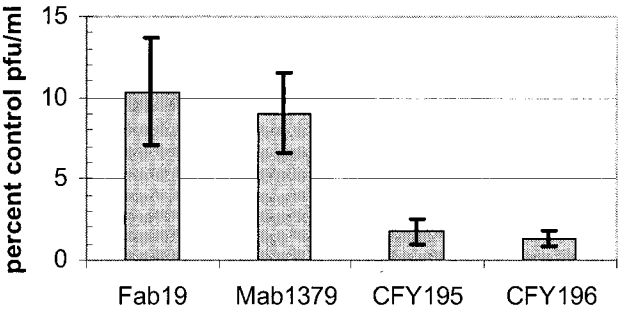

FIG. 4. Reduction of viral titer in protected respiratory epithelial cells. Viral titers from HRV serotype 15-infected BEAS-2B cells before infection and 48 h after infection are shown. Each percentage is the mean from two independent experiments measured from cells pretreated with the indicated proteins. The error bars represent the standard deviations. A value of 100% corresponds to the titer of virus produced from infected cells with no protective pretreatment.

no protection when an HRV serotype 1a, a strain that uses a different receptor was used as the infective agent (data not shown). Protection experiments were performed with other multimeric Fabs (Table 1) and HRV serotypes (HRV serotypes 14, 16 and 39; data not shown), with similar results obtained. No effect was seen when cells were grown in the presence of anti-ICAM-1 without HRV infection (data not shown).

We also tested a human respiratory epithelial cell line, BEAS-2B, as an infection target. Since these cells do not lyse upon infection, the level of infection was measured by quantifying the titer of the progeny virus accumulated in the culture medium 48 h after initial infection. Two different experiments produced titers of 3.6×10^5 and 1.5×10^6 PFU/ml from unprotected infected cells. In both experiments, the proportionate decrease was the same. Tetrameric CFY196 showed the best efficacy (Fig. 4).

DISCUSSION

Multivalency is a useful mechanism for achieving high functional affinity. It has not been widely used to design therapeutic proteins, however, due to the technical difficulties of making complex multivalent recombinant proteins. Our method represents significant progress in this area. We have designed recombinant Fab proteins based on human sequences, engineering multimerization domains to make bivalent, trivalent, and tetravalent molecules. The use of a long hinge in the design imparts both flexibility and stability. We produced these in *E. coli* and purified them from the soluble fraction as homogeneous multimers.

Application of this technology has enabled us to generate receptor blockers that prevent HRV infection in tissue culture at a concentration much lower than that which was previously attainable. We have demonstrated that the activities of our ICAM-1-binding proteins improve in proportion to their valency. In both tissue culture models presented here, we inhibited HRV infection most effectively with tetrameric CFY196. In these experiments, CFY196 was able to compete effectively with multivalent rhinovirus for ICAM-1 binding. The greatly diminished k_{off} and high avidity of CFY196 suggest potential for in vivo therapeutic effectiveness.

The final tetrameric chimera is more than 200-fold more effective than the corresponding Fab at binding to ICAM-1 and preventing HRV infection. While the particular Fab used in this study is of moderate affinity, similar improvement is to be expected for any binding interaction in which the target presents multiple ligands in reasonable molecular proximity. Natural dimeric antibodies range in affinity (K_d) from about 10^{-7} M^{-1} to below 10^{-11} M^{-1}. For the tightest natural antibodies, a 50- to 100-fold increase in affinity may be both hard to measure and functionally trivial. Nevertheless, increased avidity through multimerization provides an important engineering tool for increasing binding affinity that is completely separate and complementary to any affinity maturation process.

Our multimerization domains could be used as a cassette to improve a whole host of antibodies in development for therapeutic application. In addition, multimers may be used in any context in which greater binding efficiency is desired. Application of our method requires only the availability of a moiety that can be adapted for use as part of a chimeric protein. Furthermore, multimerization may be used in any context in which greater valency alone is desirable for improved function.

ACKNOWLEDGMENTS

We thank Andrew Philips, Andrew Miller, Ozlem Turan, and Erika Wiseman for technical assistance. We thank Tony Hunter and Michele Yelmene for critical reading of the manuscript.

Contract service work was provided by John Philo (Alliance Protein Labs) and Research Consulting Services, BIAcore, Inc. This work was partially supported by NIH SBIR grant 1 R43 AI49624-01A1.

REFERENCES

1. **Carter, P., R. F. Kelley, M. L. Rodrigues, B. Snedecor, M. Covarrubias, M. D. Velligan, W. L. Wong, A. M. Rowland, C. E. Kotts, M. E. Carver, M. Yang, J. H. Bourell, H. M. Shepard, and D. Henner.** 1992. High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment. Bio/Technology **10:**163–167.
2. **Casasnovas, J. M., and T. A. Springer.** 1995. Kinetics and thermodynamics of virus binding to receptor. Studies with rhinovirus, intercellular adhesion molecule-1 (ICAM-1), and surface plasmon resonance. J. Biol. Chem. **270:** 13216–13224.
3. **Colonno, R. J., G. Abraham, and J. E. Tomassini.** 1989. Molecular and biochemical aspects of human rhinovirus attachment to cellular receptors, p. 169–178. *In* B. L. Semler and E. Ehrenfeld (ed.), Molecular aspects of pinocornavirus infection and detection. American Society for Microbiology, Washington, D.C.
4. **Crothers, D. M., and H. Metzger.** 1972. The influence of polyvalency on the binding properties of antibodies. Immunochemistry **9:**341–357.
5. **Ellison, J. W., B. J. Berson, and L. E. Hood.** 1982. The nucleotide sequence of a human immunoglobulin C γ1 gene. Nucleic Acids Res. **10:**4071–4079.
6. **Guex, N., and M. C. Peitsch.** 1997. SWISS-MODEL and the Swiss PDB Viewer: an environment for comparative protein modeling. Electrophoresis **18:**2714–2723.
7. **Harbury, P. B., T. Zhang, P. S. Kim, and T. Alber.** 1993. A switch between two-, three, and four-stranded coiled coils in GCN4 leucine zipper mutants. Science **262:**1402–1407.
8. **Hayden, F. G., J. M. Gwaltney, Jr., and R. J. Colonno.** 1988. Modification of experimental rhinovirus colds by receptor blockade. Antivir. Res. **9:**233–247.
9. **Ito, W., and Y. D. Kurosawa.** 1993. Development of an artificial antibody system with multiple valency using an Fv fragment fused to a fragment of protein A. J. Biol. Chem. **268:**20668–20675.
10. **Kipriyanov, S. M., M. Little, H. Kropshofer, F. Breiyling, S. Gotter, and S. Dubel.** 1996. Affinity enhancement of a recombinant antibody: formation of complexes with multiple valency by a single-chain Fv fragment-core streptavidin fusion. Protein Eng. **9:**203–211.
11. **Luo, G. X., L. A. Kohlstaedt, C. H. Charles, E. Gorfain, I. Morantte, J. H. Williams, and F. Fang.** J. Immunol. Methods, in press.
12. **Nomura, N., Y. Zu, T. Maekawa, S. Tabata, T. Akiyama, and S. Ishi.** 1993. Isolation and characterization of a novel member of the gene family encoding the cAMP response element-binding protein CRE-BP1. J. Biol. Chem. **268:**4259–4266.

13. **Padlan, E. A.** 1994. Anatomy of the antibody molecule. Mol. Immunol. **31:**169–217.
14. **Palm, W., and N. Hilschmann.** 1993. The primary structure of a crystalline monoclonal immunoglobulin kappa-type L-chain, subgroup I (Bence-Jones protein Rei.); isolation and characterization of the tryptic peptides; the complete amino acid sequence of the protein; a contribution to the elucidation of the three-dimensional structure of antibodies, in particular their combining site. Z. Physiol. Chem. **356:**167–191. (In German.)
15. **Pescini, R., W. Kaszubska, J. Whelan, J. F. DeLamarter, and R. Hooft van Huijsduijnen.** 1994. ATF-a0, a novel variant of the ATF/CREB transcription factor family, forms a dominant transcription inhibitor in ATF-a heterodimers. J. Biol. Chem. **269:**1159–1165.
16. **Philo, J. S.** 1997. An improved function for fitting sedimentation velocity data for low-molecular-weight solutes. Biophys. J. **72:**435–444.
17. **Picken, R. N., A. J. Mazaitis, W. K. Maas, M. Rey, and H. Heyneker.** 1983. Nucleotide sequence of the gene for heat-stable enterotoxin II of *Escherichia coli*. Infect. Immun. **42:**269–275.
18. **Pluckthun, A., and P. Pack.** 1997. New protein engineering approaches to multivalent and bispecific antibody fragments. Immunotechnology **3:**83–105.
19. **Reddel, R. R., K. Yang, J. S. Rhim, D. Brash, R. T. Su, J. F. Lechner, B. I. Gerwin, C. C. Harris, and P. Amstad.** 5 December 1989. U.S. patent 4,885,238.
20. **Scholtissek, S., and F. Grosse.** 1987. A cloning cartridge of lambda t(o) terminator. Nucleic Acids Res. **15:**3185.
21. **Schuck, P.** 2000. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. Biophys. J. **78:**1606–1619.
22. **Schuster, T. M., and T. M. Laue (ed.).** 1994. Modern analytical ultracentrifugation. Birkhauser, Boston, Mass.
23. **Sperber, S. J., and F. G. Hayden.** 1988. Chemotherapy of rhinovirus colds. Antimicrob. Agents Chemother. **32:**409–419.
24. **Stanway, G.** 1994. Rhinovirus, p. 1253–1259. *In* R. G. Webster (ed.), Encyclopedia of virology. Academic Press, Inc., New York, N.Y.
25. **Staunton, D. E., V. J. Merluzzi, R. Rothlein, R. Barton, S. D. Marlin, and T. A. Springer.** 1989. A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses. Cell **56:**849–853.
26. **Stein, D. C., et al.** 1983. Construction and characterization of a new shuttle vector, pLES2, capable of functioning in Escherichia coli and Neisseria gonorrhoeae. Gene **25:**241–247.
27. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
28. **Wen, J., T. Arakawa, and J. S. Philo.** 1996. Size-exclusion chromatography with on-line light scattering, absorbance, and refractive index detectors for studying proteins and their interactions. Anal. Biochem. **240:**155–166.