Reprinted from Nature, Vol. 344, No. 6261, pp. 70–72, 1st March, 1990

A soluble form of intercellular adhesion molecule-1 inhibits rhinovirus infection

Steven D. Marlin*, Donald E. Staunton†, Timothy A. Springer†, Christian Stratowa‡, Wolfgang Sommergruber‡ & Vincent J. Merluzzi*

* Department of Immunology, Boehringer Ingelheim Pharmaceuticals Inc., 90 East Ridge, Ridgefield, Connecticut 06877, USA
† The Center for Blood Research, 800 Huntington Avenue, Boston, Massachusetts 02115, USA
‡ The Ernst Boehringer Institute, 5-11 Dr Boehringergasse, A-1120 Vienna, Austria

RHINOVIRUSES belong to the picornavirus family and cause about 50% of common colds[1]. Most rhinoviruses and some coxsackie viruses share a common receptor on human cells. The glycoprotein intercellular adhesion molecule-1 (ICAM-1) has recently been identified as the cellular receptor for the subgroup of rhinoviruses known as the major groups[2–4]. ICAM-1 is a member of the immunoglobulin supergene family and is a ligand for lymphocyte function-associated antigen-1 (LFA-1)[5–7]; these ICAM-1/LFA-1 interactions are critical to many cell adhesion processes involved in the immunological response[8–11]. Because anti-ICAM-1 antibodies can block binding of major-group rhinoviruses to cells, we considered that antagonism of virus–receptor interaction might be a way of preventing rhinovirus infection. We have constructed and purified a soluble form of the ICAM-1 molecule, which is normally membrane-bound, and demonstrated that it is a potent and specific inhibitor of rhinovirus infection.

The ICAM-1 molecule is composed of five immunoglobulin-like extracellular domains, a hydrophobic transmembrane domain, and short cytoplasmic domain. To produce large amounts of soluble ICAM-1 (sICAM-1), we used site-directed mutagenesis to introduce an in-frame translational stop codon at the predicted extracellular boundary of the transmembrane domain, effectively deleting the transmembrane and cytoplasmic domains of the protein. After expression and analysis of the resulting truncated gene product in transient expression assays, a stable line of ICAM-1-secreting Chinese hamster ovary (CHO) cells was produced. The sICAM-1 gene was physically linked to the hamster dihydrofolate reductase gene in an SV40-based expression vector and then transfected into CHO cells. After selection in methotrexate, clones secreting sICAM-1 were identified by enzyme-linked immunosorbent assay. Two further rounds of gene amplification gave a cell line (designated CHO118A) that secreted sICAM-1 into the culture supernate to a concentration of about 1 $\mu g\ ml^{-1}$.

We purified milligram quantities of sICAM- to >95% purity from these CH0118A cell-culture supernates by immunoaffinity chromatography with the anti-ICAM-1 monoclonal antibody R6.5. Purified sICAM-1 has an apparent relative molecular mass (M_r) of 82,000 (82K), which is consistent with the predicted size of a molecule containing all five extracellular immunoglobulin-like domains. The purified sICAM-1 binds to three distinct monoclonal antibodies raised against membrane-

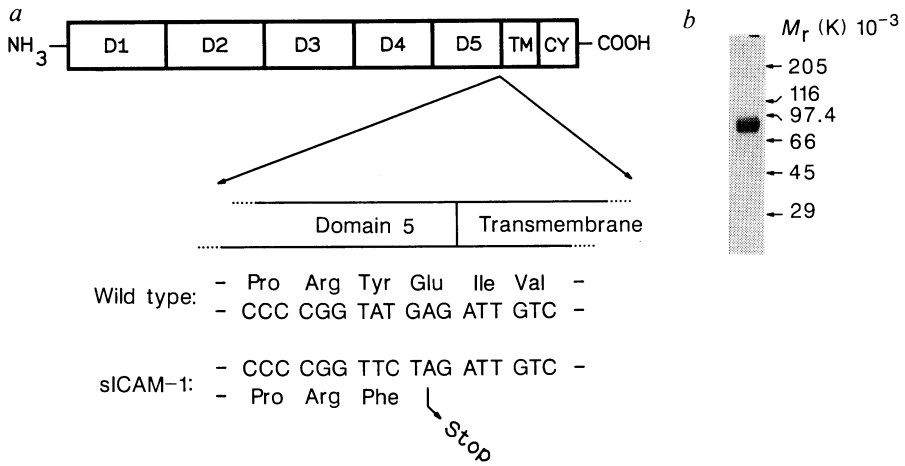

FIG. 1 Generation and purification of a soluble form of ICAM-1. *a*, Construction and expression of a mutant complementary DNA. The protein domain structure of native membrane-bound ICAM-1 is illustrated at the top: the five extracellular immunoglobulin-like domains (D1–D5), the hydrophobic transmembrane domain (TM), and the cytoplasmic tail (CY) are indicated. A soluble form of ICAM-1 was generated by mutation of the codon for Glu 453 to a translational stop codon using oligonucleotide-directed mutagenesis of the wild-type cDNA; the codon for Tyr 452 was mutated to Phe simultaneously (underlined). The plasmid containing this mutant cDNA has been designated pCDsD1-5 (D.E.S., manuscript in preparation). The truncated sequence of sICAM-1 thus differs from wild-type by a single conservative substitution at its carboxyl terminus. *b*, SDS-PAGE analysis of sICAM-1 immunoaffinity purified from culture supernates of transformed CH0118A cells. About 50 ng purified sICAM-1 was electrophoresed under reducing conditions on a 10–15% polyacrylamide gradient gel and visualized by silver staining. Migration of M_r standards is indicated on the right.

METHODS. *a*, The in-frame stop codon was generated using oligonucleotide-directed mutagenesis based on the method of Kunkel[14], as modified by Peterson and Seed[15]. The mutation was made with a single-strand uracil-containing template of the ICAM-1 cDNA subcloned into the expression vector CDM8 (pCD1.8) (ref. 7). A mutant oligonucleotide coding for a stop codon and having a unique restriction site was used to prime the second-strand synthesis reaction. After transformation into *E. coli* and confirmation of the mutation by restriction mapping, expression of a secreted form of ICAM-1 was confirmed by transfection into COS cells and analysis of the supernate by enzyme-linked immunosorbent assay (ELISA) (as described in the legend to Fig. 2) and immunoprecipitation. We constructed an expression vector consisting of the hamster dihydrofolate reductase (*DHFR*) gene and the coding region of the mutant ICAM-1 cDNA controlled by the promoter, splice signals and polyadenylation signal from the SV40 early region. The hamster *DHFR* gene was isolated from the plasmid pBR322DHFR (ref. 16) by digestion with *Fsp*I and *Hin*dIII, followed by blunt-end ligation into pSV2gpt (ref. 17) cleaved with *Bam*HI/*Hin*dIII. The mutant sICAM-1 cDNA was isolated by digestion with *Not*I, Klenow fill-in, and digestion with *Hin*dIII, then ligated into the expression vector (prepared by digestion with *Apa*I, blunting with Klenow, and digestion with *Hin*dIII to remove the guanine phosphororibosyl transferase gene). The completed vector was then transfected into CHO KI DUX-BII cells using the calcium phosphate coprecipitation method[18]. After two days of growth in non-selective medium, cells were passaged in selective medium containing methotrexate but lacking hypoxanthine and thymidine. Clones were then isolated, subcloned, and tested for sICAM-1 production by ELISA. Colonies secreting the greatest quantity of sICAM-1 were subjected to two rounds of gene amplification by methotrexate at concentrations of 0.05 to 2 μM. *b*, sICAM-1 was purified from supernates of CH0118A cells by immunoaffinity chromatography with anti-ICAM-1 monoclonal antibody R6.5. R6.5 was covalently coupled to CNBr-activated Sepharose 4B (Pharmacia LKB) to a final concentration of 5 $mg\ ml^{-1}$ packed resin. All chromatography steps were at 4 °C; buffers all contained 0.2 U per ml aprotinin and 1 mM phenylmethysulphonyl fluoride. Filtered supernate (1 litre) containing ~1 mg sICAM-1 was loaded onto a 30-ml column of R6.5-Sepharose at a flow rate of 1 $ml\ min^{-1}$. The column was then washed with 300 ml of 10 mM Tris, 0.15 M NaCl, pH 9.0, at a flow rate of 2.5 $ml\ min^{-1}$ to remove unbound material. Bound sICAM-1 was eluted with 50 mM triethylamine, 0.15 M NaCl, pH 9.0, at a flow rate of 1 $ml\ min^{-1}$. Fractions were immediately neutralized with 1 M Tris, pH 6.0, to 20 mM. sICAM-1-containing fractions were identified by SDS-PAGE (10–15% gradient gels) and silver staining, then concentrated 10-fold in a Centricon-30 microconcentrators (Amicon).

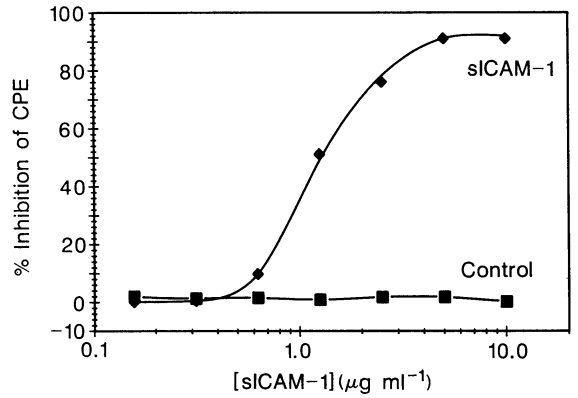

FIG. 2 sICAM-1 inhibits the cytopathic effect (CPE) induced by a major-group rhinovirus. The major-group serotype HRV54 (100 $TCID_{50}$, where one $TCID_{50}$ is the concentration required to cause 50% cytopathogenicity[3] in tissue culture was plated onto HeLa cells in the presence of sICAM-1 at the concentrations indicated (or an equivalent dilution of a buffer control from the same purification run) and the cytopathic effect determined after 4 days as described[3]. Data are representative of two experiments.

METHODS. Purified sICAM-1 was assayed for protein using a Bio-Rad kit and frozen in aliquots for use as standards. The concentration of sICAM-1 in samples was determined in a 'sandwich'-type ELISA using these reference standards and two anti-ICAM-1 monoclonal antibodies, R6.5 and R6.1 (ref. 19), which bind to non-overlapping epitopes (S.D.M., unpublished data). R6.1 was bound to 96-well plates (Nunc) by incubating 100 μl of a 10 μg ml^{-1} solution for 1 h at 37 °C. Each of the following steps used 100 μl reagent incubated at 37 °C for 30 min, followed by washes with phosphate-buffered saline: (1) binding of serial dilutions of reference standard sICAM-1 or unknowns, (2) binding of biotinylated R6.5 (1 μg ml^{-1}), and (3) binding of horseradish peroxidase-conjugated streptavidin (Zymed). After incubation for 20 min at room temperature with the substrate ABTS (2,2'-Azino-di(3-ethylbenzthiazoline) sulphonic acid; Zymed), the absorbance was read at 410 nm to obtain the concentration of sICAM-1.

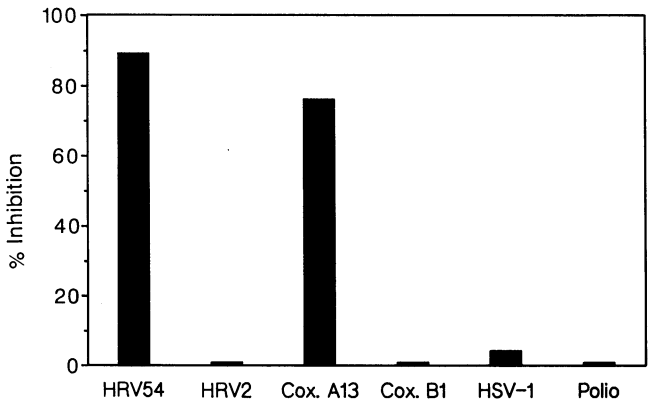

FIG. 3 Purified sICAM-1 specifically inhibits CPE induced by picornaviruses that use the major-group rhinovirus receptor. Purified sICAM-1 (5 μg ml^{-1}) was plated on HeLa cells with the viruses indicated (100 $TCID_{50}$), and cytopathic effect determined after 4 days: HRV54, major-group rhinovirus; HRV2, minor group rhinovirus; Cox. A13, coxsackie A13, a picornavirus using a major-group receptor; Cox. B1, coxsackie B1, which does not use a major-group receptor; Polio, poliovirus I; HSV-1, herpes simplex virus type-1. Data are representative of three experiments.

bound ICAM-1 (mICAM-1): RR1/1, R6.5, and CL203 (data not shown). These antibodies bind to topographically distinct sites, as assayed by competitive binding (data not shown), and because they bind to sICAM-1, we infer that the overall conformation of native mICAM-1 is maintained.

We next tested whether sICAM-1 could inhibit rhinovirus infection in a quantitative *in vitro* assay for virus cytopathic effect (CPE)[3]. As shown in Fig. 2, sICAM-1 is a potent inhibitor of human rhinovirus strain 54 (HRV54), a major-group virus: sICAM-1 at 1 μg ml^{-1} (~18 nM) significantly inhibits CPE (by ~50%), and there is >90% inhibition at 10 μg ml^{-1}. By contrast, a control derived from column fractions adjoining the sICAM-1 peak has no effect.

The specificity of inhibition by sICAM-1 was investigated using rhinoviruses from both the major and minor subgroups, other picornaviruses, and herpes simplex virus type-1 (HSV-1), an unrelated enveloped DNA virus. As shown in Fig. 3, sICAM-1 inhibits HRV54, but has no significant effect on HRV2, a minor-group strain that does not use ICAM-1 as its cellular receptor. In addition, sICAM-1 inhibits infection by coxsackie A13, another picornavirus using the major-group receptor[12]. By contrast, sICAM-1 does not inhibit poliovirus, coxsackie B1 (picornaviruses that do not bind to cells through ICAM-1) or HSV-1. The specificity of virus inhibition indicates that sICAM-1 prevents infection not as a result of generalized effects on the cell's ability to support viral replication, but rather by inhibition of virion binding, as we expected. To confirm this directly, we measured the effect of sICAM-1 on virus binding to cells using [^{35}S]methionine-labelled virus and found that the sICAM-1 inhibition of HRV14 (from the major rhinovirus subgroup) binding is dose-dependent, whereas the control has no effect (Fig. 4). As shown previously[3], the positive control anti-ICAM-1 antibody R6.5 is also effective, whereas the negative control antibody CL203 (which binds to ICAM-1, but does not inhibit its function[3]) has no significant effect. Note that the virus-binding assay uses a higher concentration of virus than the CPE assay, which could account for the drop in inhibition compared with CPE.

Although sICAM-1 inhibits virion binding, it is unclear how many of the 60 potential binding sites on the virion[13] need to be occupied by sICAM-1 to block infection efficiently. In addition, the contribution of each of the five immunoglobulin-like domains of sICAM-1 needs to be assessed. Site-specific mutagenesis and truncation indicate that domains D1 and D2

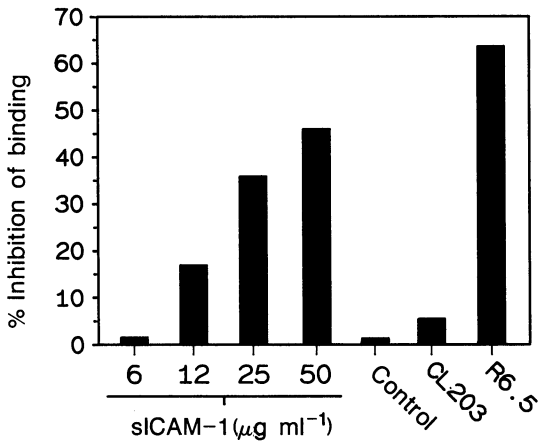

FIG. 4 Purified sICAM-1 inhibits the binding of rhinovirus virions to cells. [^{35}S]methionine-labelled HRV14 was mixed with sICAM-1, a chromatography-buffer control, or the anti-ICAM-1 monoclonal antibodies CL203 or R6.5 at 200 μg ml^{-1}. As previously shown, antibody R6.5 inhibits the interaction of ICAM-1 with either LFA-1 or major-group HRV, whereas antibody CL203 does not[3]. After preincubation for 30 min at 4 °C, the mixture was plated on HeLa cells, washed and counted for ^{35}S. Data are representative of two experiments.

METHODS. Radiolabelled rhinovirus binding was assayed using a modified method from ref. 20. Briefly, HeLa cells were infected with HRV14 for 4–6 h in methionine-free RPMI 1640 supplemented with 20 mM $MgCl_2$ and 2 mM glutamine, followed by incubation in the same medium with 2% fetal calf serum and 100 μCi ml^{-1} [^{35}S]methionine until a generalized cytopathic effect was observed (usually 18 h post-infection). After 3 cycles of freezing and thawing, virus in the supernate was precipitated with polyethylene glycol. Virus was then pelleted through a 30% sucrose step gradient (34,900 r.p.m. for 2 h in a Beckman SW41 rotor). Binding of radiolabelled virus (1×10^4 c.p.m.) to HeLa cells (confluent 24-well plates) was as described[20], except that cells were solubilized by sequential washes with 1% Triton X-100 and hot 9 M urea before scintillation counting. The total volume of virus and antibody was 90 μl; typically, 25–30% of ~10,000 input c.p.m. bound to cells.

contain the binding site for rhinovirus, and that this site overlaps with the site required for binding to LFA-1, the physiological receptor for ICAM-1 (D.E.S., manuscript in preparation). As large amounts of purified sICAM-1 can be prepared, it should be possible to resolve the three-dimensional structure of the molecule by X-ray crystallography. Finally, we need to establish how sICAM-1 might affect cell adhesion-dependent functions of the immune system.

The anti-rhinoviral activity of sICAM-1 makes it (or a derivative) an antiviral drug candidate. Soluble forms of CD4, the receptor for the human immunodeficiency virus, are now being tested for clinical efficacy against AIDS. Purified sICAM-1 should also be useful in the design and detection of anti-rhinoviral agents. The receptor binding site on the virus is conserved[13] and its mutation probably restricted: any mutation that destroys binding to ICAM-1 would be lethal unless there was compensatory binding to an alternative receptor. Thus, a drug directed at the ICAM-1–virus interaction would have the dual advantages of intervening at the sensitive first stage of virus infection, combined with restricting any escape from neutralization through the generation of drug-resistant variants. □

Received 27 November 1989; accepted 8 January 1990.

1. Sperber, S. J. & Hayden, F. G. *Antimicrob. Agents Chemother.* **32,** 409–419 (1988).
2. Greve, J. M. *et al. Cell* **56,** 839–847 (1989).
3. Staunton, D. E. *et al. Cell* **56,** 849–853 (1989).
4. Tomassini, J. E. *et al. Proc. natn. Acad. Sci. U.S.A.* **86,** 4907–4911 (1989).
5. Marlin, S. D. & Springer, T. A. *Cell* **51,** 813–819 (1987).
6. Rothlein, R., Dustin, M. L., Marlin, S. D. & Springer, T. A. *J. Immun.* **137,** 1270–1274 (1986).
7. Staunton, D. E., Marlin, S. D., Stratowa, C., Dustin, M. L. & Springer, T. A. *Cell* **52,** 925–933 (1988).
8. Dustin, M. L., Staunton, D. E. & Springer, T. A. *Immun. Today* **9,** 213–215 (1988).
9. Kishimoto, T. K. *et al. Adv. Immun.* **46,** 149–182 (1989).
10. Wawryk, S. O. *et al. Immunol. Rev.* **108,** 1035–106 (1989).
11. Springer, T. A., Dustin, M. L., Kishimoto, T. K. & Marlin, S. D. *A. Rev. Immun.* **5,** 223–252 (1987).
12. Colonno, R. J., Tomassini, J. E. & Callahan, P. L. *Positive-strand RNA Viruses* (eds Brinton, M. A. & Rueckert, R. R.) 92–102 (Liss, New York, 1987).
13. Rossman, M. G. *J. biol. Chem.* **264,** 14587–14590 (1989).
14. Kunkel, T. A. *Proc. natn. Acad. Sci. U.S.A.* **82,** 488–492 (1985).
15. Peterson, A. & Seed, B. *Cell* **54,** 65–72 (1988).
16. Mulligan, R. C. & Berg, P. *Proc. natn. Acad. Sci. U.S.A.* **78,** 2072–2076 (1981).
17. Mitchell, P. J., Urlaub, G. & Chasin, L. *Molec. cell. Biol.* **6,** 425–440 (1986).
18. Graham, F. L. & van der Eb, A. J. *Virology* **52,** 456–467 (1973).
19. Rothlein, R. *et al. J. Immun.* **141,** 1665–1669 (1988).
20. Abraham, G. & Colonno, R. J. *J. Virol.* **51,** 340–345 (1984).

ACKNOWLEDGEMENTS. We thank C. Cahill, D. Cipriano, D. Fortugno-Erickson, K. Last-Barney, S. Mayer and M. O'Neill for technical assistance. Monoclonal antibodies R6.5 and R6.1 were a gift from Dr R. Rothlein. This work was supported by Boehringer Ingelheim Pharmaceuticals Inc. and the NIH.