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Inhibition of intercellular adhesion molecule 1-dependent biological activities by a synthetic peptide analog

(leukocyte function-associated antigen 1/functional domain/sequence analysis)

JOHN V. FECONDO*†‡, STEPHEN B. H. KENT§, AND ANDREW W. BOYD*

*Lions Clinical Cancer Research Laboratory, Cellular Immunology Unit, The Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Melbourne, Victoria 3050, Australia; †Department of Applied Chemistry, Swinburne Institute of Technology, Hawthorn, Victoria 3122, Australia; and §Division of Biology, California Institute of Technology, Pasadena, CA 91125

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ABSTRACT **We have used a combination of hydropathy analysis of the intercellular adhesion molecule 1 (ICAM-1) sequence and dot-matrix comparison of the sequence with the homologous, but functionally distinct, protein myelin-associated glycoprotein to identify a putative functional binding region. One polar, and presumably surface-exposed, region of ICAM-1 showed no significant identity with myelin-associated glycoprotein. A synthetic peptide analog based on the sequence of this region (JF9) mimicked the inhibitory effects of the anti-ICAM-1 monoclonal antibody WEHI-CAM-1. These included inhibition of ICAM-1-dependent homotypic aggregation of Raji Burkitt lymphoma and phorbol-ester treated U937 cells at concentrations as low as 80 μg/ml (24 μM). In addition, at a concentration of 100 μg/ml, the peptide analog effectively inhibited cytotoxic cell activity, an ICAM-1-dependent effector function of the immune response. This simple method of sequence analysis may have general applicability to the identification of functional domains in homologous, but functionally distinct, proteins such as the translated products of gene families.**

The specific interaction between the two cell surface molecules intercellular adhesion molecule 1 (ICAM-1, CD54) and leukocyte function-associated antigen 1 (LFA-1, CD11a/CD18) has been shown to be a major adhesive mechanism in cells of the immune and hemopoietic systems (1). ICAM-1 is a 90-kDa integral membrane glycoprotein, with an extracellular region divided into five immunoglobulin-like domains of the C2 type, a transmembrane region, and a short cytoplasmic tail (2, 3). On the basis of these data and other structural similarities, ICAM-1 has been included in the immunoglobulin supergene family (2–4). The receptor for ICAM-1, LFA-1, belongs to the structurally different family of proteins termed integrins (5). The integrins are heterodimeric proteins, a number of which bind to an Arg-Gly-Asp motif in their ligands (6). There is no Arg-Gly-Asp sequence in ICAM-1 (2, 3), nor does the Gly-Arg-Gly-Asp-Ser-Pro peptide inhibit ICAM-1/LFA-1 binding (5–7).

To find amino acid sequences of possible functional importance within the ICAM-1 sequence, we compared a hydropathy analysis (8) of ICAM-1 with a dot-matrix sequence comparison (9) of ICAM-1 with the homologous but functionally distinct protein myelin-associated glycoprotein (MAG). We report the identification of a polar region within the fourth domain of the ICAM-1 sequence, which is presumably surface-exposed and shows minimal identity with MAG. We also show that a synthetic peptide analog based on this sequence mimics the effects of anti-ICAM-1 antibody in bioassays dependent upon ICAM-1/LFA-1-mediated adhesion.

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MATERIALS AND METHODS

Sequence Analysis. Hydropathy analysis of the ICAM-1 sequence was performed using the Kyte–Doolittle algorithm (8) with a window setting of 7 residues. Dot-matrix sequence comparisons were performed by using the method of Maizel and Lenk (9). Protein sequences were compared for simple identity versus nonidentity scoring of amino acids with a comparison length setting of 10 amino acids and a minimum match score of 4. The FASTA program (10) was used for short sequence homology searches of the NBRF, PRF-Japan, Swiss-Prot, and GenBank (translated) data bases¶ with a stringency set initially at ktup = 2 followed by ktup = 1.

Synthesis of Peptides. Peptides were synthesized using 4-methylbenzhydrylamine resin (United States Biochemical), or PAM-*t*-Boc-L-Pro resin (Applied Biosystems; PAM = 4-(oxymethyl)phenylacetamidomethyl, *t*-Boc = *t*-butoxycarbonyl) on an Applied Biosystems 430A automated peptide synthesizer using optimized solid-phase *t*-Boc chemistry protocols (11). An average coupling efficiency of >99.3% as determined by quantitative ninhydrin analysis (12) was achieved in all syntheses. After standard deprotection and HF cleavage from the resins (13), all peptides were purified to >95% by preparative reverse-phase HPLC using an Aquapore C_8 100 × 10 mm 20-μm Prep 10 cartridge column and analyzed on Aquapore RP-300 C_{18} 30 × 4.6 mm cartridge columns (Applied Biosystems). All peptides were characterized with respect to purity and complete covalent structure by analysis on a Sciex API-III mass spectrometer. Aqueous stocks of HPLC-pure peptides were sterilized by 0.22-μm filtration before use in the *in vitro* assays.

Monoclonal Antibodies. WEHI-CAM-1 and WEHI-B2 are both IgG2b monoclonal antibodies which recognize CD54/ICAM-1 (14) and CD21 (15), respectively. Both were purified on staphylococcal protein A-Sepharose 4B CL (Pharmacia) and eluted from the column with 0.1 M acetic acid, pH 3.0. Purity of the recovered antibodies was confirmed by SDS/polyacrylamide gel electrophoresis.

Cell Lines. All cell lines were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum.

Abbreviations: ICAM-1, intercellular adhesion molecule 1; LFA-1, leukocyte function-associated antigen 1; MAG, myelin-associated glycoprotein.

‡To whom reprint requests should be addressed at the Lions Clinical Cancer Research Laboratory, The Walter and Eliza Hall Institute of Medical Research.

¶Data bases are as follows: Protein Identification Resource (1990) Protein Sequence Database (Natl. Biomed. Res. Found., Washington), Release 26.0; PRF Protein Sequence Database (1990) (Peptide Inst., Protein Res. Found. Minoh, Osaka), Release 90/09; Swiss-Prot Protein Sequence Data Bank (1990) (Dept. Biochem. Med., Centre Medical Universitaire, Geneva and European Molecular Biology Laboratory, Heidelberg), Release 15; and GBtrans Protein Data Base (1990) (The Walter and Eliza Hall Inst. Med. Res., Melbourne, Australia), Release 9.0.

Raji is a cell line derived from a Burkitt lymphoma patient and has previously been shown to express both ICAM-1 and LFA-1 (1). GM209, a self-aggregating ICAM-1-negative small cell carcinoma line was generously provided by J. Layton (Ludwig Institute of Cancer Research, Melbourne). U937 cells were treated with phorbol 12-myristate 13-acetate (Sigma) to induce ICAM-1 expression as described previously (16, 17).

Aggregation Assay. HPLC-pure peptides (0–500 μg/ml) were added to 50,000 deaggregated Raji cells in RPMI 1640/5% (vol/vol) fetal calf serum in microtiter plate wells. Aggregation was assessed by microscopy over a 24-hr period. All scoring was performed by an observer who was blinded to the experimental design.

Cytotoxic Cell Assay. Peripheral blood mononuclear cells were activated in a two-way mixed lymphocyte response between two normal unrelated donors. The cells (10^6 per ml) were mixed in a 1:1 ratio and cultured in RPMI 1640/10% fetal calf serum for 4 days. Recombinant interleukin 2 (Cetus) was added (200 units/ml) and the cultures were continued for a further 3 days. Cells were harvested and added to a constant number (10^4 cells per well) of $^{51}CrO_4$-labeled K562 cells in a 96-well microtiter tray. ^{51}Cr release into the culture supernatants was measured after 4 hr.

RESULTS

Analysis of the ICAM-1 Sequence. The ICAM-1 sequence was first analyzed by using the Kyte–Doolittle hydropathy algorithm to predict hydrophilic potential binding regions (8). In Fig. 1*A* it can be seen that there are a number of predicted hydrophilic regions with hydropathy indices as low as −3.4, which contain clusters of both positive and negative charges. These polar regions are likely to be exposed on the surface.

The next step was to define which of these regions were most likely to be functional binding sites unique to ICAM-1. To do this, we sought such unique regions by a dot-matrix sequence comparison of ICAM-1 with the functionally distinct but homologous protein MAG (2, 19). Simple identity-versus-nonidentity scoring of amino acids is shown in the dot-matrix plot in Fig. 1*B*. A detailed analysis of the resulting plot revealed a region within the ICAM-1 sequence that had essentially no identity with MAG. This region spanned amino acid residues 369–391 and is indicated by a virtual absence of either isolated dots or strings of dots on the plot shown in Fig. 1*B*. When this sequence was analyzed in conjunction with the hydropathy plot data, there was a striking correspondence to a predicted polar surface region of the ICAM-1 sequence.

The uniqueness of this region of ICAM-1 (369–391) was further confirmed by FASTA short sequence homology searches (10) of a number of data bases with a stringency set initially at ktup = 2 followed by ktup = 1. These searches revealed no protein sequences with any significant identity to the ICAM-1 369–391 sequence (10).

Design and Synthesis of Peptides. Having defined this unique surface-exposed region of ICAM-1, we speculated that this may be critical for binding to LFA-1. To experimentally test this prediction, a 28-residue peptide analog spanning this sequence (JF9, 367–394 with Cys-378 replaced with Ala) and two control peptides of similar size, corresponding to the N and C termini (JF7B, 1–23; and JF13A, 479–507, respectively), were prepared by total chemical synthesis (Table 1). After purification and characterization, these peptides were tested for their ability to interfere with ICAM-1-mediated adhesion in several functionally distinct biological systems.

Effects of Synthetic Peptides on ICAM-1-Mediated Adhesion. Previous studies have shown that the anti-ICAM-1 monoclonal antibody WEHI-CAM-1 completely inhibits the homotypic aggregation of the Raji Burkitt lymphoma cell line

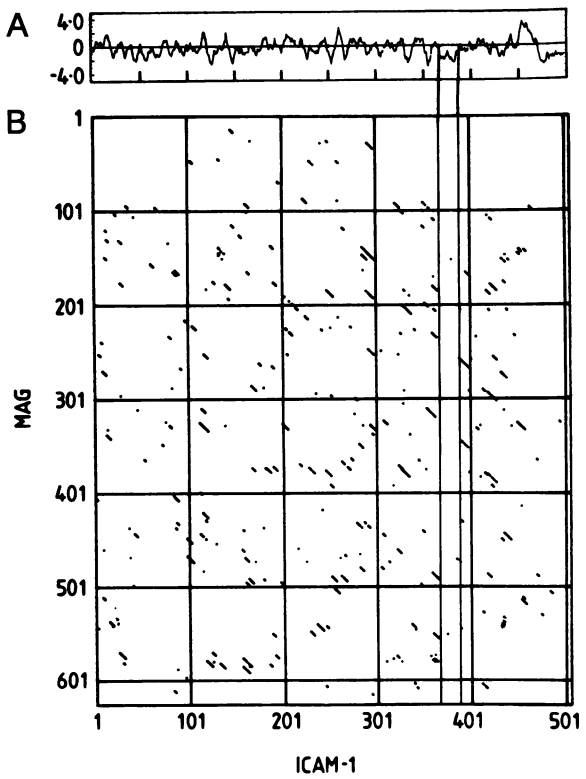

FIG. 1. Hydropathy and dot-matrix comparative sequence analysis of ICAM-1. The horizontal axes (residue number) of the two plots are to the same scale. (*A*) Hydropathy plot of ICAM-1 according to the method of Kyte and Doolittle (8) with a window setting of 7 residues. Negative numbers represent polar, presumably surface-exposed, regions of the polypeptide chain. (*B*) Dot-matrix sequence comparison of ICAM-1 (2, 3) with its homolog MAG (19), by the method of Maizel and Lenk (9). The sequences were compared for simple identity-versus-nonidentity scoring of amino acids with a comparison length setting of 10 amino acids and a minimum match score of 4. The lines extending from the dot matrix plot to the hydropathy plot indicate the correspondence between the region of nonidentity between the ICAM-1 and MAG sequences to a predicted polar and thus likely surface-exposed region of the ICAM-1 molecule.

(14). This effect could be reproduced with anti-LFA-1 antibodies (5), implying that the ICAM-1/LFA-1 linkage is critical for Raji cell aggregation. Testing of all three synthetic peptides over a concentration range of 0–500 μg/ml in the aggregation assay (see *Materials and Methods*) indicated that the ICAM-1 peptide based on the "unique" sequence (JF9) completely inhibited Raji cell aggregation at a concentration as low as 24 μM, while the two control peptides had no detectable inhibitory effect even at concentrations of 500 μg/ml (220 μM). Typical results are shown in Fig. 2. On the basis of a large number of experiments, we estimate that the IC_{50} value for the JF9 peptide lies in the range of 3–10 μM. Preliminary experiments with another ICAM-1 peptide analog (JF11, 415–439) also show no inhibitory effect on aggregation (data not shown). A time course study of the inhibition of cell aggregation by the JF9 peptide showed that the peptide still had significant inhibitory activity after 48 hr. The same pattern of inhibition was observed when the peptides were tested in an aggregation assay of phorbol myristate acetate-induced U937 cells (16, 17) (data not shown). In addition, homotypic aggregation of an ICAM-1-negative small cell carcinoma line, GM209, was not inhibited by any of the test peptides or by WEHI-CAM-1 antibody. None of the peptides were cytotoxic in any of the assays used in this work in the concentration ranges tested.

Table 1. Synthetic peptide analogs of ICAM-1

Peptide	Region of ICAM-1 spanned*	Sequence
JF7B	1–23	Asn-Ala-Gln-Thr-Ser-Val-Ser-Pro-Ser-Lys-Val-Ile-Leu-Pro-Arg-Gly-Gly-Ser-Val-Leu-Val-Thr-Cys-NH_2
JF9	367–394, [Ala-378]	Val-Leu-Tyr-Gly-Pro-Arg-Leu-Asp-Glu-Arg-Asp-Ala-Pro-Gly-Asn-Trp-Thr-Trp-Pro-Glu-Asn-Ser-Gln-Gln-Thr-Pro-Met-Cys-NH_2
JF13A	479–507	Asn-Arg-Gln-Arg-Lys-Ile-Lys-Lys-Tyr-Arg-Leu-Gln-Gln-Ala-Gln-Lys-Gly-Thr-Pro-Met-Lys-Pro-Asn-Thr-Gln-Ala-Thr-Pro-Pro-OH

*According to the sequence described by Simmons *et al.* (3).

Effects of Synthetic Peptides on ICAM-1-Dependent Cell-Mediated Cytotoxicity. Our previous studies with the WEHI-CAM-1 antibody have shown that ICAM-1 is essential for a number of cell–cell contact-mediated immune responses (14). Testing of the JF9 peptide and control peptides in a cytotoxic cell assay over a concentration range of 0–250 μg/ml demonstrated that the JF9 peptide was able to effectively inhibit cell-mediated killing of the K562 erythroid cell line at a concentration as low as 30 μM (100 μg/ml) (refer to Fig. 3). In contrast, the two control peptides had little or no effect on cytotoxic cell activity, even at a concentration of 250 μg/ml.

DISCUSSION

Using a combination of amino acid sequence hydropathy analysis and sequence comparison programs, we have identified a potential binding region in the ICAM-1 sequence (Fig. 1). We have shown that a synthetic peptide analog based on this sequence can interfere with ICAM-1-dependent processes *in vitro* (Figs. 2 and 3). In particular, the results obtained from the aggregation studies show that the JF9 peptide inhibits ICAM-1-dependent aggregation of cells, but not ICAM-1-independent aggregation (Fig. 2). Moreover, the JF9 peptide inhibits cytotoxic cell-mediated killing of K562 cells (Fig. 3), a process previously shown to require cell–cell contact mediated by the ICAM-1/LFA-1 interaction (14). In contrast, peptides JF7B and JF13A had no inhibitory effects in this assay. These results suggest that we have identified a functional binding domain within the ICAM-1 sequence.

It is of interest that the active synthetic peptide analog used in this work corresponds to a sequence located within the predicted fourth immunoglobulin-like domain (D4) on the ICAM-1 molecule (2). Staunton *et al.* (20) using domain deletion and systematic amino acid substitution techniques, have suggested that the LFA-1 binding region is located within domains 1 and 2 (D1 and D2) of ICAM-1. Moreover, the homologous ICAM-2 molecule, which also binds to LFA-1, has only three immunoglobulin-like domains, corresponding to D1, D2, and D3 of the ICAM-1 molecule (21), and lacks the unique region in ICAM-1 defined by our studies. These data suggest that interaction of ICAM-1 with LFA-1 involves multiple regions of the protein. While the mutagenesis data of Staunton *et al.* (20) do not cover the D4 region of the molecule, their data do imply that binding is mediated by

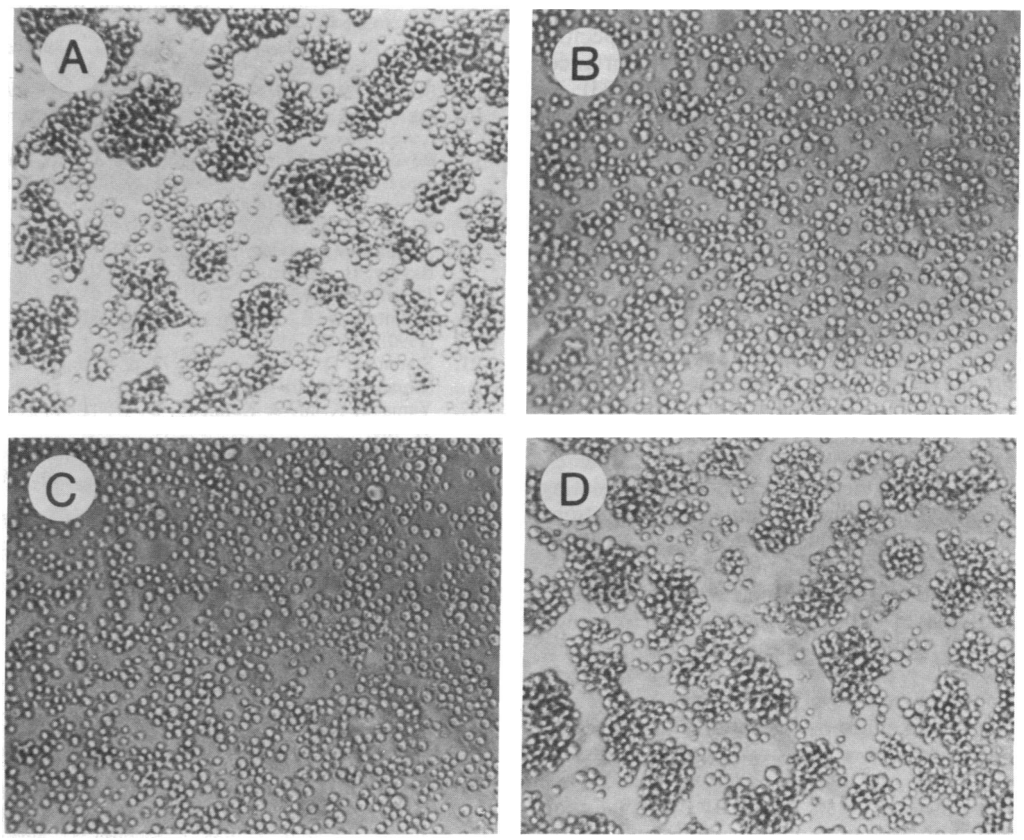

FIG. 2. Inhibition studies of ICAM-1/LFA-1-dependent homotypic aggregation of Raji cells. (*A*) Raji cells with no peptide present. Aggregation is clearly visible. (*B*) Inhibition of Raji cell aggregation by purified WEHI-CAM-1 antibody at 20 μg/ml. (*C*) Inhibition of Raji cell aggregation by JF9 peptide at 80 μg/ml. (*D*) Raji cells in the presence of control peptide JF7B at 500 μg/ml. Aggregation is clearly visible. Cells were examined after 8 hr of incubation. (×100.)

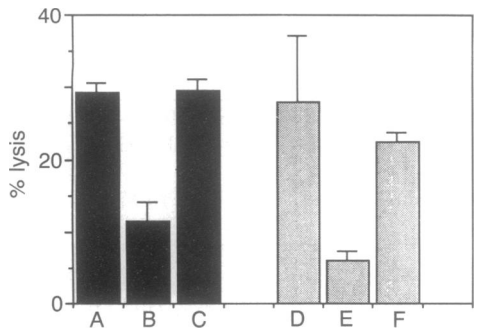

FIG. 3. Inhibition of cytotoxic cell activity by the JF9 peptide. Cell-mediated killing was measured by the release of ^{51}Cr into the medium as described previously (14). Error bars represent SEM. Bar A, negative control, no peptide or antibody present. Bar B, positive control, purified WEHI-CAM-1 antibody added at 20 μg/ml. Bar C, negative control antibody, purified WEHI-B2 antibody added at 20 μg/ml. Bar D, JF7B added at 100 μg/ml. Bar E, JF9 added at 100 μg/ml. Bar F, JF13A added at 100 μg/ml.

discontinuous segments of the ICAM-1 sequence. The inhibitory properties of the unique peptide (JF9) show that the region of the ICAM-1 molecule identified in this study is apparently a significant component of the binding process.

The peptide analog (JF9) completely inhibited ICAM-1-dependent cell adhesion at a concentration of 24 μM, indicating an IC_{50} of ≤10 μM. In contrast, the Gly-Arg-Gly-Asp-Ser peptide inhibition of fibronectin-mediated adhesion is only half-maximally effective at 78 μM (22). Synthesis and testing of a panel of shorter, overlapping peptides and isosteric analogs should identify which residues within this region of the ICAM-1 molecule are critical to the adhesion process.

Several studies using monoclonal antibodies directed against both ICAM-1 and LFA-1 have shown the specific interaction of these two molecules to be of critical importance in the homotypic adhesion of hemopoietic cells (17, 23), in migration of lymphocytes (24, 25), in cell–cell contact-mediated immune mechanisms such as T- and B-cell activation (14, 26, 27), and in certain effector functions of the immune response, including T-cell cytotoxicity and nonspecific cytotoxic cell activity (14, 26, 28). In addition to having this pivotal role in cell–cell contact-mediated immune mechanisms (14, 26, 27), ICAM-1 serves as a receptor for the major group of human rhinoviruses (29, 30) and *Plasmodium falciparum*-parasitized erythrocytes (18), raising the possibility that ICAM-1 might be a target for therapy. Blocking of ICAM-1 function may be beneficial in the treatment of diseases such as autoimmune disorders, graft rejection, the common cold, and malaria. It has now been shown that polypeptide analogs of the Arg-Gly-Asp motif can block complex *in vivo* adhesion processes such as those mediating tumor metastasis (31). Given the low molar concentrations required for inhibition by JF9, this peptide could form the basis for the design of ICAM-1-blocking drugs.

Apart from the functional significance of these observations with respect to ICAM-1, the approach used in this study has general applicability to the identification of functional domains in homologous proteins that have distinct biological activities. Our approach relies on two assumptions: (*i*) the functional domain(s) is likely to be hydrophilic and (*ii*) there will be no significant sequence identity in the domain(s) to other related but functionally distinct proteins. This method of combining a graphical analysis of the hydropathic character of a protein with sequence comparisons is a simple means for fingerprinting putative functionally important segments of biologically active proteins. The results obtained can be subjected to direct experimental verification by chemical peptide synthesis (as used here) or by genetic engineering with site-directed or deletion mutational analysis.

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