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ICAM-2 peptides mediate lymphocyte adhesion by binding to CD11a/CD18 and CD49d/CD29 integrins

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Three fifteen-amino-acid polypeptides designated peptides 1, 2 and 3 were synthesised as likely candidates for mimicking the role of ICAM-2 as a ligand. The ability of each peptide to bind lymphoid cells was tested. Peptide 2 largely mediated cell attachment of unstimulated cells and this binding was only marginally increased by stimulating the cells with phorbol dibutyrate ($P(Bu)_2$). Peptide 3 mediated minimal spontaneous cell attachment, but this binding was significantly enhanced following $P(Bu)_2$ stimulation. Peptide 1 had no effect on cell attachment with or without stimulation. The cell attachment to peptide 2 was both temperature- and cation-dependent. Studies using specific monoclonal antibodies showed that with unstimulated cells, anti-VLA-4α(CD49d) or β chain (CD29) antibodies (KD4-13 and 4B4) and anti-CD18 (1B4) each partially inhibited the cell binding. Monoclonal antibodies against CD54 (ICAM-1; 84H10 or LB2), MHC class I (W6/32) and control mouse IgG had no effect. When anti-CD29 and anti-CD18 monoclonal antibodies were used concurrently, there was almost complete inhibition of the cell attachment. These observations indicated that cell adhesion via ICAM-2 is mediated: (i) predominantly by peptide 2 in unstimulated and $P(Bu)_2$-stimulated cells, and also, to some extent, by peptide 3 in $P(Bu)_2$-stimulated cells and (ii) by binding to both CD11/CD18 and CD49d/CD29 integrins.

Cell adhesion; Integrin; CD11a/CD18; CD49d/CD29; Phorbol ester; Cell attachment assay; Monoclonal antibody; ICAM-2

1. INTRODUCTION

ICAM-1(CD54) is a cell surface adhesion molecule member of the immunoglobulin superfamily [1,2]. This molecule is found on many cell types and its expression on vascular endothelium is strongly up-regulated by the inflammatory cytokines such as; interleukin-1 (IL-1), tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) both in vitro and in vivo [3]. Its receptor is CD11a/CD18, one of a trio of heterodimeric molecules known as the β2 integrins or the CD11/CD18 molecules.

Although the CD11a/CD18-CD54 pathway plays an important role in lymphocyte adhesion to entothelial cells, monoclonal antibodies that block CD11a/CD18 only partially inhibit this adhesion [4,5]. This and several other lines of evidence have suggested the existence of a second CD11a/CD18 ligand. Homotypic adhesion of one cell line was inhibited by a monoclonal antibody to CD11a/CD18 but not by one to ICAM-1 [6]. In addition, there also exists a CD11a/CD18-dependent, ICAM-1-independent pathway of adhesion to endothelial cells [4] and there are some types of target cells in which CD11a/CD18 dependent T-lymphocyte adhesion and lysis are independent of CD54 [7]. Furthermore, in patients with a genetic deficiency of CD18 and hence of CD11a/CD18, lymphocyte recruitment into inflammatory sites is aparently normal, despite profound defects in recruitment of phagocytic cells [8], indicative of an as yet uncharacterised lymphocyte migration pathway in vivo and in vitro. Recently, an alternative CD11a/CD18-CD54-independent leukocyte homotypic adhesion pathway mediated through CD49d/CD29 has been defined [9].

A putative second ligand is designated ICAM-2. cDNA sequencing has indicated that ICAM-2 is an integral membrane protein with two immunoglobulin-like domains, whereas ICAM-1 has five such domains [1,2,10]. ICAM-1 and ICAM-2 are more closely related to each other (there being 34% homology between the two most N-terminal domains of ICAM-1 and ICAM-2) than to the other members of the immunoglobulin superfamily, demonstrating the existence of a sub-family of immunoglobulin-like ligands that bind the same integrin receptor. Significantly, the CD11a/CD18 binding region of ICAM-1 has been mapped to domains 1 and 2 by domain deletion and systematic amino acid substitution, thus the homology is both structural and functional [11].

The differences between ICAM-1 and ICAM-2 may be of potential functional importance. ICAM-1 is inducible on most cell lines using cytokines, whereas

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ICAM-2 expression is unaffected [10]. Steric and size differences may be important. The three additional domains on CD54 are expected to project its CD11a/CD18 binding site further from the cell surface than that of ICAM-2, suggesting that closer cell-cell contact would be required to effect CD11a/CD18-/ICAM-2 contact than the CD11a/CD18/ICAM-1 [11].

The binding of CD54 to CD11a/CD18, a β2 integrin, is temperature- and cation-dependent [12]. It has also been shown that the β2 integrins are not involved in binding of Jurkat or Ramos cells to cytokine-activated human umbilical vascular endothelial cells (HUVEC) as assessed by the inability of a CD18 monoclonal antibody (60.3) to inhibit the intercellular adhesion [9]. CD49d/CD29, on lymphoid cells, and VCAM-1, another member of the immunoglobulin superfamily similar to CD54 and expressed by endothelial cells, mediate this adhesion [13]. Interestingly, it has been reported that high levels of CD29, the β1 subunit shared by the VLA group of integrins are characteristic of a subset of lymphocytes enriched in chronic inflammatory sites such as the synovia of patients with rheumatoid arthritis patients [14,15]. In the present study, the ability of the synthetic peptides which were predicted from the nucleotide sequence and based on hydrophobicity, to mimic the function of ICAM-2 were studied to identify which specific regions were involved in this particular cell-cell adhesion. The data suggest that both domains of ICAM-2 can mediate adhesion, though domain 2 appears to be more relevant. Domain 1 seems to mediate adhesion following cell activation. Furthermore, the results show that ICAM-2 peptides interact not only with CD11a/CD18, but also with CD49d/CD29.

2. MATERIALS AND METHODS

2.1. *ICAM-2 peptides*

Three fifteen-amino-acid polypeptides were synthesised and purified to 99% purity by Peptide and Protein Research (PPR), University of Reading, UK. Peptide binding to plates was measured by monoclonal antibodies.

2.2 *Monoclonal antibodies*

Monoclonal antibody 4B4 (CD29) and control mouse IgG, both purified IgG were purchased from Coulter Immunology, Luton, UK. Monoclonal antibody 1B4 (CD18) was kindly provided by Dr S. Wright (Rockefeller Univ., NY). Monoclonal antibody W6/32 (MHC class 1) was used as ascites fluid and purchased from Sera Lab (London, UK). KD4-13 (anti-CD49d or anti-VLA-4α) and 84H10 (anti-ICAM-1) were kindly provided by S. Shaw (NIH, Bethesda, MD).

2.3 *Cell attachment test*

The peptides were reconstituted in the coating buffer at a 10 mg/ml concentration and used finally, at (100 μl; 30 μg/well) by adding to the ELISA plates (Maxisorb, Nunc, Germany) which were incubated at room temperature overnight. At the end of the incubation, the peptide solution was shaken out and the plate was washed once with PBS (100 μl/well). Blocking solution containing human serum albumin (HSA) (0.2% w/v) in PBS was then added (100 μl/well) to saturate the unbound sites and incubated for 2 h at room temperature. At the end of this incubation, the plate was washed 4 times in PBS and allowed to drain.

A lymphoblastoid B-cell line (LCL) called NAD 20 was used in this assay. Immunofluoresence flow cytometry studies demonstrated that CD11a/CD18 and CD49d/CD29 are expressed on 90 and 70% of the cells, respectively (data not shown). Cells were split one day prior to the day of assay, harvested, washed twice with PBS and counted. They were then resuspended in RPMI medium (0.5 × 10⁶/ml) containing BSA (0.2% w/v). The cell suspension (2 ml) was then aliquotted into tubes or added to the coated plate which had been equilibrating in RPMI medium containing BSA (0.2% w/v). The plate was incubated at 37°C for 1 h.

At the end of this incubation, the plate was washed 4 times first with RPMI containing 0.2% BSA, and then with PBS. The cells attached were observed under the microscope and then fixed by adding 3% (w/v) paraformaldehyde for approximately 15 min. Filtered Toluidine blue dye (Merck, 0.5% w/v in 3.7% v/v formaldehyde) was added for a minimum of 1 h at room temperature. The plate was then washed with copious amounts of distilled water. The attached cells can now be counted by observing through the microscope or quantified in a microplate reader at 690 nm by first releasing the blue dye by lysing the cells with 2% (w/v) SDS [16].

In another experiment, the cells were treated with 4 β-phorbol 12,13-dibutyrate ($P(Bu)_2$) (Sigma) dissolved in dimethyl sulfoxide (60 nM final concentration) for 10 min. After this time, the $P(Bu)_2$-treated and non-treated cells were added to the plate and the experiment repeated as above. The inhibition experiments were performed by adding the monoclonal antibodies (10 μg/ml) to the cells in a tube and incubating for ten minutes at 37°C and then performing the cell attachment test in the same manner as above.

2.4. *Specificity of the assay*

The specificity of ICAM-2 peptide-cell adhesion was studied for cation-dependence using 10 mM EDTA (Kebo, Stockholm) and temperature requirement by incubations at 37 or 4°C. Furthermore inhibition of adhesion was tested in the continuous presence of monoclonal antibodies directed against several well-defined adhesion receptors.

3. RESULTS AND DISCUSSION

Three peptides were selected based on computer predictions as likely candidates for antigenicity to raise antibodies for functional and structural studies of ICAM-2. The peptides shown in Fig. 1 are designated peptide 1, 2 and 3. The sequences indicate similarity between peptides 2 and 3. In studies of the mixed lymphocyte reaction, peptides 1 and 3 singly or in combination inhibited. Peptide 2, in contrast, enhanced this reaction. Furthermore, the inhibitory role of peptides 1 and 3 was always abrogated in the presence of peptide 2 (R.S. and M.W.M., manuscript in preparation). These results suggested functional differences in the three peptides.

To further elucidate the roles of the peptides they were tested in a cell adhesion assay. The results in Fig. 2a show that peptide 2 mediates most of the cell binding. However when cells are treated with $P(Bu)_2$, the adhesion mediated by peptide 3 was significantly increased. Phorbol esters are known to induce CD11/CD18-dependent leukocyte adhesion [17]. Peptide 1 did not mediate adhesion whether the cells are untreated or $P(Bu)_2$-treated. The data suggest that a major adhesion binding site on ICAM-2 is located on peptide

THE ICAM-2 PEPTIDES PREDICTED FROM cDNA SEQUENCE

Peptide 1 (PGI)

GNIFHKHSAPKMLEI

Peptide 2 (PGP)

GKSFTIECRVPTVEP

Peptide 3 (PLN)

LQCHFTCSGKQESMN

PEPTIDES 2 AND 3 OF ICAM-2

GKSFTIECRV PTVEP (PGP)

LQCHFTCSGKQESMN (PLN)

The probable location of the epitope

Fig. 1. The amino acid sequences of the three peptides used in the cell attachment assay. There is a similarity between peptides 2 and 3. This similarity may indicate the probable binding site.

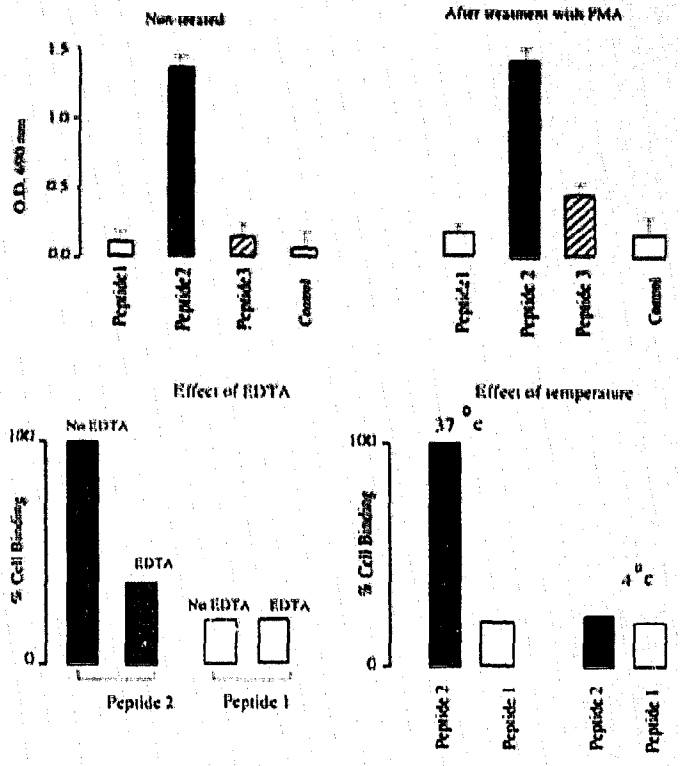

Fig. 2. (a) The binding of the lymphoblastoid NAD-20 cells to the various ICAM-2 peptides. (b) The effect of EDTA and temperature on cell attachment to peptide 2. Peptide 1 was used as the negative control.

2 which is found on the second immunoglobulin-like domain. When cells are activated with $P(Bu)_2$, cell attachment can be mediated to some extent by peptide 3 which is located on domain one of the ICAM-2 molecule. Thus there are two independent adhesion mediating sites, located on the two domains which appear to function depending on the activation status of the adhering cell. Polysera that bound peptide 1 failed to inhibit in the cell aggregation assay, confirming that this peptide has no role in adhesion (unpublished data) and was therefore used as a control in the present study.

The specificity of adhesion was studied for both temperature and cation-dependence. The results in Fig. 2b show that EDTA inhibited adhesion. Furthermore adhesion occurred only at 37°C and was completely abolished at 4°C. Both features are characteristic of integrin-receptor mediated adhesion [12,17].

To test the integrins involved in this cell attachment a panel of monoclonal antibodies against various cell surface molecules was tested. We expected that anti-CD18 mab would completely inhibit the cell attachment since ICAM-2 is a known ligand for CD11a/CD18 [10]. The latter molecule is the major $\beta 2$ integrin of NAD-20 cells (only 2% and 2.7% of the cells express CD11b/CD18 and CD11c/CD18, respectively [18]). However, the results in Fig. 3 show that anti-CD18 always partially inhibited. Anti-CD29 also partially inhibited. Furthermore, anti-VLA-4α mab inhibited to the same extent as the anti-CD29 mab. The combination of both anti-CD18 and anti-CD29 almost completely inhibited adhesion. Mabs against other cell surface molecules such as CD54 (ICAM-1) and MHC Class I were not inhibitory. This observation suggests that adhesion mediated by peptide 2 is not only via binding to the CD11a/CD18 molecule but also to the CD49d/CD29 (VLA-4) molecule. Therefore the ligand ICAM-2 uses two integrin receptors to mediate adhesion.

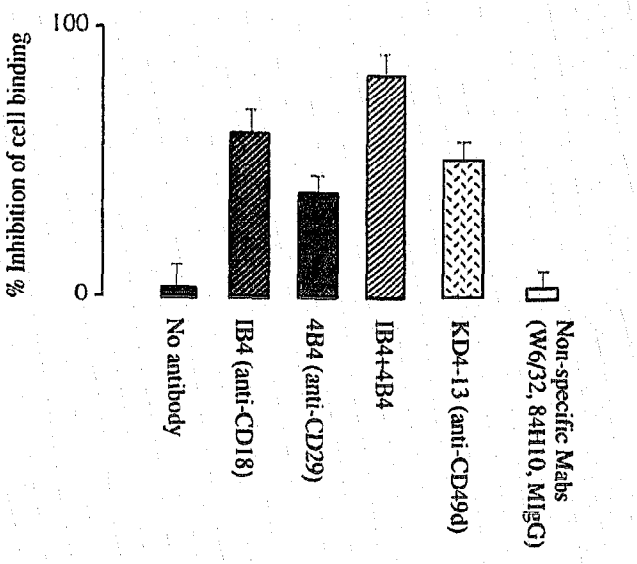

Fig. 3. The inhibition of cell binding to peptide 2 by a panel of antibodies. MIgG is mouse immunoglobulin.

Several lines of evidence have suggested the existence of a second CD11a/CD18 ligand designated ICAM-2. This ligand was cloned, and sequencing indicated an integral membrane protein with two immunoglobulin-like domains [10]. Recently, antibodies to recombinant ICAM-2 have confirmed that native ICAM-2 is a cell surface glycoprotein with an apparent MW of 55 000 expressed by vascular endothelium and some leukocytes [19]. To date, its functional role in antigen-specific T-cell recognition remains poorly defined. We have used a novel approach of using the synthetic peptides, predicted from its cDNA sequence, to mimic its functional role as a ligand in order to identify the sites involved in cell adhesion.

The results indicate that ICAM-2 has a minimum of two binding sites located on each of the two immunoglobulin-like domains. A major adhesion site for cells is on domain 2 since it accounts for most of the binding of unstimulated and $P(Bu)_2$-stimulated cells. With cell activation following $P(Bu)_2$, domain 1 also mediates adhesion. Peptides 2 mediates adhesion by interacting with CD11a/CD18 which is expected since ICAM-2 was identified in this way. The finding that the peptide also interacts with CD49d/CD29 is novel and may be due to structural homology between ICAM-2 and VCAM-1, since both molecules are members of the immunoglobulin superfamily.

The binding of the integrin receptors to the peptide seems to be an active cellular process. We have found that binding of cells to ICAM-2 peptides via CD49d/CD29 and CD11a/CD18 molecules is a temperature-sensitive and cation-dependent process, consistent with the interaction of ICAM-2 with an integrin-like receptor.

CD49d/CD29 and CD49e/CD29 integrins both bind to fibronectin (FN) [20,21], and CD49d/CD29 also recognises a sequence on FN which has previously been shown to be recognised by a variety of neural crest cells [22,23]. Recently CD54 has been shown to be a ligand for both CD11a/CD18 and CD11b/CD18 [24]. There are striking similarities between CD11a/CD18 and CD49d/CD29-integrins. The cell binding is enhanced in both by $P(Bu)_2$ treatment. Increased binding is not accompanied by increased cell surface expression of the receptors but by a change in receptor affinity for the ligand. The significance of the differential usage of the same ligand by two integrin receptors remains unclear but is consistent with the promiscuous nature of integrin receptors and the functional diversity which is necessary in this recognition system.

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