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Servei d'Immunologia[▲], Hospital Clínic, Barcelona, Division of Basic Sciences[◇], Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver and Committee on Immunology[■], Department of Pathology, Harvard Medical School and Center for Blood Research, Boston

CDw50 and ICAM-3: Two names for the same molecule*

CDw50 differentiation antigen is a molecule broadly expressed on hematopoetic cells but not on other cells. Previous experiments showed that CDw50 monoclonal antibodies (mAb) inhibited primary mixed lymphocyte culture (MLC). To understand the function of CDw50 better, we purified it and obtained peptide sequence. At the same time, intercellular adhesion molecule (ICAM)-3, the third ligand of lymphocyte function-associated molecule 1, was described by mAb and subsequent cDNA cloning. Immunochemical, functional, and protein sequencing studies show that ICAM-3 and CDw50 are the same glycoprotein, a 120-kDa surface molecule with presumably an important role in the immune responses.

1 Introduction

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At the same time, the study of the lymphocyte function-associated molecule 1 (LFA-1), an integrin that mediates a wide range of leukocyte interactions with other cells in immune and inflammatory responses, led to the discovery of its ligands, intercellular adhesion molecule (ICAM)-1, -2 and -3 [3–6]. Anti-ICAM-3 mAb have been raised [6], and recently the molecule has been cloned [7–9]. ICAM-3 was found to be a member of the immunoglobulin (Ig)-like supergene family, containing five Ig-like domains that are highly homologous to those found in ICAM-1 and ICAM-2. The pattern of reactivity of the CDw50 and ICAM-3 mAb seemed to be strikingly similar with a few differences. Both studies [2, 5] indicated that CDw50 and ICAM-3 may be restricted to hematopoetic cells and that both molecules have a similar molecular mass (M_r) of approximately 120–130 kDa.

[I 11533]

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Key words: CDw50 / ICAM-3 / LFA-1

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2 Materials and methods

2.1 Monoclonal antibodies

The following murine mAb to human antigens were used: CBR-IC3/1 (anti-ICAM-3, IgG1) [6], CBR-IC3/2 (anti-ICAM-3, IgG2a) [9], 101-1D2 (anti-CDw50, IgG1) [2], 140-11 (anti-CDw50, IgG2b) [2], 152-2D11 (anti-CDw50, IgG1), Cris-1 (anti-CD5, IgG2a) [10] and 134-2C2 (IgM, CD26) [11], W6/32 (anti-HLA-A, B, C, IgG2a) [12], TS1/22 (anti-CD11α, IgG1) [13], NS1 and nonbinding control mAb X63 (IgG1).

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PBMC and cell lines (10^6/0.1 ml) were washed with IF buffer (PBS containing 0.02 mM NaN_3 and 1% BSA) and incubated with mAb at saturating concentration for 30 min at 4 °C, washed twice with IF buffer, and then incubated with FITC-conjugated goat anti-mouse Ig (Sigma, St. Louis, MO) for 30 min at 4 °C. Cells were analyzed on a FACStar-plus (Becton Dickinson, Mountain View, CA). In the cross-blocking experiment with CDw50 and ICAM-3 mAb, PHA-stimulated PBMC were first incubated with saturating concentrations of each antibody, washed twice with IF buffer, and then stained with fluorescein-conjugated CDw50 mAb. The Cris-1 (a CD5 mAb) and NS1 ascites were utilized as controls. The positive cell percen-

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tages were established by marker positioning so that negative control gives less than 2% positive cells. Immunofluorescence FCM of L cell transfectants was performed on an EPICS V analyzer (Coulter Diagnostics, Hialeah, FL) as previously described [5].

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For radiolabeling with ^{125}I, PBMC were surface labeled with ^{125}I using Iodogen (Pierce, Rockford, IL) [14]. After labeling, 2.5×10^7 cells were lysed in 1 ml lysis buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP40, 1 mM NaF, 3 mM NaN_3, 1 mM benzamidine HCl, 10 mM iodoacetate, 1 mM PMSF, 20 μg/ml egg white trypsin inhibitor, 1 μg/ml chymostatin, 1 μg/ml leupeptin and 1 μg/ml pepstatin) for 45 min on ice. Nuclei insoluble debris were removed by centrifugation at 14000 × g for 10 min and lysates precleared with normal rabbit serum, plus an unrelated mAb and protein A-Sepharose (Pharmacia). All preclearing reagents were prewashed in lysis buffer. Immunoprecipitation was performed with the indicated mAb (Fig. 1) for 1 h at 4 °C and immune complexes fixed on rabbit anti-mouse immunoglobulins (Dako, Denmark) pre-coupled to protein A-Sepharose, washed five times with washing buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 8.0, 0.05% NP40 and 0.05% SDS) and eluted with 2 × SDS sample buffer and then analyzed on 8% polyacrylamide slab gels [15]. For sequential immunoprecipitation studies ^{125}I-labeled PBMC lysates were precleared six times with one of either 101-1D2, CBR-IC3/1, CBR-IC3/2 or 134-2C2 mAb and immunoprecipitated with each of the other three remaining mAb. The immune complexes were fixed on rabbit anti-mouse immunoglobulins (Dako) pre-coupled to protein A-Sepharose, washed, eluted with SDS sample buffer and analyzed on 10% polyacrylamide gels, as mentioned above. SDS-PAGE molecular weight standards (Bio-Rad, Richmond, CA) were used. Gels were stained with Coomasie blue, dried and exposed to Kodak X-Omat AR films with Dupont intensifying screens at − 80 °C.

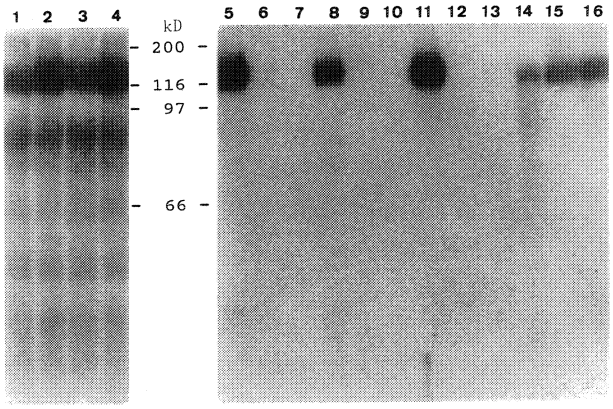

Figure 1. Sequential immunoprecipitation analysis. Lysates from ^{125}I-labeled PBMC were precleared six times with one of either 101-1D2 (anti-CDw50), CBR-IC3/1 (anti-ICAM-3), CBR-IC3/2 (anti-ICAM-3) or 134-2C2 (anti-CD26) mAb, and immunoprecipitated with each of the other three remaining mAb. Lanes 1–4: immunoprecipitates with 134-2C2, 101-1D2, CBR-IC3/1 and CBR-IC3/2 mAb, respectively. Lanes 5–7: immunoprecipitates with 101-1D2 mAb after preclearing with 134-2C2, CBR-IC3/1 or CBR-IC3/2 mAb, respectively. Lanes 8–10: immunoprecipitates with CBR-IC3/1 mAb after preclearing with 134-2C2, 101-1D2 or CBR-IC3/2 mAb, respectively. Lanes 11–13: immunoprecipitates with CBR-IC3/2 mAb after preclearing with 134-2C2, 101-1D2 or CBR-IC3/1 mAb, respectively. Lanes 14–16: immunoprecipitates with 134-2C2 mAb after preclearing with 101-1D2, CBR-IC3/1 or CBR-IC3/1 mAb, respectively. All immunoprecipitates were analyzed by 10% SDS-PAGE and subjected to autoradiography. Molecular mass of standards are shown between lanes 4 and 5.

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ICAM-3^+ L cell stable transfectants were generated as described [16, 17]. Briefly, murine L cells were transfected by calcium phosphate with ICAM-3 cDNA in Ap^rM9 [9] (pCDIC-3; 8 μg) and pneo plasmid containing the G418 selection marker (100 ng). Transfectants were selected and maintained in a selection media that consists of 10% FBS/DMEM containing 400 μg/ml G418 (Geneticin, Gibco Laboratories, Grand Island, NY). Untransfected L cells are maintained in DMEM without G418. L cells were detached with 10 mM EDTA/HBSS, washed three times in 10% FBS/DMEM, and then used for either flow cytometric analysis or binding to LFA-1-coated plates.

2.5 Adhesion assay

Adherence of L cell transfectants to purified LFA-1 absorbed onto 96-well polystyrene microtiter plate was as previously described [5, 17]. Briefly, fluoresceinated L cells were resuspended in PBS, 2 mM $MgCl_2$, 5% FBS (assay media) at a concentration of 10^6 cells/ml. Cells were pretreated with 20 μg/ml of purified mAb for 30 min at room temperature, and 5×10^4 cells were transferred to each well. Some wells were pretreated for 30 min at room temperature with 20 μg/ml of purified TS1/22 (anti-LFA-1a mAb). Cells were allowed to settle and adhere to the solid-phase LFA-1 for 1 h at 37 °C. Unbound cells were washed with four aspirations through a 25-gauge needle followed by two aspirations through a 23-gauge needle. Bound cells were quantitated in the 96-well plates using a Pandex fluorescence concentration analyzer (IDEXX Corp., Westbrook, ME). The number of LFA-1 sites/microtiter well was determined using saturating amounts of ^{125}I-labeled TS1/22 mAb [18]. Assuming monovalent binding of the mAb [18], the density was 700 sites/μm^2.

2.6 Peptide sequence of CDw50

CDw50 protein purification was based on a method previously described with some modifications [19]. Briefly, CDw50 was affinity purified from 8×10^9 PBMC lysed with 80 ml of 0.5% NP40 lysis buffer (with protease inhibitors) on a 101-1D2 mAb-coupled immunoaffinity column. The column was washed with 150 ml of lysis buffer and 15 ml of 1 M $MgCl_2$. CDw50 protein was selectively eluted with 4 M $MgCl_2$. After concentration (Centricon 30, Amicon, Beverly, MA) the sample was submitted to SDS-PAGE on an 8%-gel, under reducing conditions, and semi-dry electroblotted (4 mA/cm^2) with tricine transfer buffer (39 mM tricine, 48 mM Tris-base, 1.3 mM SDS and 20% methanol) to nitrocellulose, the band was visualized by Ponceau S, and

Table 2. Cross-competition of CDw50 and ICAM-3 mAb[a)]

Second incubation	First incubation (ascites fluid or purified mAb)						
	Cris-1	101-1D2	140-11	152-2D11	CBR-IC3/1	CBR-IC3/2	NS1
Cris-1	30	92	90	90	90	92	92
101-1D2	99	20	99	99	99	10	99
152-2D11	99	99	99	50	99	99	99

a) PHA-stimulated PBMC were first incubated with saturating concentrations of each antibody and the second incubation performed with affinity-purified fluorescein-conjugated mAb. Cris-1 (a CD5 mAb) and NS1 mAb-containing ascites fluid were utilized as controls. The positive cell percentages were established by marker positioning so that negative background gives less than 2% positive cells on a FACStar-plus (Becton Dickinson).

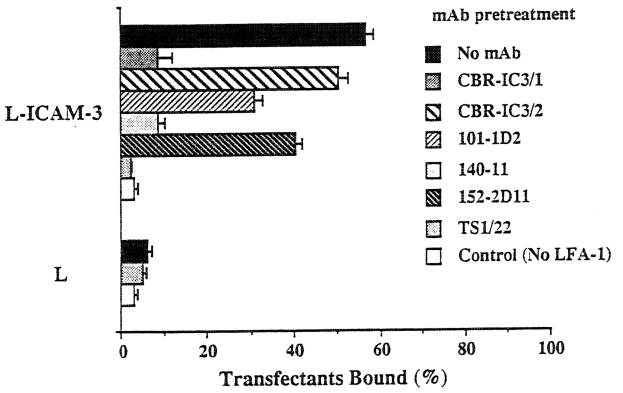

Figure 3. Adhesion of ICAM-3 transfected cells to purified LFA-1. Murine L-cells either stably transfected with ICAM-3 cDNA or untransfected were allowed to bind to LFA-1-coated microtiter wells for 60 min at 37 °C and washed four times by aspiration through a 25-gauge needle followed by two aspirations through a 23-gauge needle. Control wells lacked LFA-1. Cells were pretreated with saturating concentrations of one of the following mAb: W6/32 (anti-HLA, A, B, C), CBR-IC3/1 (anti-ICAM-3), CBR-IC3/2 (anti-ICAM-3), 101-1D2 (anti-CDw50), 140-11 (anti-CDw50), or 152-2D11 (anti-CDw50). Alternatively, the absorbed purified LFA-1 was pretreated with mAb TS1/22 (anti-LFA-1α). One of four representative experiments is shown and errors bars indicate one standard deviation.

CDw50 mAb recognize the expressed ICAM-3 cDNA and are capable of inhibiting LFA-1:ICAM-3 interactions.

Lastly, comparison between the deduced amino acid sequence derived from the ICAM-3 cDNA and the sequence of three peptides obtained through microsequencing of purified CDw50 reveal them to be identical (Fig. 4).

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ICAM-3 (167-188)   M Q P Q G L G L F V N T S A P R Q L R T F V
CDW50  (PEPTIDE 2)   Q P Q G L G L F V X T S A P R Q L R T F V

ICAM-3 (239-266)  M N H G D T L T A T A T A T A R A D Q E G A R E I V
CDW50 (PEPTIDE 3)   N X G D T L T A T A T A T A X A X Q E G A R E I V

ICAM-3 (308-336)  M A G A R V Q V T L D G V P A A A P G Q P A Q L Q L N A T
CDW50 (PEPTIDE 1)   A G A T V Q V T L D G V P A A A P G Q P A Q L Q L N A T
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Figure 4. Identity between deduced ICAM-3 protein sequence from the cDNA and CDw50 partial peptide sequence. Numbering of ICAM-3 amino acid positions are from de Fougerolles et al. [9]. Assignment of X in the CDw50 peptide sequence represent non-identifiable signal in the sequence analysis.

4 Concluding remarks

Immunochemical, functional, and protein sequencing studies confirm that ICAM-3 and CDw50 are indeed the same molecule. An important role for ICAM-3 in the initiation of immune responses is implied by the finding that adhesion of resting T lymphocytes to purified LFA-1 occurs primarily via ICAM-3, combined with the fact that ICAM-3 is much better expressed than other LFA-1 ligands on resting monocytes and lymphocytes [6]. In support of this hypothesis is the finding that anti-CDw50 mAb are capable of partially inhibiting primary allogeneic responses [2]. Also of interest is the finding that upon stimulation with PMA or a physiologic stimuli, such as T cell receptor cross-linking, CDw50 becomes rapidly and transiently phosphorylated on serine residues [21]. The functional significance of this phosphorylation is unknown, but interestingly, although all ICAM are very homologous to each other in their extracellular domains, their cytoplasmic domains are poorly conserved. Indeed, neither mouse nor human ICAM-1 or -2 contain any serine residues in their cytoplasmic region, whereas human ICAM-3 contains five such residues. By gaining information about ICAM-3 and CDw50, a clearer picture should emerge of the role of ICAM-3 in immune responses.

We thank J. Freed (National Jewish Center, Denver, CO) for peptide sequencing from PVDF.

Received February 4, 1993; in revised form March 25, 1993.

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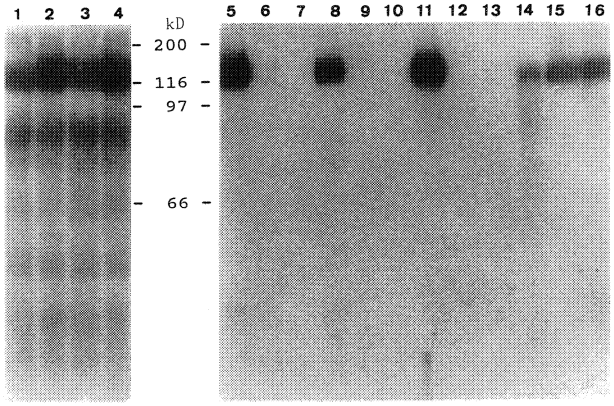

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CDw50 protein purification was based on a method previously described with some modifications [19]. Briefly, CDw50 was affinity purified from 8×10^9 PBMC lysed with 80 ml of 0.5% NP40 lysis buffer (with protease inhibitors) on a 101-1D2 mAb-coupled immunoaffinity column. The column was washed with 150 ml of lysis buffer and 15 ml of 1 M $MgCl_2$. CDw50 protein was selectively eluted with 4 M $MgCl_2$. After concentration (Centricon 30, Amicon, Beverly, MA) the sample was submitted to SDS-PAGE on an 8%-gel, under reducing conditions, and semi-dry electroblotted (4 mA/cm^2) with tricine transfer buffer (39 mM tricine, 48 mM Tris-base, 1.3 mM SDS and 20% methanol) to nitrocellulose, the band was visualized by Ponceau S, and

cut out. The excised nitrocellulose membrane was digested with cyanogen bromide in formic acid. After drying and redissolving, peptides were separated by 20% SDS-PAGE and semidry electroblotted as above to polyvinylidene difluoride (PVDF). The PVDF membrane (PVDF-Problott, Applied Biosystems, Forster City, CA) was stained with amido black and peptide bands were cut out and microsequenced directly from PVDF on an Applied Biosystems 470A Microsequencer.

3 Results and discussion

CDw50 and ICAM-3 mAb showed similar reactivities on all cell lines tested (Table 1). Both molecules are leukocyte restricted in their expression, and are negative on nonlymphoid cell lines U937, K562 and HeLa. Some differences between this collaborative analysis and previous data of each group were observed: the HPB-ALL T cell line and the B cell lines Daudi, Raji, and Nalm-6 were positive (Table 1). These differences can be accounted by variations in these cell lines.

In the CD Guide of the Fourth International Workshop on Human Leukocyte Differentiation Antigens, CDw50 was described as a heterodimer of 140/108 kDa which was potentially phosphatidylinositol (PI)-linked [20]. As ICAM-3 is a single-chain molecule of M_r 124 000 and is not PI linked, it was not immediately recognized to be identical to CDw50. Immunoprecipitates of ICAM-3 and CDw50 were carried out and revealed a protein of M_r 124 000 (Fig. 1, lanes 2–4). Sequential immunoprecipitation were performed to determine whether the mAb identified the same protein (Fig. 1). Two ICAM-3 mAb (CRB-IC3/1 and CBR-IC3/2), one CDw50 mAb (101-1D2) and a control CD26 mAb (134-2C2) were utilized. The anti-CDw50 mAb, 101-1D2, was able to completely preclear all the ICAM-3 antigen (Fig. 1, lanes 9 and 12) and both anti-ICAM-3 mAb were able to preclear all the CDw50 antigen (Fig. 1, lanes 6 and 7). We concluded that anti-CDw50 and anti-ICAM-3 mAb recognize the same molecule.

Cross-blocking studies were then performed with CDw50 and ICAM-3 mAb to determine if similar epitopes on the molecule were recognized (Table 2). PHA-stimulated PBMC were incubated with an unlabeled blocking antibody, followed by a fluoresceinated test antibody and immunofluorescence FCM. In all cases, staining by the fluoresceinated mAb was inhibited after pretreatment with the same unfluoresceinated mAb. The ability of the CBR-IC3/2 mAb to diminish staining of the fluoresceinated 101-1D2 mAb shows that these two mAbs recognize similar epitopes on the same molecule. The epitopes recognized by mAb 140-11, 152-2D11, and CBR-IC3/1 seem to be different.

To demonstrate further that CDw50 and ICAM-3 are the same molecule, immunofluorescence FCM was performed on mouse L cells stably transfected with the ICAM-3 cDNA (Fig. 2). The ICAM-3$^+$ L cells reacted specifically with both anti-ICAM-3 (CBR-IC3/1) and anti-CDw50 (101-1D2) mAb. The transfectant also reacted with five other anti-ICAM-3 mAb, including CBR-IC3/2, as well as with the CDw50 mAb, 140-11 and 152-2D11 (data not shown). The ICAM-3$^+$ L cells were then tested for binding to purified LFA-1 and for the ability of anti-ICAM-3 and anti-CDw50 mAbs to inhibit this adhesion (Fig. 3). L cell transfectants expressing ICAM-3 bound efficiently to purified LFA-1 on plastic, while untransfected L cells bound poorly, showing equivalent binding to substrates bearing LFA-1 and BSA (Fig. 3). Binding of ICAM-3$^+$ L cells to LFA-1 was blocked by LFA-1 mAb, and to varying degrees by anti-ICAM-3 and anti-CDw50 mAbs (Fig. 3). In agreement with the finding that CBR-IC3/2 and 101-1D2 mAb recognize the same epitope, both molecule are inefficient at inhibiting interaction to LFA-1. The other CDw50 mAb and CBR-IC3/1 each partially block adhesion to LFA-1. This demonstrates that

Table 1. Comparison of CDw50 and ICAM-3 cell distribution

Cell type	Percent positive cells[a]			
	101-1D2	140-11	CBR-IC3/1	CBR-IC3/2
T cell lines				
CEM	+++	+++	+++	+++
MOLT-4	+++	+++	+++	+++
JURKAT	+++	++	++	+++
HSB2	+++	+++	+++	+++
HPB-ALL	++	++	++	++
B cell lines				
DAUDI	+++	+++	+++	+++
RAJI	+	+	+	+
RPMI-8226	+++	+++	+++	+++
NALM-6	+	++	+	+
KM3	+	+	+	+
RAMOS	+	+	+	+
Non lymphoid cell lines				
HL-60	++	++	++	++
U-937	0	0	0	0
K-562	0	0	0	0
HELA	0	0	0	0

a) Membrane expression determined by indirect immunofluorescence FCM with CDw50 (101-1D2, 140-11) and ICAM-3 (CBR-IC3/1 and CBR-IC3/2) mAb. +++ = > 90%, ++ = 50–90%, + = 20–50%, 0 = < 20%. Positive cell percentages were established by histogram substraction on a FACStar-plus (Becton Dickinson).

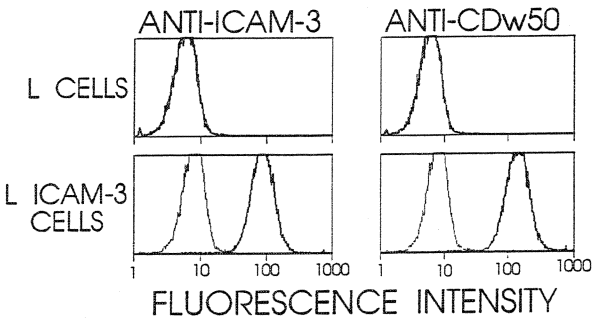

Figure 2. ICAM-3 cDNA transfectants are recognized by anti-CDw50 and anti-ICAM-3 mAb. L cells stably transfected with ICAM-3 cDNA or untransfected L cells labeled with saturating amounts of control mAb × 63 (thin line) or mAb 101-1D2 (anti-CDw50) or mAb CBR-IC3/1 (anti-ICAM-3) followed by FITC-anti-mouse Ig. Cells were subjected to immunofluorescent FCM.

Table 2. Cross-competition of CDw50 and ICAM-3 mAb[a)]

Second incubation	First incubation (ascites fluid or purified mAb)						
	Cris-1	101-1D2	140-11	152-2D11	CBR-IC3/1	CBR-IC3/2	NS1
Cris-1	30	92	90	90	90	92	92
101-1D2	99	20	99	99	99	10	99
152-2D11	99	99	99	50	99	99	99

a) PHA-stimulated PBMC were first incubated with saturating concentrations of each antibody and the second incubation performed with affinity-purified fluorescein-conjugated mAb. Cris-1 (a CD5 mAb) and NS1 mAb-containing ascites fluid were utilized as controls. The positive cell percentages were established by marker positioning so that negative background gives less than 2% positive cells on a FACStar-plus (Becton Dickinson).

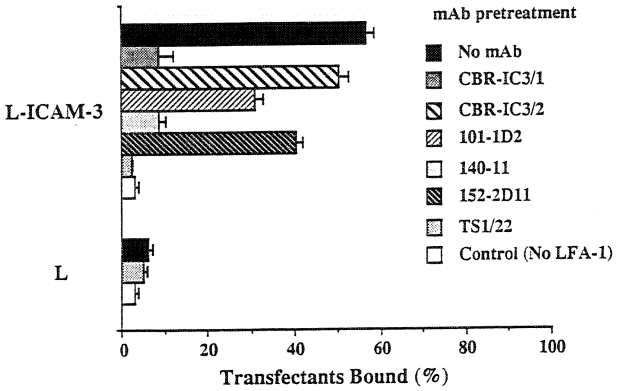

Figure 3. Adhesion of ICAM-3 transfected cells to purified LFA-1. Murine L-cells either stably transfected with ICAM-3 cDNA or untransfected were allowed to bind to LFA-1-coated microtiter wells for 60 min at 37 °C and washed four times by aspiration through a 25-gauge needle followed by two aspirations through a 23-gauge needle. Control wells lacked LFA-1. Cells were pretreated with saturating concentrations of one of the following mAb: W6/32 (anti-HLA, A, B, C), CBR-IC3/1 (anti-ICAM-3), CBR-IC3/2 (anti-ICAM-3), 101-1D2 (anti-CDw50), 140-11 (anti-CDw50), or 152-2D11 (anti-CDw50). Alternatively, the absorbed purified LFA-1 was pretreated with mAb TS1/22 (anti-LFA-1α). One of four representative experiments is shown and errors bars indicate one standard deviation.

CDw50 mAb recognize the expressed ICAM-3 cDNA and are capable of inhibiting LFA-1:ICAM-3 interactions.

Lastly, comparison between the deduced amino acid sequence derived from the ICAM-3 cDNA and the sequence of three peptides obtained through microsequencing of purified CDw50 reveal them to be identical (Fig. 4).

```
ICAM-3 (167-188)   M Q P Q G L G L F V N T S A P R Q L R T F V
CDW50  (PEPTIDE 2)   Q P Q G L G L F V X T S A P R Q L R T F V

ICAM-3 (239-266)  M N H G D T L T A T A T A T A R A D Q E G A R E I V
CDW50 (PEPTIDE 3)   N X G D T L T A T A T A T A X A X Q E G A R E I V

ICAM-3 (308-336)  M A G A R V Q V T L D G V P A A A P G Q P A Q L Q L N A T
CDW50 (PEPTIDE 1)   A G A T V Q V T L D G V P A A A P G Q P A Q L Q L N A T
                        R
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Figure 4. Identity between deduced ICAM-3 protein sequence from the cDNA and CDw50 partial peptide sequence. Numbering of ICAM-3 amino acid positions are from de Fougerolles et al. [9]. Assignment of X in the CDw50 peptide sequence represent non-identifiable signal in the sequence analysis.

4 Concluding remarks

Immunochemical, functional, and protein sequencing studies confirm that ICAM-3 and CDw50 are indeed the same molecule. An important role for ICAM-3 in the initiation of immune responses is implied by the finding that adhesion of resting T lymphocytes to purified LFA-1 occurs primarily via ICAM-3, combined with the fact that ICAM-3 is much better expressed than other LFA-1 ligands on resting monocytes and lymphocytes [6]. In support of this hypothesis is the finding that anti-CDw50 mAb are capable of partially inhibiting primary allogeneic responses [2]. Also of interest is the finding that upon stimulation with PMA or a physiologic stimuli, such as T cell receptor cross-linking, CDw50 becomes rapidly and transiently phosphorylated on serine residues [21]. The functional significance of this phosphorylation is unknown, but interestingly, although all ICAM are very homologous to each other in their extracellular domains, their cytoplasmic domains are poorly conserved. Indeed, neither mouse nor human ICAM-1 or -2 contain any serine residues in their cytoplasmic region, whereas human ICAM-3 contains five such residues. By gaining information about ICAM-3 and CDw50, a clearer picture should emerge of the role of ICAM-3 in immune responses.

We thank J. Freed (National Jewish Center, Denver, CO) for peptide sequencing from PVDF.

Received February 4, 1993; in revised form March 25, 1993.

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