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A Novel Leukointegrin, αdβ2, Binds Preferentially to ICAM-3

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Summary

The leukocyte-restricted β2 (CD18) integrins mediate cell adhesion in a variety of events essential for normal immune function. Despite extensive research in this field, only three members of this integrin subfamily have been described: CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), and CD11c/CD18 (p150,95). We have identified a cDNA encoding a fourth α chain, αd, that associates with CD18. The αd subunit is more closely related to CD11b and CD11c than to CD11a. This integrin is expressed at moderate levels on myelomonocytic cell lines and subsets of peripheral blood leukocytes, and more strongly on tissue-compartmentalized cells such as foam cells, specialized macrophages found in aortic fatty streaks that may develop into atherosclerotic lesions. The αd/CD18 molecule exhibits preferential recognition of ICAM-3 over ICAM-1.

Introduction

The integrins are a family of heterodimeric membrane-bound glycoproteins that mediate homotypic and heterotypic cell–cell adhesion in a broad range of biological contexts (Hynes, 1992; Larson and Springer, 1990). A subfamily of integrins expressed in leukocytes is defined by a common beta chain, β2 (CD18), that pairs with a distinct subgroup of α chains (Hynes, 1987). These α chains contain a region of approximately 200 aa residues, designated the I (inserted) or A domain, that shares structural homology to ligand-binding domains in Von Willebrand factor and other proteins (Larson and Springer, 1990; Michishita et al., 1993; Lee et al., 1995) and has recently been implicated in ligand recognition (Landis et al., 1993; Diamond et al., 1993; Randi and Hogg, 1994; Zhou et al., 1994). The leukointegrins are critical to immune function: absence of CD18 cell surface expression in patients with leukocyte adhesion deficiency (LAD) results in impairment of a variety of immune functions, including neutrophil transendothelial migration, macrophage oxidative burst and phagocytosis, and lymphocyte proliferation (Springer et al., 1984; Anderson and Springer, 1987).

To date, the leukointegrin subfamily includes CD11a/CD18, CD11b/CD18, and CD11c/CD18, also known as LFA-1, Mac-1, and p150,95, respectively (Kishimoto et al., 1987; Corbi et al., 1987, 1988; Larson et al., 1989). LFA-1 cell surface expression is constitutive on a broad range of leukocytes, while expression of Mac-1 and p150,95 is more restricted and inducible by inflammatory mediators (Larson and Springer, 1990). LFA-1 and Mac-1 mediate cell–cell contact by interacting with the intercellular adhesion molecules (ICAMs), immunoglobulin superfamily members (Hynes, 1992; Larson and Springer, 1990). Whereas both LFA-1 and Mac-1 bind to ICAM-1, only LFA-1 has been demonstrated to bind to ICAM-3. ICAM-3 is highly expressed on resting lymphocytes and its linkage to intracellular signal transduction pathways contributes to lymphocyte activation (Vives, 1994). Here, we report the identification of a novel fourth leukointegrin α chain, αd, that contains an I or A domain and is most homologous to Mac-1 and p150,95. The αd protein is expressed on myelomonocytic cell lines in a pattern distinct from that of CD11b and CD11c. This molecule is expressed predominantly in splenic red pulp on macrophages and granulocytic cells in an overlapping but nonidentical pattern with the other leukointegrin α chains. The protein can be detected at low levels relative to CD11a and CD11b on subsets of peripheral blood cells. In addition, it is present on lipid laden macrophages in aortic fatty streaks. This α chain forms a heterodimer with β2 that binds ICAM-3 but not ICAM-1 when expressed in Chinese hamster ovary (CHO) cells.

Results and Discussion

For analyses of β2 integrin function in the dog, Danilenko et al. (1992) generated a panel of monoclonal antibodies (MAbs) against canine CD18 complexes. Interestingly, one of these canine-specific antibodies recognized a CD18-associated protein with histologic distribution and apparent molecular weight distinct from that of the known leukointegrins (Danilenko et al., 1995). The expression pattern of this molecule was restricted mainly to tissues and differed from the distribution of CD11b and CD11c. Amino-terminal sequence derived from this α chain indicated leukointegrin homology; however, this protein could not be defined as the distinct product of a novel gene since no sequence information regarding canine CD11a, CD11b, or CD11c was available for comparison. A priori, these findings might also be explained by cell-type specific mRNA splicing, posttranslational modification, or unexpected pairing of CD18 with known integrin α chains from other subfamilies.

We presumed that DNA fragments amplified from canine cDNA could be used in cross-species hybridizations to identify a human homolog, thus permitting direct comparisons with known α subunits. We obtained peptide sequences for polymerase chain reaction (PCR) primer design from fragmentation of the αd protein isolated from

canine spleen. Degenerate oligonucleotides based on 2 of 9 peptides sequenced were used as primers in a PCR with canine splenic cDNA to amplify a 1 kb DNA fragment. This fragment was used as a probe to identify a homologous clone (19A2) in a human spleen cDNA library under stringent hybridization conditions. Although no exact matches with any sequence in the National Center for Biotechnology Information databases were found, sequence homologies to the known leukointegrin α chains suggested that the protein encoded by clone 19A2 belonged to this integrin subfamily. This clone appears to share sequence homology with a partial genomic clone identified independently (D. Wong, personal communication). We have designated this previously unidentified α chain αd.

As shown in Figure 1, clone 19A2 encompasses the entire coding region for the mature protein, including 13 residues of leader sequence. Two additional clones have been isolated and used to confirm this sequence and to indicate that a methionine precedes the 19A2 leader sequence. The putative amino acid sequence shares greater identity to CD11b (60%) and CD11c (66%) than to CD11a (36%). The molecular mass of the mature core protein is predicted to be 125 kDa with an extracellular domain encompassing 1084 residues followed by a hydrophobic 23-residue potential transmembrane domain and a 38-residue cytoplasmic tail. There are eleven potential N-linked glycosylation sites. The seven homologous internal repeats observed in CD11a, CD11b, and CD11c are also conserved in αd.

Also present in αd is a 204-residue segment homologous to the I or A domain recently implicated in ligand recognition (Landis et al., 1993; Diamond et al., 1993; Randi and Hogg, 1994; Edwards et al., 1995). The I domain

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                  I
mtvlllsvlas yhgFNLDVEE PTIFQEDAGG FGQSVVQFGG SRLVVGAPLE VVAANQTGR 60
                 II
LYDCAAATGMC QPIPLHIRPE AVNMSLGLTL AASTNGSRLL ACGPTLHRVC GENSYSKGS 120
CLLLGSRWEII QTVPDATPEC PHQEMDIVFL IDGSGSIDQN DFNQMKGFVQ AVMGQFEGT 180
DTLFALMQYS NLKIHFTFTQ FRTSPSQQSL VDPIVQLKGL TFTATGILTV VTQLFHHKN 240
GARKSAKKLL VITDGQKYKD PLEYSDVIPQ AEKAGIIRYA IGVGHAFQGP TAREELNTI 300
                                              III
SSAPPQDHVFK VDNFAALGSI QKQLQEKIYA VEGTQSRASS SFQHEMSQEG FSTALTMDG 360
                    IV
LFLGAVGSFSW SGGAFLYPPN MSPTFINMSQ ENVDMRDSYL GYSTELALWK GVQNLVLGA 420
                  V
PRYQHTGKAVI FTQVSRQWRK KAEVTGTQIG SYFGASLCSV DVDSDGSTDL ILIGAPHYY 480
                      VI
EQTRGGQVSVC PLPRGQRVQW QCDAVLRGEQ GHPWGRFGAA LTVLGDVNED KLIDVAIGA 540
                         VII
PGEQENRGAVY LFHGASESGI SPSHSQRIAS SQLSPRLQYF GQALSGGQDL TQDGLMDLA 600
VGARGQVLLLR SLPVLKVGVA MRFSPVEVAK AVYRCWEEKP SALEAGDATV CLTIQKSSL 660
DQLGDIQSSVR FDLALDPGRL TSRAIFNETK NPTLTRRKTL GLGIHCETLK LLLPDCVED 720
VVSPIILHLNF SLVREPIPSP QNLRPVLAVG SQDLFTASLP FEKNCGQDGL CEGDLGVTL 780
SFSGLQTLTVG SSLELNVIVT VWNAGEDSYG TVVSLYYPAG LSHRRVSGAQ KQPHQSALR 840
LACETVPTEDE GLRSSRCSVN HPIFHEGSNG TFIVTFDVSY KATLGDRMLM RASASSENN 900
KASSSKATFQL ELPVKYAVYT MISRQEESTK YFNFATSDEK KMKEAEHRYR VNNLSQRDL 960
AISINFWVPVL LNGVAVWDVV MEAPSQSLPC VSERKPPQHS DFLTQISRSP MLDCSIADC 1020
LQFRCDVPSFS VQEELDFTLK GNLSFGWVRE TLQKKVLVVS VAEITFDTSV YSQLPGQEA 1080
FMRAQMEMVLE EDEVYNAIPI IMGSSVGALL LLALITATLY KLGFFKRHYK EMLEDKPED 1140
                      ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^
TATFSGDDFSC VAPNVPLS                                          1159
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Figure 1. Amino Acid Sequence of the αd Subunit

Leader sequence is denoted by lowercase letters. Additional cDNA clones have confirmed the single methionine preceding the leader sequence from clone 19A2. Homologous internal repeats are labeled with roman numerals at their amino-terminal ends. The I domain is underlined. Putative cation binding sites are indicated in bold letters; the MIDAS motif is included in the first cation binding site. Potential N-linked glycosylation sites are underlined. The transmembrane domain, denoted by carets, immediately precedes the cytoplasmic domain. The cytoplasmic KLGFFKR motif is in italics. The nucleotide sequence of clone 19A2 can be obtained from the Genbank database (accession number U37028).

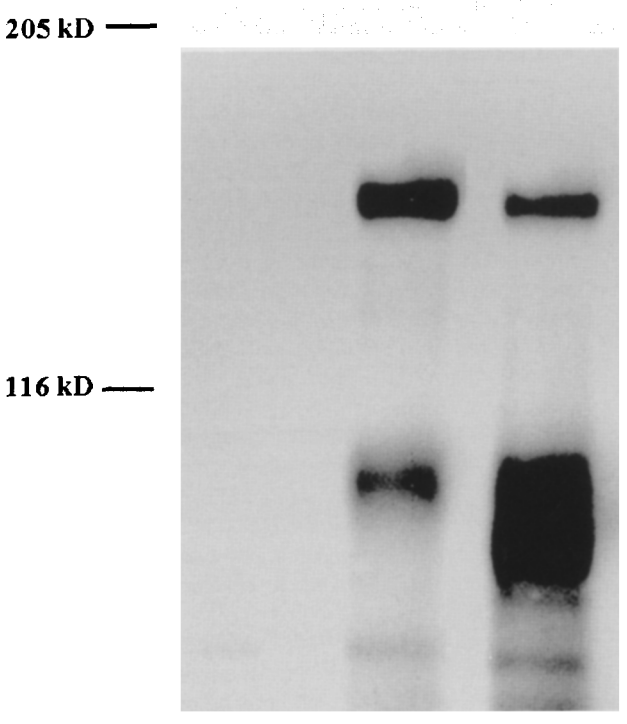


Figure 2. αd Forms a Heterodimer with β2, CD18

Heterodimeric complexes immunoprecipitated from lysates of biotinylated αd/CD18 CHO transfectants with either αd- or CD18-specific MAb were visualized with peroxidase-conjugated streptavidin. Lane 1, αd/CD18 CHO plus mouse IgG; lane 2, αd/CD18 CHO plus TS1/18; lane 3, αd/CD18 CHO plus 169A. Cell surface expression of CD18 required cotransfection of the αd cDNA: transient transfections of the β chain construct alone did not result in detectable CD18 expression by flow cytometry and in immunoprecipitation experiments (data not shown).

amino acid sequence is 36%, 62%, and 57% identical to the same regions in CD11a, CD11b, and CD11c, respectively. αd also contains identical residues defined in the I domain of CD11b as a metal ion-dependent adhesion site (MIDAS) necessary for ligand binding (Michishita et al., 1993, Lee et al., 1995), as well as three additional cation binding sites conserved in leukointegrin α subunits. There is similarity between the peptide sequences from CD11b, which have been demonstrated to interact with complement component iC3b (Ueda, et al., 1994) and the homologous region in αd. The moderate level of sequence conservation in these regions suggests that while αd may share ligands with the known leukointegrins, it may also recognize distinct cell surface or extracellular matrix proteins.

The cytoplasmic domains of integrin α subunits are implicated in integrin activation. These domains are typically unrelated to one another, but conserved across species. The putative cytoplasmic sequence of αd differs markedly from that of CD11a, CD11b, and CD11c (10%, 16%, and 18% identity, respectively), except for the membrane proximal KXGFFKR motif, conserved among all α integrins, that is involved in affinity regulation from the cytoplasmic side, or inside-out signaling (Ginsberg et al., 1992, Rojiani et al., 1991). Engagement of αd, even in a similar cellular context as other leukointegrins, may involve interactions with a distinct subset of cytosolic proteins and may have functional consequences specific to αd.

Cotransfection of the cDNAs encoding αd and CD18 into CHO cells resulted in cell surface expression of a heterodimeric molecule, both chains of which were immunoprecipitated by either anti-CD18 or anti-αd MAb (Figure 2).

The αd-specific MAb used in this experiment was generated from mice injected with CD18 complexes isolated from human splenic lysates precleared of CD11a and CD11b. Supernatants from resulting immunoglobulin G (IgG)-secreting hybridomas were screened by flow cytometry for reactivity with αd/CD18 CHO transfectants but not with JY cells, which had been previously determined by Northern blot analysis to be αd negative. The IgG1 antibody secreted by clone 169A was determined to be specifically reactive with αd only by flow cytometric analysis of stable αd/CD18, CD11a/CD18, CD11b/CD18, and CD11c/CD18 CHO transfectants (Table 1, stable transfectants). This antibody was not observed to have inhibitory activity in the functional assays presented here.

To ascertain cell-type expression of αd and its relationship to expression of other leukointegrins, the presence of αd protein on human lymphoid and myelomonocytic cell lines and on freshly isolated peripheral blood leukocytes (PBLs) was evaluated by flow cytometry using the MAb 169A and compared with expression of CD11a, CD11b, and CD11c (Table 1). αd expression is modulated with differentiation of the myeloid cell lines HL60 and THP-1 (Table 1, cell lines). DMSO treatment, which induces granulocyte differentiation in the promyelomonocytic line HL60 (Collins, 1987) did not affect the percent of cells positive for αd, which was expressed on more cells than CD11c

Table 1. Flow Cytometric Analysis of αd Surface Expression on Cell Lines, PBL, and Transfectants

Cell lines	αd	CD11a	CD11b	CD11c	PBL	αd	CD11a	CD11b	CD11c	Stable Transfectants	αd	CD11a	CD11b	CD11c
Myeloid					Lymphocytes	25 (83)	120 (93)	67 (45)	27 (23)	αd/CD18 CHO	64 (95)	19 (1)	11 (1)	9 (1)
HL60										LFA-1 CHO	10 (1)	45 (70)	8 (1)	12 (1)
No stimulation	36 (82)	102 (98)	19 (38)	13 (3)	Monocytes	51 (98)	132 (99)	418 (90)	84 (99)					
PMA	27 (42)	45 (78)	54 (71)	31 (72)										
DMSO	20 (87)	74 (78)	18 (87)	28 (15)										
THP-1					Granulocytes									
No stimulation	42 (66)	55 (72)	81 (52)	82 (35)	No stimulation	19 (94)	46 (99)	330 (99)	23 (98)					
PMA	59 (26)	55 (35)	170 (99)	101 (95)	PMA	54 (95)	26 (95)	500 (96)	31 (95)					
					fMLP	70 (92)	71 (91)	403 (98)	62 (97)					
Lymphoid														
Jurkat (T cell)	7 (7)	16 (88)	9 (42)	8 (4)										
Ramos (B cell)	5 (5)	9 (84)	5 (11)	5 (5)										
JY (B cell)	11 (13)	17 (66)	12 (19)	17 (8)										
KU812 (mast)	11 (16)	14 (40)	19 (47)	4 (30)										

MAbs were used to detect surface expression of αd, CD11a, CD11b, or CD11c, respectively, on cell lines, peripheral blood leukocytes, and stable CHO transfectants. Data shown are from a single experiment representative of 3–5 separate experiments. Gates were set to exclude isotype-matched irrelevant controls with a background of 5% positive cells. Results are expressed as a mean fluorescence intensity of 10^5 positive cells per determination, with percent positive in parentheses.

both before and after treatment. However, phorbol myristate acetate (PMA) stimulation of both HL60 and THP-1 cells along a macrophage-like differentiation pathway resulted in increased CD11b and CD11c expression levels, with a corresponding reduction in percent positive for αd. The majority of peripheral blood lymphocytes express low levels of αd relative to CD11a and CD11b (Table 1, PBL); these patterns do not vary significantly among donors. Granulocytes were also observed to express αd, although at significantly lower levels than CD11b. Expression appeared to increase rapidly on granulocytes with PMA or f-Met Leu–Phe (fMLP) stimulation, consistent with recruitment of an intracellular pool of αd in these cells. Thus, levels of αd expression may differ from that of CD11a, CD11b, or CD11c depending on the cell type and activation or differentiation state.

Comparative expression of the previously identified leukointegrin α chains and αd was assessed by immunohistology in the spleen because of the highly ordered structure of the lymphoid domains and the presence of diverse cell types. The overall pattern of αd distribution in the red pulp of spleen was overlapping with but not identical to that of CD11a, CD11b, and CD11c (Figure 3). Whereas CD11a was diffusely expressed by leukocytes in all splenic domains, including lymphoid domains of the white pulp and marginal zone and cords and sinuses of the red pulp, CD11b, CD11c, and αd expression patterns were more restricted. CD11b staining was prominent on granulocytes in the red pulp cords and sinuses. CD11c was expressed by dendritic cells in the periarterial lymphoid sheaths, marginal zones, and follicles of the white pulp. The distribution of αd was predominantly localized to the red pulp cords and sinuses on small and large mononuclear cells and granulocytes. Expression of αd in the white pulp was limited to scattered dendritic cells occurring less frequently than $CD11c^+$ cells. Although relative intensity of splenic integrin staining appeared to vary among three tissue donors (data not shown), probably owing to differences in age, health, or mortality factors, the distribution patterns did not vary.

A preliminary evaluation of αd expression in pathologic tissue with macrophage-specific etiology was performed using sections of abdominal aorta selected for the incidence of fatty streaks, which histologically consisted of subintimal infiltrates of smaller leukocytes and aggregates of foamy macrophages (Pathobiological Determinants of Atherosclerosis in Youth [PDAY] Research Group). These highly specialized macrophages are implicated in the establishment of subintimal aortic fatty streaks, which may progress to fulminant atherosclerotic lesions (Ross, 1993). Foamy macrophages can be identified in fatty streaks by detection of ingested lipid with the Oil Red O stain and differentiated from lipid-containing smooth muscle cells by specific markers. In the Oil Red O–positive sections we examined, a subset of the large lipid-laden cells reactive with an antibody to CD68, a macrophage-specific marker, expressed moderate levels of αd (Figure 3). These cells were not stained by an antibody to smooth muscle actin, confirming that the $αd^+$ foam cells were not of smooth muscle origin (data not shown). In addition, these macrophage-like cells did not stain with CD3, CD8, or CD20 antibodies (data not shown). Double-labeled aortic sections confirm a significant overlap of CD68 and αd staining. The αd-specific antibody did not react with aortic sections that were Oil Red O negative (data not shown).

Typically, the leukocyte integrins promote cell–cell contact by binding to the intercellular adhesion molecules (ICAMs) (Hynes, 1992; Larson and Springer, 1990). Soluble forms of ICAM-1 and ICAM-3 have been observed in serum with increased levels in certain pathologic conditions (Martin et al., 1995). Therefore, the ability of CHO cells expressing αd/CD18 or LFA-1 to bind soluble ICAM-3/Ig, ICAM-1/Ig, and VCAM-1/Ig chimeric proteins was assessed by flow cytometry (Figure 4A). The αd/CD18, LFA-1, and VLA-4 transfectants used in subsequent binding experiments were 60%–90% CD18 or α4 positive, with equivalent cell surface expression in individual experiments. In replicate experiments, ICAM-3/Ig, but not ICAM-1/Ig or VCAM-1/Ig, bound to αd/CD18 transfectants. The CD18 dependence of this binding was confirmed by its sensitivity to treatment with anti-CD18 antibody. Similar results were obtained for αd/CD18 cotransfectants in adhesion assays using immobilized ICAM-1/Ig or ICAM-3/Ig chimeras (Figure 4B). The αd/CD18 transfectants bound 2- to 4-fold greater to ICAM-3/Ig than to the bovine serum albumin (BSA) control, with no significant binding to ICAM-1/Ig.

Treatment of αd/CD18 transfectants with PMA or manganese, positive regulators of LFA-1 activation, did not significantly affect binding to CAMs in either of the adhesion assays described. CD11a/CD18 transfectants exhibited a PMA-dependent 3- to 5-fold increase in binding to ICAM-1 (Figure 4B), consistent with the activation requirement and apparent avidity reported for LFA-1/ICAM-1 interactions in this cellular context (Dustin and Springer, 1989; van Kooyk et al., 1989). Differences in αd and CD11a regulation may reflect unique cytosolic interactions mediated by their nonhomologous cytoplasmic tails.

In summary, we have described a previously unknown human leukointegrin α subunit that forms a functional heterodimer with CD18. Although structurally similar to the known α chains, divergence in key regions of this protein implies that αd/CD18 plays a different role in immune responses. For example, the distinct sequences of the αd I domain and cytoplasmic tail region suggest the potential for unique ligands, or unique binding sites on shared ligands, and affinity regulation specific to this integrin.

The predominant expression of αd observed on specialized cells in tissues suggests that the major functions of αd may be restricted to particular microenvironments. For example, the presence of αd on splenic red pulp macrophages may indicate a role for this integrin in phagocytosis of effete erythrocytes, bloodborne pathogens, and particulate matter from the blood. The involvement of foam cells in establishment of vascular lesions in certain types of atherosclerosis (Ross, 1993), combined with the observation that αd is expressed on those cells in fatty streaks, suggest that αd may contribute to macrophage-specific

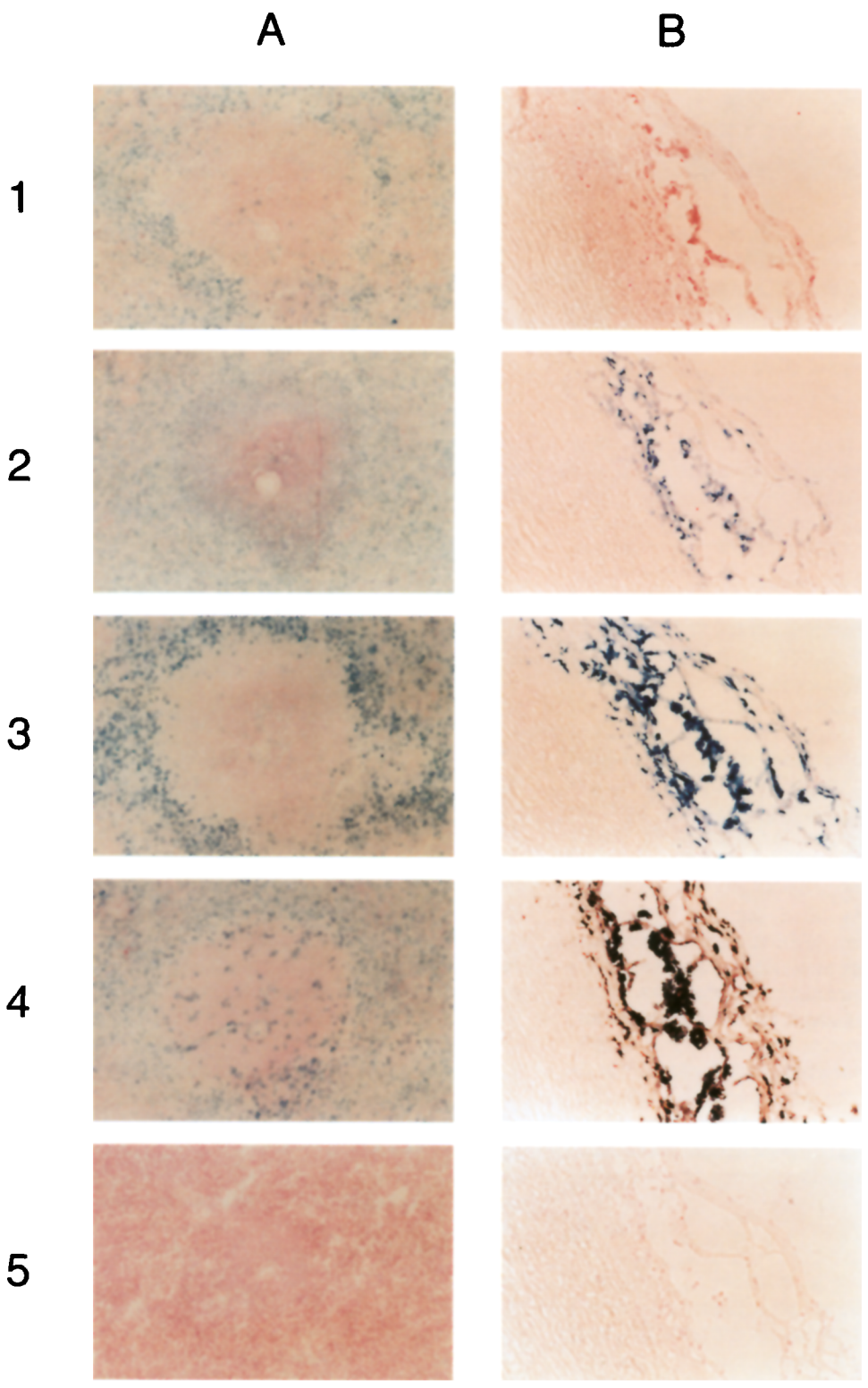
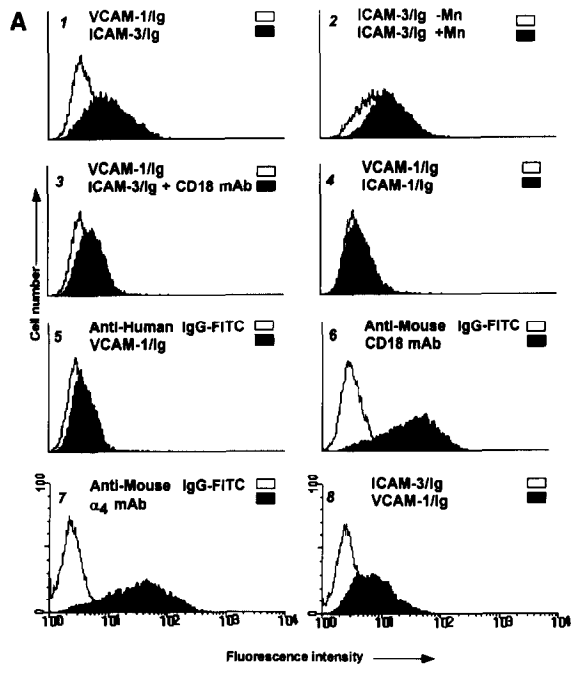
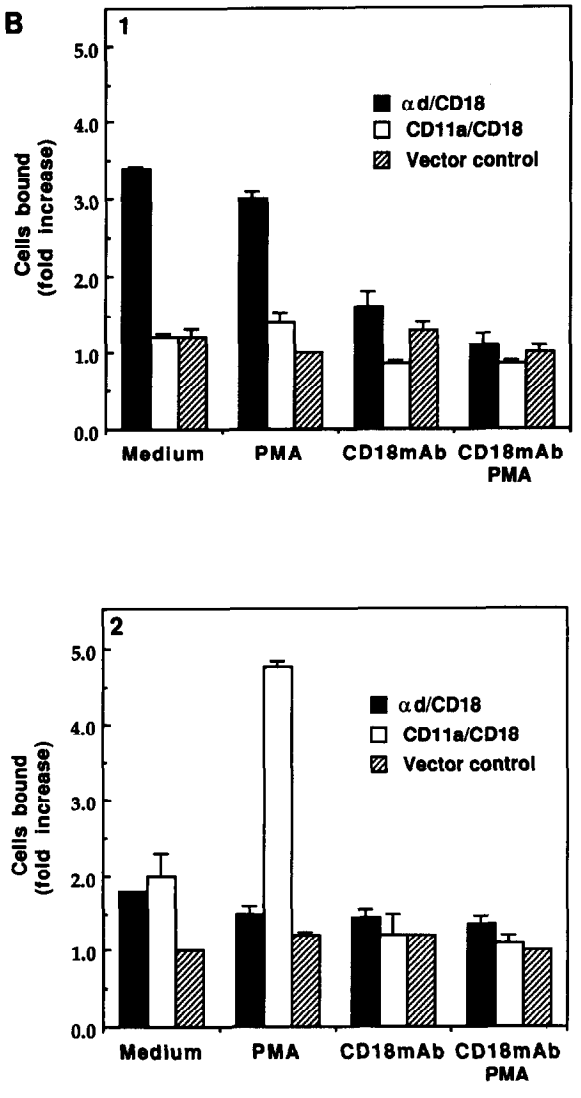


Figure 3. Expression of αd in Human Spleen and Aorta

(A) Fresh frozen splenic sections were acetone fixed and treated with antibodies against (1) αd, (2) CD11a, (3) CD11b, (4) CD11c, or (5) irrelevant mouse IgG1.

(B) Abdominal aortic sections were acetone fixed and treated with (1) Oil Red O, (2) MAb 169A (αd) (blue), (3) MAb EBM11 (CD68) (blue), (4) both 169A (blue) and EBM11 (brown), and (5) irrelevant mouse IgG1.





activities such as phagocytosis of modified lipoproteins in these lesions. Therefore, animal models of atherosclerosis may present one interesting venue to explore the in vivo function of αd.

The relevance of the low levels of αd observed on granulocytes, peripheral blood T cells, and monocytes may become evident when the full range of αd functions is defined. The presence of αd protein in CD18 immunoprecipitations from PBL may easily have been overlooked in the past due to overwhelming levels of other leukointegrins and similarity in apparent molecular weights of the α chains. It is possible that ligand specificity, activation regulation, and increased expression levels in particular environments may confer to αd different functions than other leukointegrins expressed by these cells.

The cell type and tissue distribution of human and canine αd protein expression is only partially conserved. As in the dog, αd is expressed on splenic red pulp macrophages (Danilenko et al., 1995) but not on Kupffer cells, resident macrophages of the liver (data not illustrated). However, the presence of αd on peripheral blood and splenic granulocytes from humans contrasts with the dog. This cross-species variation is perhaps unsurprising considering the presence of CD4 on canine, but not human granulocytes (Moore, et al., 1992). Other tissues that differ in αd distribution or intensity or both between the human and other species may be attributed either to species differences or to the variable characteristics of human donor tissues.

The capacity of αd/CD18 to bind both soluble and immobilized ICAM-3 implies a higher affinity interaction than that observed for LFA-1, which does not bind ICAM-3 in

Figure 4. αd/CD18 Binds to Soluble and Immobilized ICAM-3

The ability of stable αd/CD18 CHO transfectants to recognize soluble (4A) and immobilized (4B) ICAM-3 and ICAM-1 was determined.

(A) Transfectants were incubated in buffer with ICAM-3/Ig, ICAM-1/Ig, or VCAM-1/Ig. Bound protein was detected with FITC-conjugated anti-human immunoglobulin and subsequent flow cytometric analysis. (1) Comparison of ICAM-3/Ig and VCAM-1/Ig binding; (2) relative binding of ICAM-3/Ig in the presence or absence of manganese; (3) inhibition of ICAM-3/Ig binding by TS1/18 relative to VCAM-1/Ig binding; (4) comparison of ICAM-1/Ig and VCAM-1/Ig binding; (5) VCAM-1/Ig binding to transfectants relative to anti-human immunoglobulin tracer antibody only; (6) surface CD18 expression on αd/CD18 CHO transfectants, detected with the anti-CD18 MAb TS1/18; (7) α4 expression on VLA-4 CHO transfectants, detected with the antibody A4.1; and (8) binding of VCAM-1/Ig and ICAM-3/Ig to VLA-4 CHO transfectants. Mock-transfected and untransfected cells were not recognized by any fusion protein or by the FITC-conjugated tracer antibody (data not shown).

(B) CHO transfectants labeled intracellularly with calcein were incubated on microtiter plates coated with ICAM-3/Ig or ICAM-1/Ig. Fluorescence before and after washing was determined. Data is presented as fold increase of binding to ICAM/Ig over BSA control. Maximal binding of αd/CD18 CHO to ICAM-3 was 30%–50% of cells added to plates; for LFA-1 (PMA^+) and ICAM-1, 20%–30%; and 5%–10% for all transfectants to BSA. (1) Binding of CHO transfectants to ICAM-3/Ig; (2) binding of CHO transfectants to ICAM-1/Ig. The CD11a/CD18 transfectants did not show significant binding to ICAM-3, perhaps owing to modest surface expression of LFA-1. No binding of αd/CD18 or CD11a/CD18 transfectants to VCAM-1/Ig was observed (data not shown).

a similar cellular context. In addition, the failure of αd/CD18 to bind soluble or immobilized ICAM-1 or VCAM-1 suggests an exclusive ICAM-3 recognition by this integrin. However, the αd subunit, like other leukointegrin α chains, probably recognizes multiple ligands and may exhibit additional CAM binding specificities when expressed in other cellular contexts in vivo. We are currently attempting to define the unique functional range of the αd molecule. Moreover, reevaluation of many leukointegrin-dependent immune phenomena may now be warranted to determine the contribution of this previously unrecognized family member.

Experimental Procedures

Isolation of the αd cDNA

αd/CD18 complexes affinity purified from detergent lysates of canine spleen (Danilenko et al., 1992) were subjected to preparative SDS–PAGE in 7% polyacrylamide and transferred to PVDF membranes. The Coomassie-stained 150 kDa band was fragmented with cyanogen bromide (CNBr) in 70% formic acid. CNBr-cleaved protein was eluted once with 60% acetonitrile, 0.1% TFA, and once with 80% acetonitrile, 0.08% TFA. The CNBr fragments were dried by vacuum, reduced and alkylated, and digested with trypsin (Stone et al., 1992). Resulting peptides were separated on a Waters Associates high pressure liquid chromatography system using a Vydac C-18 column and sequenced by Edman degradation.

Degenerate oligonucleotides were designed on the peptide sequences (FNLDVEEPMVFQED) and (FQEGFSSVLT). Double-stranded cDNA was generated from canine splenic poly $(A)^+$ mRNA (Gubler and Hoffman, 1983). A PCR containing the oligonucleotides (RAANCCYTCYTGRAAACTYTC) and (TTYAAYYTNGAYGTNGARGARCC) and 200 ng cDNA yielded a 1 kb product, which was ligated into the pCRII vector (Invitrogen, San Diego, California) and sequenced by the Sanger dideoxy termination method.

Oligo dT-primed double-stranded cDNA was prepared from poly$(A)^+$ RNA isolated from normal human spleen. BstXI linkers were added to the cDNA prior to cloning into pCDNA.Amp (Invitrogen). The ^{32}P-labeled canine αd fragment was added to colony replicas in prehybridization buffer (5× SSC, 10× Denhardt's, 1% SDS, 30% formamide), and allowed to hybridize overnight at 42°C. The final stringency wash was at 65°C in 1× SSC, 0.1% SDS. A single clone, 19A2, was identified and sequenced on the sense strand by the nested deletions technique. The antisense strand was sequenced for confirmation.

Expression and Analysis of the αd cDNA

An oligonucleotide was designed to include a 5′ EcoRI site, modified Kozak consensus sequence (Kozak, 1987), an adenosine, and sequence overlapping the 5′ end of clone 19A2 (AGTTACGAATTCGCCACCATGACCTTC GGCACTGTG). A 706 bp fragment was amplified from clone 19A2 using this oligo and a specific internal oligonucleotide from the antisense strand (CCACTGTCAGGATGCCCGTG). The PCR product was restricted with EcoRI and BamHI and ligated to the 3.2 kb BamHI–XbaI fragment from 19A2. The resulting EcoRI–XbaI fragment was ligated into pcDNA3 (Invitrogen) resulting in the plasmid pATM.D12. The cDNAs encoding human CD11a, CD18, and CD29 (β1) were each subcloned into the expression vector pDC1. A pCDM8 construct containing the CD11c cDNA was obtained from F. Sanchez-Madrid (Universidad Autonoma de Madrid, Spain). The CD49d (α4) and CD11b encoding cDNAs were subcloned into the pcDNA.1 vector (Invitrogen). The plasmids pATM.D12 and pDC1.CD18 were used to transfect DHFR–DG44. CHO cells (obtained from L. Chasen, Columbia University, New York) by electroporation. The plasmid pDC1.CD11a was transfected along with pDC1.CD18 as a positive control. Vectors expressing α4 and β1 were cotransfected to provide a positive control for VCAM-1 binding experiments. Stably transfected clones were selected in nucleoside-deficient medium.

Biotinylated (Cole et al., 1987) CD18 complexes were precipitated from detergent lysates of CHO transfectants with the monoclonal antibody TS1/18 (American Type Culture Collection [ATCC] HB203) or 169A (anti-αd). Biotinylated protein was isolated under reducing conditions on 8% polyacrylamide gels and transferred to PVDF membranes. Biotinylated proteins were detected with Strepavidin POD (Boehringer Mannheim) diluted 1:6000 in TBS-T, 0.3% BSA. Hyperfilm (Amersham) was used to expose the membranes for 0.5–2 min after development with enhanced chemiluminescence reagents (Amersham).

Generation of αd-Specific MAbs

Detergent lysates were made as previously described (Danilenko et al., 1992) from 100 g of human spleen obtained from the University of Utah. Lysates were clarified by centrifugation and sequentially precleared of LFA-1 and Mac-1 with Affigel 10 resin to which the antibody TS1/22.1 (ATCC HB202) or 44aacb (ATCC HB249) was bound. Remaining CD18 complexes were affinity purified with the murine antibody TS1/18. BALB/c mice were immunized three times at 21 day intervals with 30 μg/mouse protein in adjuvant. The spleen from a single mouse was fused with NS-1 myeloma cells and hybridomas were obtained according to standard procedures. Culture supernatants were analyzed by flow cytometry for the ability to react with αd/CD18 CHO cells but not with JY cells, which in our hands express only CD11a/CD18. The clone 169A (IgG1) was isolated after two rounds of subcloning. Antibody purified from exhausted hybridoma supernatant on a PROSEP-A column (Bioprocessing Limited) according to package directions was used in the flow cytometric experiments presented; histologic analysis was performed using supernatants.

Flow Cytometric Analysis of αd Expression

Surface expression of leukointegrin α chains on cell lines, PBLs, and transfectants was detected with the murine MAbs 169A (αd), TS1/22 (CD11a, ATCC), 44aacb (CD11b, ATCC), and BU15 (CD11c, Dako). Human PBLs were isolated from blood on a Histopaque 1077 (Sigma) gradient using standard procedures. Granulocytes were isolated on Ficoll–Hypaque gradients (Ferrante and Thong, 1982). Cellular Fc were blocked with 1 mg/ml human IgG (Sigma) in RPMI with 2% fetal bovine serum and 0.01% sodium azide (FACS buffer) for 30 min on ice. Cells (10^6) per group were incubated on ice in 100 μl FACS buffer with primary antibody at 10 μg/ml or 100 μl hybridoma supernatant. Bound antibody was detected with sheep anti-mouse IgG–fluorescein isothiocyanate (FITC) (Sigma), followed by washing and analysis on a Becton-Dickinson FACscan using Lysis II software.

Immunohistology

Frozen sections from human abdominal aorta (PDAY Research Group) were stained with irrelevant murine IgG or MAb 169A (αd). Bound antibody was detected using rabbit anti-mouse IgG (Dako) and mouse APAAP reagent (Dako) with subsequent development with Substrate Kit III (Vector). Sections were counterstained with Nuclear Fast Red (Vector). Human spleen and liver sections (National Disease Research Interchange) were treated in the same fashion, but with the addition of MAbs against CD11a (TS1/22, ATCC), CD11b (44aacb, ATCC), and CD11c (BU15, Dako). Aortic sections stained with Oil Red O were dehydrated with 100% propylene glycol before staining. For double staining, MAb EBM11 (CD68)-treated sections were incubated successively with supersaturating concentrations of peroxidase-conjugated anti-mouse IgG (1:100, Dako) and peroxidase-conjugated swine anti-rabbit IgG (1:30, Dako). Sections were blocked with swine anti-rabbit IgG before addition of MAb 169A. Slides were developed with the DAB (3.3 diaminobenzidine) kit (Vector) and the AP (alkaline phosphatase) Substrate III kit (Vector).

CAM Binding Assays

To analyze αd/CD18–CAM interactions in solution, 10^6 (per group) αd/CD18 CHO transfectants from suspension cultures were incubated for 20 min at room temperature in 100 μl buffer only (Tris-buffered saline, 0.1% BSA), buffer with 10 μg/ml MAb, or buffer with 2 mM $MgCl_2$. After washing, cells were incubated in buffer only or buffer (± $MgCl_2$) with 10 μg/ml ICAM-3/Ig, ICAM-1/Ig, or VCAM-1/Ig (Sadhu et al., 1994) for 30 min at room temperature. Bound fusion protein was detected with goat anti-human IgG–FITC (Jackson Labs, West Grove, Pennsylvania) diluted 1:100 in buffer, followed by washing and subsequent analysis on a Becton-Dickinson FACscan.

For analysis of integrin binding to immobilized CAM/Ig, microtiter

plates were coated with 10 μg/ml soluble ICAM-3/Ig or ICAM-1/Ig, or 1% BSA and blocked with 1% BSA. CHO transfectants (1×10^7) per group were labeled with 8 μg calcein dye (Molecular Probes, Eugene, Oregon) in phosphate-buffered saline. Calcein-labeled cells were washed and resuspended in buffer with or without 10 μg/ml TS1/18 and incubated at 37°C for 25 min in triplicate wells/group on microtiter plates. After washing by immersion in phosphate-buffered saline with 0.1% BSA for 20 min, remaining fluorescence was determined with an automatic fluorescence reader (Cyto Fluor 2300–2350, Millipore).

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