Reprinted from Nature, Vol. 326, No. 6111, pp. 400-403, 26 March 1987

The T lymphocyte glycoprotein CD2 binds the cell surface ligand LFA-3

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CD2 (known also as T11 (ref. 1), LFA-2 (ref. 2) and the erythrocyte rosette receptor (ref. 3)) is a functionally important T lymphocyte surface glycoprotein of relative molecular mass 50,000 to 58,000[4] (M_r 50–58 K) which appears early in thymocyte ontogeny and is present on all mature T cells[5]. Monoclonal antibodies to CD2 inhibit cytotoxic T-lymphocyte (CTL)-mediated killing by binding to the T lymphocyte and blocking adhesion to the target cell[2–4,6]. Such antibodies also inhibit T helper cell responses including antigen-stimulated proliferation, interleukin-2 (IL-2) secretion, and IL-2 receptor expression[2–4,7–9]. Certain combinations of monoclonal antibodies to CD2 epitopes trigger proliferation of peripheral blood T lymphocytes[1], cytotoxic effector function[10] and expression of IL-2 receptors by thymocytes, resulting in thymocyte proliferation in the presence of exogenous IL-2 (ref. 11). These findings suggest that CD2 can function in signalling as well as being an adhesion molecule. To understand the role of CD2 in T-cell adhesion and activation, it is essential to define its natural ligand. Our previous observation that purified CD2 inhibits rosetting of T lymphocytes with sheep erythrocytes and can be absorbed by sheep erythrocytes[12] suggested it also might bind with detectable affinity to human cells. We now report that CD2 binds to a cell-surface antigen known as lymphocyte function-associated antigen-3 (LFA-3) with high affinity, and can mediate adhesion of lymphoid cells via interaction with LFA-3.

We established an assay of purified CD2 binding to human lymphoid cells. CD2 was solubilized with the detergent Triton X-100 and purified to near homogeneity from the human T lymphoblast line SKW3 by affinity chromatography as described[12,13] and labelled with ^{125}I (Fig. 1*a* inset). CD2 was shown to be the major component of this preparation by immunoblotting the unlabelled CD2 preparation with ^{125}I-labelled anti-CD2 monoclonal antibody. The sequence of the N-terminus and tryptic peptides of this CD2 preparation are reported elsewhere[13]. CD2 preparations were diluted with buffers containing bovine serum albumin (BSA) (which binds detergent)[14] to a final detergent concentration of 0.001–0.05% depending on the experiment. Under these conditions, at 4 °C, there was no cell lysis. ^{125}I-labelled CD2 binding was tested with the B lymphoblastoid cell line JY, which is an excellent target in CTL-mediated killing[2,4]. Binding of ^{125}I-labelled CD2 to JY cells was dependent on the concentration of ^{125}I-labelled CD2 and JY cells. At the highest concentration of JY cells tested, 19% of input ^{125}I-llabelled CD2 bound (Fig. 1*a*). Binding was specific, as it was competed out by unlabelled purified CD2 but not by a control preparation of purified lymphocyte function-associated antigen-1 (LFA-1) (Fig. 1b). Binding was half-maximally inhibited at 90 mM CD2, suggesting an association constant of $\sim 10^7$ M^{-1}.

Several recent findings suggested that the ligand for CD2 might be the LFA-3 molecule, a glycoprotein of M_r 55–70K that is broadly distributed on both nonhaematopoietic and haematopoetic cells[2,4]. Monoclonal antibodies (mAb) against LFA-3 and CD2 inhibit a similar spectrum of antigen-specific helper T and CTL functions[4]. The anti-CD_2 antibodies inhibit CTL-mediated killing by binding to the T cells, whereas anti-LFA-3 mAb inhibit by binding to target cells[4]. Both types of antibody block T lymphocyte-target cell adhesion[6]. Studies on antigen-independent conjugation of CTL to B lymphoblastoid target cells have suggested that CD2 and LFA-3 participate in the same adhesion-strengthening functional pathway[15]. Antibodies to each antigen partially (about 50%) inhibit conjugate formation. Combinations of saturating concentrations of mAb to LFA-1 and CD2 or LFA-1 and LFA-3 inhibit conjugate formation totally, and thus are additive, whereas the combination of antibodies to CD2 and LFA-3 is no more effective than either antibody alone[4,15]. CD2 and LFA-3-dependent adhesion can occur in the absence of Mg^{2+} and at 4 °C, whereas LFA-1-dependent adhesion is Mg^{2+} and temperature dependent. Furthermore, thymocyte rosetting with thymic epithelial cells is dependent on CD2 on the thymocyte and LFA-3 on the thymic epithelial cell[16].

Table 1 Inhibition of ^{125}I-labelled CD2 binding to JY cells by anti-LFA-3 antibody

Antibody specificity	CD2 bound (mean c.p.m. ± s.d.)
X63	6,249 ± 595
Anti-LFA-1	6,900 ± 493
Anti-HLA-A, B	6,818 ± 681
Anti-CR1	6,599 ± 355
Anti-LFA-3	165 ± 11
Anti-CD2	64 ± 12

The ^{125}I-labelled CD2 was prepared as described (Fig. 1, legend). Specific activity was 3.8×10^8 c.p.m. nmol^{-1}. JY cells (3.3×10^5) were incubated with 50 μl of antibody (hybridoma culture supernatant) for 45 min at 4 °C. Then 50 μl of ^{125}I-labelled CD2 (diluted to 4,000 c.p.m. μl^{-1} with 10% fetal bovine serum (FBS)/RPMI 1640/2 mM HEPES, *p*H 7.4/3% BSA) was added and the incubation continued for another 2 h at 4 °C. After incubation the cells were washed three times with 10% FBS/RPMI 1640/2 mM HEPES, *p*H 7.4 and gamma counted. Antibodies used: anti-CD2 (TS2/18), anti-LFA-3(TS2/9), anti-LFA-1(TS1/22)[2], anti-HLA-A,B(W6/32)[27], control (P3X63myeloma IgG_1) and anti-CR1(D44)[28].

Pretreatment of JY cells with the LFA-3 mAb completely inhibited^{125}I-labelled CD2 binding (Table 1). ^{125}I-labelled CD2 binding was not inhibited by antibodies against LFA-1, HLA-A,B, or complement receptor type 1 (CR1), but was inhibited by anti-CD2 mAb, confirming the specificity of inhibition and that ^{125}I-labelled CD2 molecules had retained the TS2/18 epitope after affinity purification.

These findings suggest that CD2 and antibodies against LFA-3 compete for binding to cell surface LFA-3 molecules. This was confirmed with the reciprocal approach: inhibition of antibody binding to LFA-3 by CD2 (Fig. 2). JY cells were incubated with purified CD2 or the purified LFA-1 membrane protein as control. The subsequent binding of LFA-3 mAb to cells was determined by immunofluorescence flow cytometry. Purified CD2 inhibited LFA-3 mAb binding by 91% at 420 nM during the preincubation (Fig. 2*a,b*). Half the maximal inhibition was obtained at 60 nM CD2 (Fig. 2*d*). Preincubation with CD2 did not affect binding of mAb to intercellular adhesion molecule-1 (ICAM-1), a putative ligand for LFA-1[17] (Fig. 2*c,d*). Preincubation with purified LFA-1 or a matched detergent buffer had no effect on binding of antibodies to either LFA-3 or ICAM-1 (Fig. 2*d*).

To obtain an accurate measurement of the affinity of soluble CD2 for JY cell surface LFA-3, saturation binding experiments were carried out (Fig. 1*c*). CD2 showed saturable binding to JY cells. Binding which could not be inhibited by 217 μg ml^{-1} LFA-3 mAb (100-fold the saturating concentration) was taken to be nonspecific and was linearly dependent on the concentration of CD2 added. Scatchard analysis of specific binding shows the association constant $K_a = 1.9 \times 10^7$ M^{-1} (Fig. 1*d*). At saturation, 680,000 molecules of CD2 were bound per JY cell. The number of CD2 binding sites per JY cell is slightly higher (2- to 3-fold) than an approximate estimate of the number of LFA-3 sites obtained by fluorescence flow cytometry (data not shown). Whether the hydrophobic region of CD2 influences affinity and

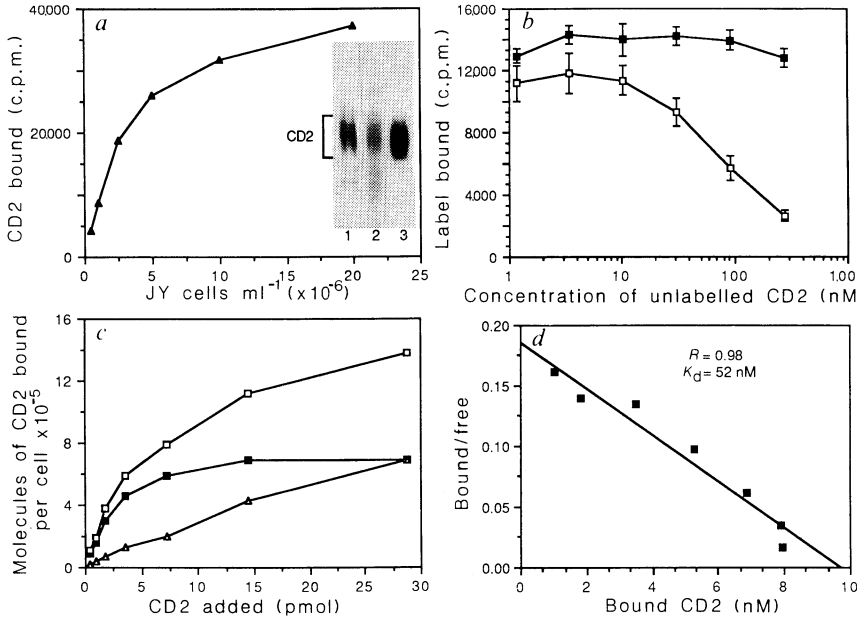

Fig. 1 ^{125}I-labelled CD2 binding to JY cells. *a*, Binding of ^{125}I-labelled CD2 to JY cells at various cell concentrations. The input of CD2 was 2×10^5 c.p.m. in 50 μl, and the binding assay was carried out as described (Table 1 legend). The specific activity of ^{125}I-labelled CD2 was 3.8×10^8 c.p.m. nmol^{-1}. Inset, purified CD2 subjected to SDS 8% PAGE (polyacrylamide gel electrophoresis) under reducing conditions. Lane 1, polyacrylamide gel after silver staining[29]; lane 2, autoradiogram of ^{125}I-labelled CD2; lane 3, autoradiogram after immunoblotting with ^{125}I-labelled anti-CD2 mAb[12]. *b*, Competitive inhibition of ^{125}I-labelled CD2 binding to JY cells. The specific activity of labelled CD2 was 7×10^7 c.p.m. nmol^{-1}. JY cells (5×10^5 in 100 μl) were incubated with ^{125}I-labelled CD2 (10^5 c.p.m.) and unlabelled CD2. The binding assay was as described (Table 1). As a control, purified LFA-1 at the same detergent and buffer concentration as CD2 (but 2.4-fold higher in concentration at each point) was serially diluted in parallel to unlabelled CD2 and mixed with labelled CD2. □, Unlabelled CD2 added; ■, LFA-1 added. *c*, Saturation binding of ^{125}I-labelled CD2 to JY cells. The specific activity of the ^{125}I-labelled CD2 was 1.8×10^7 c.p.m. nmol^{-1}. JY cells (5×10^5) were preincubated in 10 μl of 10% FBS/RPMI 1640 with or without purified anti-LFA-3 antibody (1.3 mg ml^{-1}) for 30 min at 4 °C. Then 50 μl of varying concentrations of ^{125}I-labelled CD2 was added and the binding of CD2 was assayed as described above. Nonspecific binding represents binding in presence of anti-LFA-3 mAb. The specific binding was obtained by subtracting nonspecific binding from total binding. Note that at the higher CD2 concentrations used here, the proportion of binding which could not be competed out with antibody to LFA-3 was higher than in other experiments with high specific activity ^{125}I-labelled CD2 preparations. CD2 molecules bound per cell was calculated taking the M_r of CD2 as 50K. ■, Specific binding; □, total bound; △, nonspecific binding. *d*, Scatchard plot. The values obtained as specific binding (Fig. 1*c*) were used for Scatchard analysis[30]. The dissociation constant (K_d) was obtained from slope $=-1/K_d$.

Methods. CD2 was purified from the SKW3 T lymphoma cell line by immunoaffinity chromatography as described[12,13]. The CD2 was eluted from a column of TS2/18 mAb CD2-Sepharose using 0.1 M glycine HCl buffer *p*H 2.75 containing 0.2 M NaCl and 0.2% Triton X-100. The eluate was immediately neutralized to *p*H 7.4 with 0.1 vol. of 1 M Tris HCl *p*H9.0. Protein was determined on ethanol-precipitated CD2 according to Lowry. Aliquots were labelled with ^{125}I using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril[31] and extensively dialysed against 10 mM Tris HCl *p*H 8.0, 0.14 M NaCl, 0.02% NaN_3. LFA-1 was purified from the same SKW3 cell lysate using a TS1/22 monoclonal antibody[2]-Sepharose column linked in series to the CD2 antibody-Sepharose column under identical chromatography and elution conditions.

site number measurements can only be determined when extracellular-domain fragments of CD2 become available.

Functional studies with purified CD2 demonstrate that it mediates adhesion of lymphoid cells (Fig. 3). Although JY cells (LFA-1$^+$, ICAM-1$^+$, CD2$^-$, LFA-3$^+$) aggregate at 37 °C by LFA-1-dependent adhesion[18], they do not spontaneously aggregate at 4 °C (Fig. 3*a*). At 4 °C, the CD2/LFA-3 pathway operates but the LFA-1 pathway does not[15]. Addition of purified CD2 (400 nM) resulted in substantial homotypic adhesion of JY cells at 4 °C (Fig. 3*b*) whereas heat-denatured CD2 was without effect (Fig. 3*c*). Anti-LFA-3 inhibited adhesion (Fig. 3*d*). As a specificity control, aggregation induced by the anti-LFA-1 IgM mAb, RDF4 could not be inhibited by anti-LFA-3 mAb (not shown). These results show that purified CD2 can directly mediate cell–cell adhesion by binding to LFA-3. Although we do not know the physical form of CD2 which mediates cell–cell adhesion in this assay, we believe that a significant amount of CD2 integrates into the JY cell membrane by means of a hydrophobic region at the high concentration we have used for aggregation studies (see the binding which can not be competed by antibody against LFA-3 in Fig. 1*c*). Sedimentation of CD2 in detergent-free sucrose gradients suggested that the antigen is monomeric (data not shown), and that the aggregation of JY cells was not due to multimeric CD2.

Previous findings that antibodies against CD2 and LFA-3 inhibit T-cell functions and T-cell conjugation to target cells have been interpreted to suggest either that CD2 and LFA-3 are adhesion proteins[2,4,6,15,16,19,20] or that CD2 and LFA-3 may have a general role in cell function unrelated to adhesion, and that binding of antibody delivers a 'negative signal'[7,20]. Our results clearly define CD2 as a T-cell adhesion protein. Furthermore, we have found that LFA-3 is a cellular ligand for CD2. Thus both a T-cell adhesion receptor and its ligand have been defined. Thus far, the mechanism of action of other T-cell 'accessory molecules' including LFA-1, CD4 (T4) and CD8 (T8) is unclear. Although functional studies have led to the proposal that they interact with ligands on other cells (ICAM-1, HLA class I, and HLA class II, respectively[21]), in no case has a physical interaction with a ligand, or of a purified molecule with whole cells, been demonstrated. This report is the first demonstration that a purified T-cell surface protein can bind to a cell-surface ligand and mediate cell adhesion.

We are unaware of any previous determinations of the affinity of a cell adhesion molecule for a cell-surface ligand. The affinity of CD2 for LFA-3 is high. The association constant of 1.7×10^7 M^{-1} is within the range of affinities of antibodies for antigens. Affinities of this order of magnitude may be important in stabilizing adhesion between motile cells and in overcoming repulsion between cells due to net negative surface charge, the glycocalyx and other factors.

We have further found that, as with antigen-independent adhesion of T lymphocytes to target cells, rosetting of activated T lymphocytes with autologous erythrocytes is mediated by the interaction between CD2 and LFA-3 (refs 22, 23). We have also found that purified CD2 mediates aggregation of human erythrocytes, and this is inhibited by antibody to LFA-3 (ref. 22). The interaction between CD2 and LFA-3 appears analogous to that between CD2 and the sheep T11TS molecule in rosetting of human T lymphocytes with sheep erythrocytes[24]. Studies with purified LFA-3 reciprocal to those reported here have confirmed that CD2 is a receptor for LFA-3. Furthermore, we have found that purified LFA-3 reconstituted into planar membranes mediates T lymphocyte adhesion by interaction with CD2[25].

Monoclonal antibodies against CD2 can stimulate T-cell

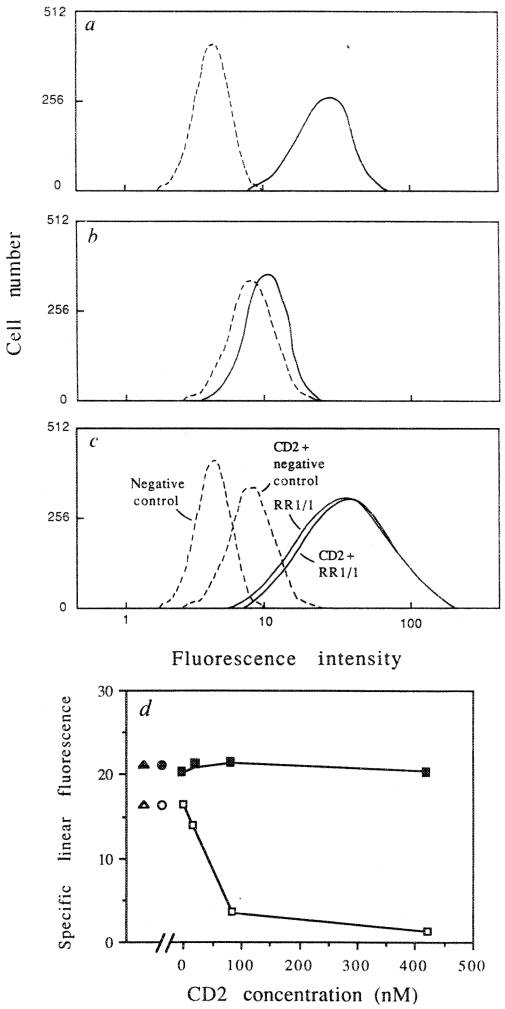

Fig. 2 Inhibition by CD2 of antibody binding to LFA-3. *a*, Anti-LFA-3 mAb (solid line) and nonbinding IgG_1 control antibody (dashed line) staining of JY cells pretreated with control buffer; *b*, as in *a*, but JY cells were pretreated with 420 nM CD2. CD2 caused a concentration-dependent increase in negative control fluorescence which was not given by detergent controls; this may result from nonspecific association of IgG with the hydrophobic portion of bound CD2. *c*, Anti-ICAM-1 mAb RR1/1 (ref. 17; solid line) and nonbinding control antibody (dashed line) staining of JY cells with and without pretreatment with 420 nM CD2. *d*, Inhibition of antibody binding as a function of CD2 concentration. Monoclonal anti-ICAM-1 and LFA-3 antibodies with CD2: ■ and □, respectively; anti-ICAM-1 and anti LFA-3 controls (no detergent): ▲ and △; anti-ICAM-1 and anti-LFA-3 controls with 1,000 nM LFA-1: ● and ○.

Methods. JY cells (10^5) were incubated with purified CD2, purified LFA-1, or control buffer with identical detergent concentration (0.015% or less) in 15% BSA, Hanks balanced salt solution (HBSS) at 4 °C in a volume of 20 μl. After 60 min, 20 μl of mAb in HBSS, 15% BSA was added. The lowest concentration of antibody giving optimal staining (2 μg ml^{-1} for the monoclonal anti-LFA-3 antibody TS2/9) was used. After 30 min at 4 °C the cells were washed ×3 with HBSS 10% FBS and stained with fluoroscein isothiocyanate (FITC)-goat anti-mouse IgG for 30 min at 4 °C. Cells were then analysed on a Coulter Epics V flow cytometer.

proliferation and effector function[1,10,11], suggesting that interaction of CD2 with its physiological ligand, LFA-3, may mediate T-cell activation as well as adhesion. Interaction with $LFA\text{-}3^+$ thymic epithelial cells may be important in driving the proliferation of immature, $CD2^+$, antigen-receptor negative thymocytes[16,21,26]. Further studies are required to determine

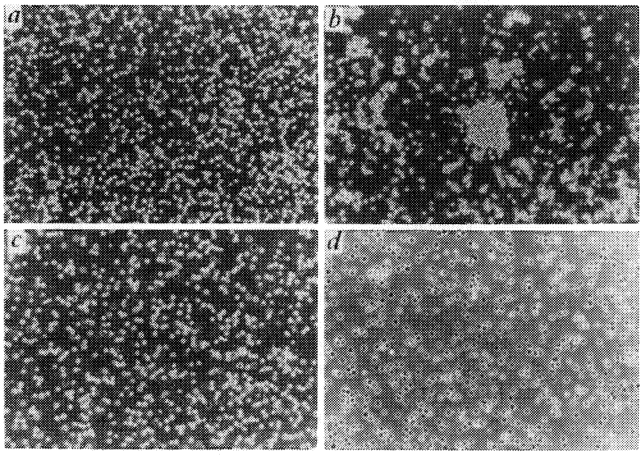

Fig. 3 CD2 and LFA-3-dependent cell adhesion. *a*, JY cells; *b*, JY cells + CD2; *c*, JY cells + heat denatured CD2 (100 °C for 15 min) *d*; JY cells + CD2 + anti-LFA-3 mAb.

Methods. Purified CD2 (84 μg ml^{-1}) or control buffer (24 μl), 30% BSA (50 μl), and 0.25 mg ml^{-1} antibody (2 μl) or buffer (2 μl) were mixed on ice and added to 10^6 JY cells in 24 μl. The mixture was centrifuged for 2 min at 200*g* and incubated on ice for 2 h. The cells were gently resuspended and photographed with an inverted microscope at ×100 magnification.

whether receptor–ligand interaction between CD2 on mature T lymphocytes and LFA-3 stimulates the 'alternative pathway'[1] of T-cell activation or can synergize with the classical antigen receptor pathway.

Supported by NIH grant CA31798 and an American Cancer Society Faculty Award (T.A.S.).

Received 5 November 1986; accepted 29 January 1987.

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