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Two antigen-independent adhesion pathways used by human cytotoxic T-cell clones

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Cell–cell adhesion is essential for many immunological functions[1–4], including interaction of cytotoxic T lymphocytes (CTLs) with their targets[5–8]. We have explored CTL-target interactions using well-characterized cloned human CTLs[9,10]. Conjugate formation between these CTLs and many antigen-negative targets is almost as efficient as with specific target cells, but does not lead to target-cell lysis. Thus, on specific target cells, adhesion by antigen-independent pathways may occur concurrently with or precede antigen recognition. The molecules LFA-1, CD2 (T11, LFA-2) and LFA-3 have been shown[11–15] to be involved in human CTL conjugation with and lysis of specific target cells. Here we describe monoclonal antibody inhibition studies using individual monoclonal antibodies and mixes which demonstrate (1) that LFA-1, CD2 and LFA-3 are involved in antigen-independent conjugate formation; and (2) suggest that CD2 and LFA-3 are involved in one pathway and LFA-1 in another. We confirmed the existence of distinct pathways by the demonstration that LFA-1-dependent adhesion requires divalent cations and is temperature-sensitive whereas CD2- and LFA-3-dependent adhesion does not require divalent cations and is temperature-insensitive. Together with previous data, our studies suggest that CD2 on the effector interacts with LFA-3 as its ligand on targets.

We have analysed the specificity of conjugate formation and of cell-mediated lysis (CML) for a panel of CTLs specific for an allogeneic HLA class II molecule[16] (HLA-DPw2). The antigen specificity of target-cell lysis is shown in Table 1 for a typical CTL clone (designated 8.9); target cells which express the DPw2 allele are lysed and DPw2-negative targets are not. We measured conjugate formation between CTL and targets by a two-colour fluorescence technique that exploits flow microfluorometry to enumerate conjugates objectively[17,18]. In contrast to target lysis, the ability of this clone to form conjugates is largely independent of whether or not the target expresses DPw2. Antigen-independent conjugate formation is observed with all DPw2-negative lymphoblastoid B-cell lines (LCLs) tested; conjugate formation is comparable on LCLs from normal donors and LCLs derived from patients with a defect in LFA-1 expression[19], demonstrating that LFA-1 expression on the target is not required for conjugate formation.

Antigen-independent conjugate formation is also observed in various other cell lines; for example, high conjugate formation on U266 (a plasmacytoid cell line grown spontaneously from cells of a patient with multiple myeloma[20]) and on class II-negative cells such as the cervical carcinoma line HeLa. Conjugate formation also occurs between CTLs and subpopulations of peripheral blood mononuclear cells such as plastic adherent cells or non-adherent non-T cells (E−). Little conjugate formation is seen with resting T (E+) cells as targets. Lysis by this CTL is dependent on target expression of DPw2 but conjugate formation is largely independent of that expression (Table1).

Antigen-independent conjugates are not artefacts of long-term *in vitro* propagation as they are observed not only with the five clones tested here but also with short-term alloantigen-stimulated T cells[21] (data not shown). The finding that conjugate formation can occur without target expression of specific antigen and even in the absence of class II expression suggests that, on antigen-positive targets, adhesion precedes or occurs simultaneously with antigen recognition. Defects in this interaction between cells of different species[22] may contribute to the inability of many T-cell clones to recognize their specific alloantigen expressed in xenogeneic cells[23,24].

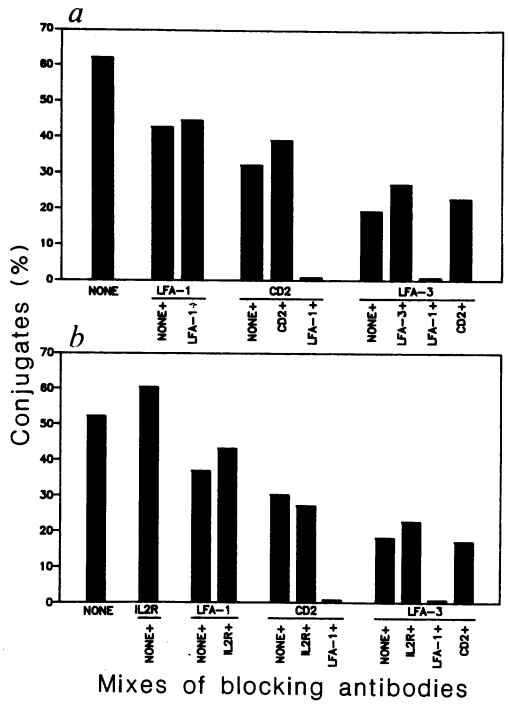

Fig. 1 Antibody-inhibition of antigen-independent conjugate formation of two different DPw2-specific CTL clones (8.9 in *a*; 8.2 in *b*) with target U266. MAb was added to the effectors for a 20-min pre-incubation at 37 °C and then remained during the assay of conjugate formation (performed as described in Table 1). The αLFA-1 antibody was MHM23 (refs 13, 34) which is specific for a determinant (also termed CDw18) on the β-chain shared by LFA-1, Mac-1 and p150/95; the αCD2 antibody was 95-5-49 (R.Q. and R.E.G., manuscript in preparation); the αLFA-3 antibody was TS2/9 (refs 11, 12); and the negative control antibody was 7G7/B6 which is specific for the interleukin-2 receptor[35]. Antibody concentrations were based on results of earlier experiments to give optimal inhibition and were a 1/100 final concentration of ascites fluid for MHM23, TS2/9 and 7G7/B6 and 1/200 ascites for 95-5-49. Each antibody was present at that same concentration in mixes with other antibodies. Results for mixes are shown next to the results for the antibody which was most inhibitory by itself.

Blocking studies with monoclonal antibodies (MAbs) known to inhibit CML demonstrated that αLFA-1, αCD2 and αLFA-3 consistently inhibit antigen-independent conjugate formation (Fig. 1). Potential for antibody-mediated crosslinking between effector and target was minimized by choice of a target (U266) with minimal LFA-1 expression and no CD2; crosslinking by αLFA-3 is not expected as the effectors express only low levels of LFA-3 (data not shown). Blocking by optimal concentrations of these MAbs typically ranges between 20 and 70% and is often less effective at increased antibody concentrations. Previously, antigen-independent conjugate formation has often been ignored on the assumption that it represents an unexplained *in vitro* artefact. But, the probable physiological relevance of the phenomenon is emphasized by our present finding that the molecules involved in antigen-independent conjugate formation are precisely those which are involved in conjugate formation with antigen-positive targets[15] and which are critical in the lytic interaction as a whole[11–14]. Recent studies by Spits and coworkers[25] support our hypothesis of a critical role of antigen-independent conjugate formation in CML.

When mixes of these MAbs are assayed for inhibitory capacity, additive (or even synergistic) effects are observed with a

combination of αLFA-1 and either αCD2 or αLFA-3, but the mix of αCD2 and αLFA-3 is no more inhibitory than αLFA-3 alone. One previous study[11] found a similar pattern of additivity among these MAbs in inhibition of CML. These results are consistent with the hypothesis that the CD2 and LFA-3 molecules are involved in one binding pathway whereas the LFA-1 molecule is involved in a distinct pathway. Our data (Fig. 1) indicate that there are no additional pathways in this model system because the mixture of αLFA-1 and αCD2 or αLFA-3 inhibits completely; with these effectors, no role has been demonstrated for the CD4 molecule in physical conjugate formation (S.S. and G.E.G.L., unpublished observations). If CD2 and LFA-3 participate in the same pathway, then antibodies against both would not have additive effects at optimal concentrations because either alone would block the postulated CD2/LFA-3 pathway. In contrast, αCD2 or αLFA-3 have enhanced effectiveness when mixed with αLFA-1, because each antibody in the mix is inhibiting a different pathway.

Studies of other agents known to inhibit CML show that some manipulations (chilling to 4 °C and removal of divalent cations) may partially inhibit antigen-independent conjugate formation (Fig. 2); this suggests that only part of the process of conjugate formation is temperature-sensitive or divalent cation-dependent. When conjugate formation is analysed using these agents in combination with MAb blocking, the data confirm this hypothesis. LFA-1-mediated conjugate formation (which remains after αCD2 or αLFA-3 inhibition) is temperature-sensitive and depends on a cation, probably magnesium, which is bound by EDTA but not EGTA. CD2- and LFA-3-dependent conjugate formation (which remains after αLFA-1 inhibition) is temperature-insensitive and divalent cation-independent. The cation-dependence of the LFA-1 pathway is consistent with evidence that magnesium rather than calcium is required for conjugate formation[26].

Table 1 Lysing of DPw2+ targets and antigen-independent conjugation by clone 8.9

Target cell type	Lysis (%) DPw2+	Lysis (%) DPw2−	Conjugates (%) DPw2+	Conjugates (%) DPw2−
Experiment 1				
Lymphoblastoid B cell lines				
from normal donors	29	2	54	36
from LFA-1$^-$ patients	26	1	45	34
Experiment 2				
U266, Plasmacytoid cell line	—	1	—	65
HeLa, Class II$^-$ cervical carcinoma	—	2	—	49
Peripheral leukocyte populations				
plastic adherent	32	3	59	49
non-adherent E$^-$	33	1	31	21
non-adherent E$^+$	5	0	8	4

Results shown are for individual targets but are representative of results with other clones and targets. Cytotoxic T-cell clones were propagated and assayed for CML by ^{51}Cr-release as described elsewhere[9]. Conjugate formation was measured following pelleting of effector and target together at a ratio of 4:1, incubation for 6 min at 37 °C and resuspension in cold phosphate buffered saline. Conjugates were enumerated by a flow microfluorometric assay: effectors were stained to fluoresce green, targets to fluoresce red and conjugates detected as particles that fluoresce at both wavelengths[17]; 10,000 particles were analysed and the results expressed as the percentage of targets found in conjugates. We also determined the background caused by coincident detection of red and green cells in a cell mix lacking conjugates (that is, an effector/target mix analysed immediately after mixing); this background ranges from 2 to 13% and is subtracted from experimental values. Target cells were prepared as follows: donor cells were typed for presence of DPw2 as described previously[32]; lymphoblastoid B cell lines were standard Epstein-Barr virus-transformed lines derived from local normal donors or from patients with <1% of normal LFA-1 expression[19]; U266 is a plasmacytoid cell line[20] provided by Dr S. Korsmeyer; mononuclear cells were prepared by density separation of fresh peripheral blood, fractionated by adherence to plastic and then the nonadherent cells fractionated by rosetting with 2-aminoethyl-isothiouronium bromide hydrobromide-treated sheep red blood cells[33].

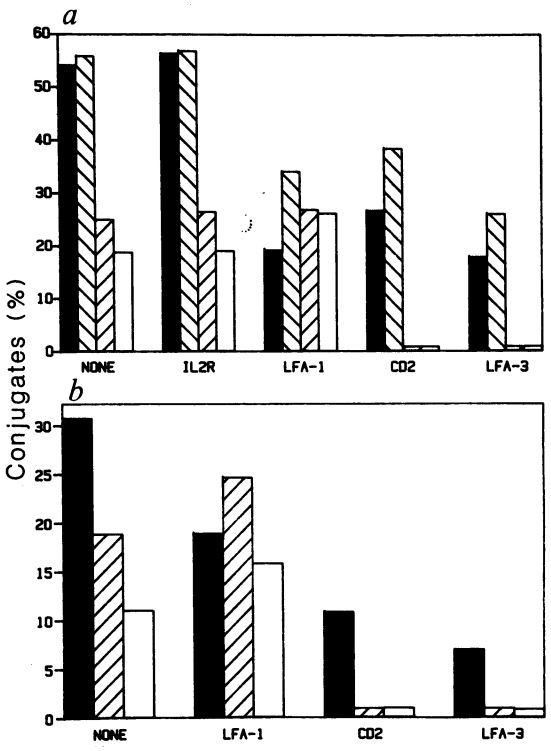

Fig. 2 Differences between pathways of conjugate formation in their requirements for divalent cations and their ability to support conjugate formation at 4 °C. Conjugates were formed between two DPw2-specific clones on two different DPw2-negative targets (clone 8.2 on target U266 in *a*; clone 8.9 on a normal LCLs in *b*). Conjugate formation was measured in the presence of various MAbs whose specificity is indicated below the relevant group of bars; antibody concentrations and incubation conditions were identical to those described in Fig. 1 legend. Chelation of divalent cations was achieved by pre-incubation of both effector and target for 20 min at 37 °C with: ■, no inhibitor in the presence of 1.8 mM Mg^{2+} and 1.3 mM Ca^{2+}; ▨, ▧, in the presence of an excess (5 mM) EDTA or EGTA, respectively; □, 4 °C with no inhibitor. Conjugate formation was measured following pelleting, incubation for 6 min at 37 °C (standard conditions) or for 20 min at 4 °C and resuspension.

It has previously been shown[11] that the inhibitory effect of both αLFA-1 and αCD2 is exerted by binding to the effector cell, whereas the inhibitory effect of αLFA-3 is mediated by binding to the target cell. Our present data suggest that LFA-3 is the ligand for CD2. Additional supporting evidence comes from studies by Wolf and co-workers[27] demonstrating that the binding of thymocytes to cultured thymic epithelium is inhibited by αCD2 binding to the thymocyte or αLFA-3 binding to the thymic epithelium. Two pieces of evidence conflict with the hypothesis that CD2 binds LFA-3 (1) high conjugate formation is not observed between CTL and many cell types that express LFA-3 (data not shown), but interaction between CD2 and LFA-3 may depend on post-translational modification of the LFA-3 molecule, as has been dramatically demonstrated for the neural cell adhesion molecule, N-CAM[28]. (2) Trypsin pre-treatment of target LCL eliminates binding by the αLFA-3 MAb, but cytotoxic interactions still occur and are still blocked by αCD2 (ref. 29). This can be explained if the αLFA-3 antibody binds to a trypsin-sensitive epitope different from the binding site for CD2 and prevents interaction of LFA-3 with CD2 either by steric hindrance or by an allosteric effect.

What is the ligand for LFA-1? It is apparent that homotypic interaction between LFA-1 molecules on the effector and target is not critical. This has been demonstrated by previous studies[30,31], and is evident from the present studies in which conjugate formation is as good with LFA-1-deficient LCL as with LFA-1-sufficient LCL (Table 1).

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Note added in proof: Recent studies[36] suggest that I-CAM-1 may be an LFA-1 ligand.

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