Short paper

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Functional evidence that intercellular adhesion molecule-1 (ICAM-1) is a ligand for LFA-1-dependent adhesion in T cell-mediated cytotoxicity

Although intercellular adhesion molecule-1 (ICAM-1) has been implicated as a ligand in some LFA-1-dependent adhesion, its importance to T cell function has not been established. The present studies investigate the importance of ICAM-1 for human cytotoxic T lymphocytes (CTL), both in their formation of antigen-independent conjugates (AIC) and in their lysis of targets. Analysis of monoclonal antibody (mAb) inhibition of AIC formation indicate that ICAM-1 mAb 1 blocks (a) AIC formation with some but not all targets; (b) the LFA-1 pathway but not the CD2/LFA-3 pathway of adhesion; (c) by binding to the target cell, not the T cell. In studies of cell-mediated lysis (CML) ICAM-1 mAb inhibited lysis of some targets, such as U-937, that use ICAM-1 predominantly in AIC formation; CML on some other targets is not inhibited by ICAM-1 mAb. These data indicate that ICAM-1 is a ligand for AIC formation, antigen-specific CTL recognition and cytolysis of particular target cells. The data also indicate that ICAM-1 is not used in LFA-1-dependent CTL interactions with all kinds of target cells, suggesting the existence of alternative ligands for LFA-1.

1 Introduction

Although the essential role of LFA-1 in cytotoxic T lymphocyte (CTL) recognition was demonstrated 5 years ago (reviewed in [1, 2]) the ligand(s) recognized on the target in CTL-mediated lysis has not been identified. LFA-1 is a leukocyte adhesion receptor utilized not only by CTL but also by other T cells, B cells and granulocytes [1, 2]. Recently, a molecule designated intercellular adhesion molecule 1 (ICAM-1) was proposed as a ligand for LFA-1 [3, 4]. It was identified as a cell surface glycoprotein involved in LFA-1-dependent phorbol myristate acetate (PMA)-induced homotypic adhesion of lymphoblastoid B cell lines [3]. ICAM-1 is an 80–114-kDa sialoglycoprotein expressed on a variety of cells. Its expression increases with either interferon-γ-induced activation of fibroblasts and endothelium or PMA-induced differentiation of U-937 [2].

Previous studies with the ICAM-1 monoclonal antibody (mAb) RR1/1 in T cell interactions indicated that ICAM-1 participates in the LFA-1-dependent adhesion of T cell tumors, and PMA- or phytohemagglutinin-activated T cells, and that it does so by binding not to the T cell but to the opposing cell [3, 4]. Such data suggest that the ICAM-1 molecule could be relevant to T cell recognition and function. However, to establish such relevance it is essential to demonstrate that ICAM-1 mAb inhibits both (a) adhesion of functionally defined T cells, and (b) T cell function. The present studies demonstrate a role for ICAM-1 both in antigen-independent conjugate (AIC) formation and lysis by CTL.

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Abbreviations: **AIC:** Antigen-independent conjugates **CD:** Cluster determinant (standard nomenclature of World Health Organization) **CML:** Cell-mediated lysis **CTL:** Cytotoxic T lymphocyte **ICAM-1:** Intercellular adhesion molecule-1 **LCL:** Lymphoblastoid B cell line **mAb:** Monoclonal antibody(ies)

2 Materials and methods

2.1 Cell lines

G12B and F2B are lymphoblastoid B cell lines (LCL) which had been generated by transformation of normal donors' PBML with Epstein-Barr virus. The cervical carcinoma HeLa and the promocytic line U-937 are available from the American Type Culture Collection, Rockville, MD. The Reed-Sternberg line L428 was kindly provided by Dr. J. Cossman (NIH, Bethesda, MD).

2.2 Human cellular reagents

DPw2-specific CTL clones are those described previously [5]. Bulk populations of allo-immune T cells were prepared by two cycles of *in vitro* stimulation of peripheral blood mononuclear leukocytes (PBML) with 10 000 rds-irradiated L428 or U-937 stimulator cells. Monocytes purified from PBML by elutriation were kindly provided by Dr. L. Wahl (NIH, Bethesda, MD). This population assessed by CD11b expression was >98% monocytes.

2.3 Antibodies

The LFA-1 IgG_1 mAb (MHM23 [6], a gift from Dr. J. Hildreth (Johns Hopkins University, Baltimore), is specific for the CD18 determinant on the β chain shared by LFA-1, Mac-1 and p150/95. The ICAM-1 IgG_1 mAb RR1/1 and the LFA-3 IgG_1 mAb TS2/9 have been described previousy [3, 7]. The CD21 IgG_2 mAb 95-5-49 (R. Quinones, R. Gress, manuscript submitted) was a gift from Dr. R. Quinones (NIH, Bethesda). LFA-1 and LFA-3 mAb were used (a) as Fab fragments at 300 μg/ml in all conjugate assays with continuous antibody; (b)

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as purified IgG at 100 μg/ml for conjugate assays with preincubation; (c) at 25 μg/ml in assays of cell-mediated cytotoxicity (CML). ICAM-1 mAb was always used as 1/40 dilution of an ascites fluid. Cell surface expression of ICAM-1 was measured by flow cytometry as previously described [8] and results were expressed in the units millivolts (mV) which are linearly related to antigen density; values in excess of 5 mV are easily detectable.

2.4 CML and conjugate formation

The ^{51}Cr-release assay was standard except that effector and target were not centrifuged together to initiate the assay; standards errors were always less than 10% of the mean. Formation of conjugates was measured by two-color flow cytometry generally as described [9, 10]. Briefly, suspensions of sulfofluorescein diacetate-labeled effectors and hydroethidine-labeled targets were mixed at a 4 : 1 ratio and allowed to settle for an hour at 4 °C. The tubes were incubated at 37 °C for 6 min, vortexed and the contents transferred into media at 4 °C. Conjugates were enumerated by two-color flow cytometry and the results expressed as percent of targets in conjugates. Since 10 000 events are analyzed counting errors are small and differences between samples of 2–3% conjugates are generally reproducible [10]. In most assays of mAb inhibition, saturating concentrations of mAb were added before the settling and present continuously thereafter. In designated experiments, effector and/or target cells were preincubated with mAb for 10 min at 4 °C and the unbound antibody was removed by three washes.

3 Results and discussion

3.1 Involvement of ICAM-1 in AIC formation

To study the role of ICAM-1 in T cell adhesion, we analyzed the ability of ICAM-1 mAb to inhibit AIC formation between a DPw2-specific CTL clone and the DPw2$^-$ target cell HeLa (Fig. 1A). As has been shown previously, AIC formation with HeLa (like many other targets) occurs via two molecular pathways: one mediated by LFA-1 on the effector and a putative ligand on the target, and the other mediated by CD2 on the effector and LFA-3 on the target [5, 10]. The operation of these two pathways is evident in the partial inhibition by LFA-1 mAb or LFA-3 mAb individually, but by complete inhibition by those antibodies in combination. Results with ICAM-1 mAb demonstrated that (a) it inhibited AIC, but since inhibition was partial it suggested inhibition of only one pathway; (b) ICAM-1 participated in the LFA-1 and not the CD2/LFA-3 pathway of AIC, since ICAM-1 mAb caused complete inhibition in a mix with LFA-3 mAb but no enhanced inhibition in a mix with LFA-1 mAb.

Preincubation studies demonstrated that ICAM-1 mAb inhibited primarily by binding to the target not the effector (Fig. 1B). The results shown are in the presence of LFA-3 mAb so that AIC formation is exclusively via the LFA-1 pathway. Preincubation of the target with ICAM-1 mAb was almost as inhibitory as the continuous presence of ICAM-1 mAb; preincubation of effector alone resulted in no inhibition. Preincubation of both effector and target with ICAM-1 mAb may be slightly more inhibitory than target only, probably because pretreatment of both cells prevents the ICAM-1 IgG

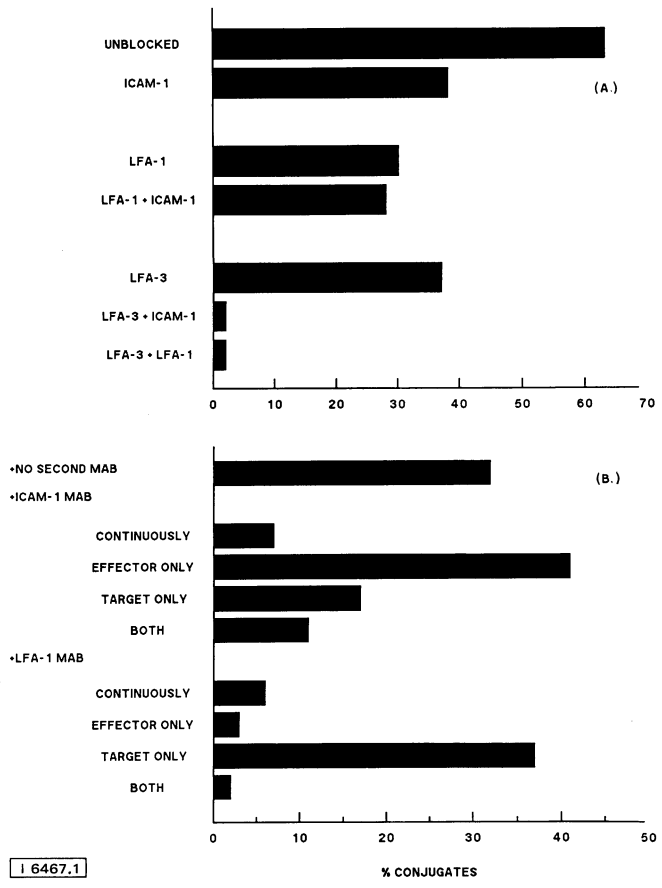

Figure 1. Inhibition of AIC between CTL clone 8.2 and antigen-negative target HeLa. (A) Conjugate formation was measured in the continuous presence of the indicated mAb. (B) To isolate the LFA-1 pathway, LFA-3 mAb was present continuously in all conditions. In addition, the indicated mAb was present continuously or used to pretreat the effector or target or both.

bridging between cells which can occur when only one cell type is pretreated. LFA-1 mAb conversely inhibited AIC by binding to effectors (Fig. 1B) as has been shown previously [10]. Thus, ICAM-1 is essential on the target side in the LFA-1 pathway of AIC formation, suggesting it is a ligand for LFA-1.

3.2 ICAM-1 utilization in AIC formation differs between targets

A variety of target cells were surveyed to assess their use of ICAM-1 as a ligand for LFA-1 in AIC formation. Results are shown for a representative experiment (Fig. 2). Effectiveness of blocking by LFA-1 and ICAM-1 can be assessed on total AIC formation (1st bar control *vs.* and 3rd) or on the LFA-1 pathway alone (4th bar control *vs.* 5th and 6th). Inhibition of AIC by ICAM-1 mAb varied for different targets, from almost complete inhibition with U-937 to no inhibition with monocytes. ICAM-1 must be the major functional ligand on U-937. Failure of inhibition by ICAM-1 mAb of AIC on monocytes suggests the existence of alternative LFA-1 ligands utilized on that target (see discussion below).

3.3 Role of ICAM-1 in CML

Adhesion is a critical step in lysis of targets by antigen-specific T cells (reviewed in [1, 2]) and both pathways of adhesion

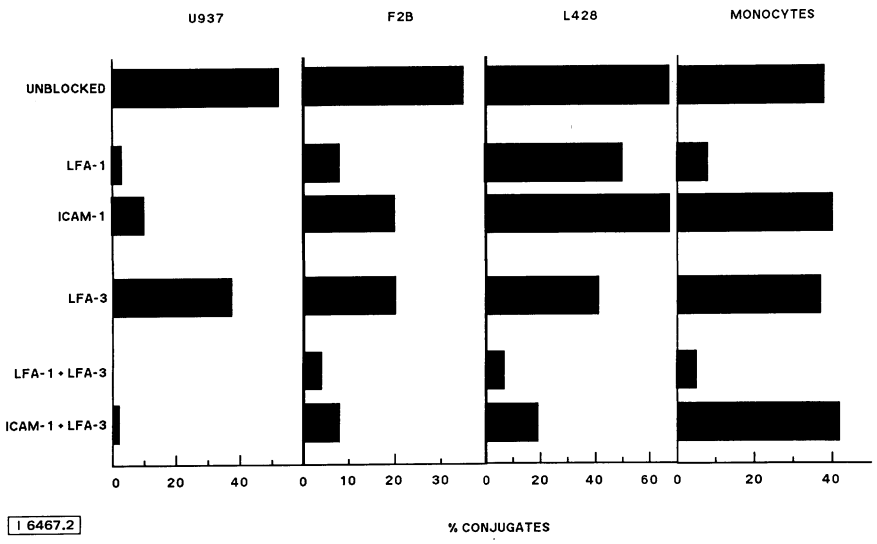

Figure 2. mAb blocking of AIC between CTL clone 8.2 and different antigen-negative targets. Target cell lines and representative values for their ICAM expression are as follows: promonocytic line U-937 (102 mV), Epstein-Bar transformed LCL F2B (813 mV), Reed-Sternberg line L428 (2441 mV) and peripheral blood monocytes (266 mV). ICAM-1 expression on each of these cell populations is characterized by a unimodal profile.

(LFA-1 and CD2/LFA-3) are functionally involved in such CML [7, 10]. To assess the role of ICAM-1 in CML, ICAM-1 mAb was tested for its ability to inhibit allo-specific T cell-mediated lysis on target cells known to utilize ICAM-1 in AIC formation. Representative data shown in Fig. 3 demonstrate that the pattern of CML inhibition varied between different target cells. ICAM-1 mAb alone efficiently inhibited the lysis of U-937, as did LFA-1 mAb; this lysis depends largely on LFA-1/ICAM-1 interaction rather than CD2/LFA-3, since CD2 mAb and LFA-3 mAb inhibit minimally alone or in combination. Dramatic inhibition by ICAM-1 mAb of lysis on target U-937 has been seen with each of four different CTL populations in separate experiments. In contrast, CML on L428 could proceed via either pathway of adhesion, since neither LFA-1 mAb nor the CD2, LFA-3 mAb alone could inhibit. Complete inhibition of CML on L428 was achieved only with mixes of LFA-1 mAb with either CD2 or LFA-3 mAb.

ICAM-1 did not have a critical role in CML on L428 since mixes of ICAM-1 mAb plus CD2 or LFA-3 mAb did not inhibit. CML on an LCL G12B showed yet a third pattern of mAb inhibition: partial inhibition by LFA-1 mAb or by CD2, LFA-3 mAb and complete inhibition by mixes of LFA-1 mAb with CD2 or LFA-3 mAb. Although ICAM-1 mAb alone was unable to inhibit CML on LCL G12B, small but reproducible inhibition by ICAM-1 mAb could be detected in mixes with CD2 mAb (74% inhibition increased to 93% by addition of ICAM-1 mAb) and with LFA-3 mAb (63% inhibition increased to 77% by addition of ICAM-1 mAb) but not in mixes with LFA-1 mAb (60% inhibition *vs.* 56% in combination with ICAM-1 mAb). With regard to the more general concept of two pathways, the total inhibition of CML by mixes of LFA-1 mAb with either CD2 or LFA-3 mAb establishes the absolute requirement for these molecular pathways in all three of these diverse effector/target combinations.

An interesting pattern is suggested by comparisons of inhibition of AIC formation (Fig. 2) with inhibition of CML (Fig. 3) on the same targets (U-937 and L428) or target cell type (LCL F2B and G12B): mAb (or mixes of mAb) which cause partial inhibition of AIC cause less inhibition of CML in these and other experiments. One explanation could be that our assay of AIC requires stronger adhesion (*i.e.* vortex resistance) than is

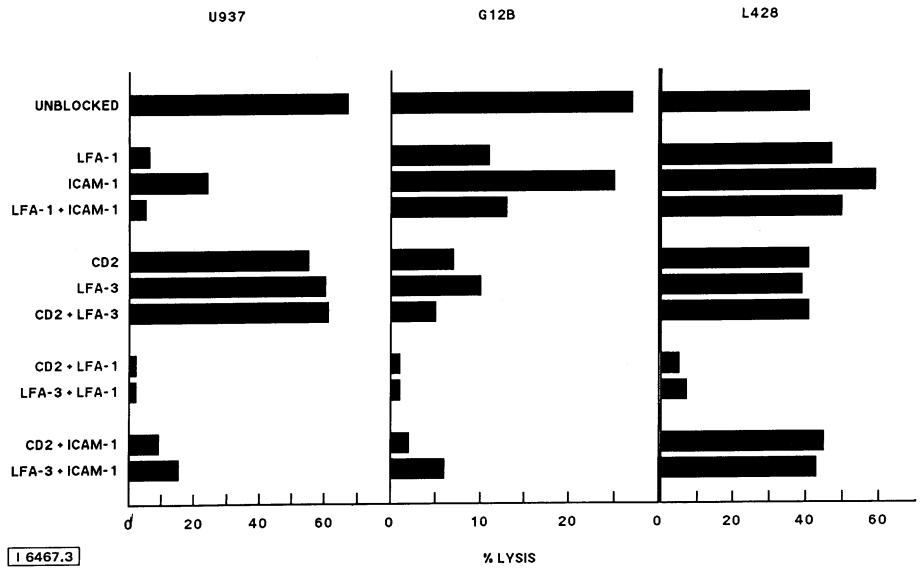

Figure 3. mAb blocking of CML. Effector/target combinations tested include allospecific bulk T cell populations sensitized against and tested on U-937 and L428, and a DPw2-specific CTL clone 8.2 tested on DPw2$^+$ LCL G12B. All combinations were tested at a 10 : 1 E : T ratio in a 4-h ^{51}Cr-release assay in the continuous presence of the indicated mAb.

required to achieve CML and, therefore, conditions which inhibit AIC will not necessarily be inhibitory for CML as previously reported by Shortman and Golstein [11].

3.4 Inference that ICAM-1 is one LFA-1 ligand

Thus, both studies of AIC and CML demonstrate that ICAM-1 mAb inhibits LFA-1-dependent interactions with some target cells. These studies demonstrate that ICAM-1 and LFA-1 function in the same pathway of CTL adhesion to target cells and are consistent with the interpretation that ICAM-1 can serve as a ligand for LFA-1. Studies showing that LFA-1-dependent homotypic adhesion is inhibited by ICAM-1 mAb, and that adhesion of T lymphoblasts to fibroblasts is inhibited by LFA-1 pretreated of lymphocytes or ICAM-1 mAb pretreatment of fibroblasts previously suggested that ICAM-1 could be an LFA-1 ligand [4]. Similar functional evidence prompted the inference that LFA-3 is the ligand for CD2 [5] which has now been confirmed biochemically [12, 13]. The ability of ICAM-1 to act as a ligand for the LFA-1-dependent adhesion pathway has recently been demonstrated with purified ICAM-1 either incorporated into phospholipid vesicles [14] or bound to microtiter plates [15]. The binding of purified ICAM-1 to LFA-1 [14, 15] or the inhibition of conjugates that form via LFA-1/ICAM-1 binding [15] has distinctive requirements for Mg^{2+} and Ca^{2+} [14, 15].

3.5 Inference that there are other LFA-1 ligands

The failure of ICAM-1 mAb inhibition of other LFA-1-dependent CTL interactions cannot be explained by (a) lack of ICAM-1 on the target since all targets used express large amounts of ICAM-1 (see legend to Fig. 2), or (b) subsaturating concentrations of ICAM-1 mAb since concentration of the mAb was at least 25-fold greater than that required for maximal binding. Failure of ICAM-1 mAb inhibition is probably not due to incomplete blocking of the function of the ICAM-1 molecule because two more mAb have been identified with similar blocking properties and blocking is no greater in mixes of mAb than with mAb individually (M. W. Makgoba, manuscript in preparation). Instead, the data indicate that there are alternative ligands for the LFA-1 receptor as suggested by Rothlein et al. [3] based on the ability of ICAM-1 mAb to inhibit some but not all LFA-1-dependent homotypic adhesions.

3.6 Comparisons of two pathways

A number of important differences were apparent previously between the two adhesion pathways used by T cells: LFA-1 is structurally related to the cytoadhesion family of cell adhesion molecules [16] while CD2 has homology with members of the immunoglobulin supergene family [17]. LFA-1 is widely expressed and used for adhesion on a variety of cells [1, 2], while CD2 is virtually T cell specific in its expression and utilization. KNowledge regarding ICAM-1 expands understanding of the differences between the pathways. LFA-3 is the only defined ligand for CD2 [12, 13]. In contrast, ours and previous findings [3] argue that ICAM-1 is one of multiple ligands for LFA-1; if so, this would be similar to findings on the cytoadhesin family of receptors which can often use several different ligands [16]. Differences in tissue distribution of LFA-3 and ICAM-1 are also noteworthy. LFA-3 is expressed on almost all cells and is generally not increased with activation [2]; in contrast, ICAM-1 is expressed on rersting cells of few lineages, such as endothelium and monocytes, but is up-regulated on those cells and induced on other cells with cytokines and cell activation [2, 4]. Thus, although the LFA-1/ICAM-1 pathway and the CD2/LFA-3 pathways overlap in their functional role in cell adhesion, they will undoubtedly have important functional differences. For example, because ICAM-1 (unlike LFA-3 on most tissues) is up-regulated or induced during cell activation and inflammation [4], the LFA-1 pathway may be particularly effective in mediating T cell surveillance of cells undergoing activation in inflammatory sites or by neoplastic transformation.

4 Concluding remarks

Thus, the characteristics of the ICAM-1 molecule are fully consistent with its role as a ligand for LFA-1 in CTL interactions with certain targets. We expect that the functional relevance of ICAM-1 will not be restricted to CTL, but will be generally true for many or all T cells. It remains to be determined whether the interaction of ICAM-1 with LFA-1 is primarily important for establishing adhesion, or whether that interaction also generates signals which are important for subsequent triggering of the T cell.

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