DIRECT EVIDENCE FOR BINDING OF CD8 TO
HLA CLASS I ANTIGENS

BY YVONNE ROSENSTEIN,* SHELDON RATNOFSKY,* STEVEN J. BURAKOFF,* AND STEVEN H. HERRMANN*§

From the *Division of Pediatric Oncology, Dana-Farber Cancer Institute, †Departments of
Pediatrics and §Pathology, Harvard Medical School, Boston, Massachusetts 02115;
and the †Departamento de Immunologia, Instituto de Investigaciones Biomedicas,
Universidad Nacional Autonoma de Mexico, Mexico DF

The activation of T lymphocytes appears to be a multiple step procedure initiated
by direct cell-cell contact between T lymphocytes and antigen-bearing or target cells
(1). Initial T cell-target cell interaction progresses to T cell activation if the target
expresses an antigen or some other ligand able to deliver an activation signal through
the antigen-specific TCR-CD3 complex (2-4). The initial interaction, as well as the
triggering process, seems to involve several receptor-ligand pairs in addition to the
TCR-target cell interaction.

Cell surface proteins thought to play a role in T lymphocyte-target cell adhesion
include CD8 (5, 6), CD4 (7, 8), LFA-1 (9, 10), and CD2 (10, 11). Recent experiments
have begun to delineate the target cell ligands for these receptor molecules. A variety
of experimental approaches have indicated that LFA-3 and ICAM-1 are target cell
ligands for CD2 and LFA-1, respectively (12-14). CD4 and CD8 were initially be-
lieved to be phenotypic markers for functional T cell subsets (15-17). However, the
existence of CD8+ Th specific for MHC class I products and of CD4+ cytotoxic
T cells specific for MHC class II products led to the hypothesis that phenotype corre-
lates with the class of MHC protein recognized. This suggested that CD4 and CD8
might be receptors for class II and class I MHC molecules, respectively (18-21). The
ability to block T cell functions with mAbs directed against CD4 and CD8 is consis-
tent with these mAbs disrupting a receptor-ligand interaction (22, 23). There is also
evidence, however, that these mAbs may be capable of delivering an "off signal" to
the cells (24, 25).

Evidence that the interaction of CD4 and class II molecules augments T cell activa-
tion has been provided by infecting a murine T cell hybridoma that produces IL-2
in response to human HLA-DR antigens with a retroviral vector containing human
CD4 cDNA (26). Similar results were reported with another murine T cell hybridoma,
specific for H-2 class I molecules and transfected with a cDNA clone for human
CD4 (27). A direct interaction between HLA-DR and CD4 was demonstrated by
showing that monkey CV1 cells, transiently expressing high levels of CD4, bind to
human B cells bearing MHC class II molecules (28).

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Gene transfer techniques have also offered a complementary approach to elucidate the role of the CD8 molecule in T cell activation. Reconstitution of MHC class I specificity by transfer of the TCR and Lyt-2 genes to recipient T cells demonstrates that the expression of the CD8 molecule is necessary for these T cells to interact with their targets (29, 30). The functional involvement of CD8 in T cell triggering was also demonstrated by showing that a murine T cell hybridoma specific for HLA class II antigens transfected with a cDNA clone for human CD8 specifically interacts with class I+ cells (31). These data support the idea that class I MHC molecules encode a ligand for CD8.

In a previous report we describe the preparation of lipid-sealed spherical nylon vesicles with the size and density of an intact cell (13). These vesicles, termed artificial target cells (ATCs),1 were reconstituted with purified cell surface glycoprotein LFA-3. ATC bearing LFA-3 formed conjugates with CD2+CD8+ T cells, whereas ATCs prepared without surface proteins or with purified HLA class I protein failed to interact with the CD2+ cells. The specificity of the interaction between LFA-3 ATCs and CD2+ cells was demonstrated by the ability of mAb against CD2 or LFA-3 to block the vesicle cell adhesion (13). In the present study, we have used ATCs bearing purified CD8 and ATCs bearing HLA class I proteins to demonstrate a direct receptor-ligand interaction between CD8 and MHC class I molecules capable of mediating adhesion. We also used ATCs and purified proteins to determine if membrane-associated CD2 will bind to membrane-associated LFA-3. These data provide direct evidence that an interaction between CD2 and LFA-3 or between CD8 and HLA class I molecules can mediate cell-cell adhesion.

Materials and Methods

Cell Lines and mAbs. The cell lines used were JY, an EBV-transformed B cell line expressing high amounts of HLA and LFA-3; Jurkat, a T cell line expressing CD2; 16T8-15, a CD8+ hybridoma derived from the infection of the murine T cell hybridoma By155.16 with a defective retrovirus containing a cDNA clone for human CD8, as described (31). The mAbs used were anti-CD8, OKT8 (IgG2a) (32); antiframework HLA, W6/32 (IgG2a) (33); anti-HLA-A2 MA2.1 (IgG1), PA2.1 (IgG1) (34, 35); anti-HLA-B7 ME1 (IgG1) and MB40.2 (IgG1) (36, 37) (all purchased from American Type Collection, Rockville, MD); anti-LFA-3, TS2/9 (38); and anti-CD2, TS2/18, (38). W6/32, TS2/18, and OKT8 were purified from ascites on an Affi-gel protein A column (Biorad Laboratories, Richmond, CA). TS2/9 was purified by preparative ion-exchange high-pressure liquid chromatography (13). Anti-A2 and anti-B7 were used as ascites. In adhesion assays all mAbs were used at a final concentration of 10 μg/ml.

Affinity Purification of the Different Cell Surface Molecules. Purified mAbs were coupled to Sepharose 4B-CNBr (Pharmacia Fine Chemicals, Piscataway, NJ), according to the instructions of the manufacturer. HLA class I molecules and LFA-3 were purified from JY cells by using a Sepharose 4B-W6/32 and a Sepharose 4B-TS2/9 column, respectively (13). The CD2 molecule was purified as described (39), by passing the cell lysate from Jurkat cells over a Sepharose 4B-TS2/18 column. CD8 was isolated from the murine T cell hybridoma 16T8-15, which expresses a high density of human CD8 on the cell surface as a result of infection with a defective retrovirus containing a cDNA for human CD8 (31). Optimal elution conditions for CD8 purification from 16T8-15 cell lysates were determined as follows. Sepharose 4B-OKT8 beads were loaded with 10^9 16T8-15 cellular equivalents in 10 mM tris, pH 8, 0.15 M NaCl, 0.5% Triton X-100. After washing with the same buffer, the beads were divided in three aliquots, and eluted under different conditions: pH 3, pH 11, or 3.5 M KSCN. Eluted

Abbreviation used in this paper: ATC, artificial target cells.
samples (100–150 μl) were analyzed by SDS-PAGE (10%) under reducing conditions, using the buffer system of Laemmli (40). The gels were stained with Coomassie blue and silver stain. The best approach, with respect to yield, purity, and serological activity of CD8 were as follows. The lysate-loaded column was washed with 10 vol of 10 mM tris, 0.15 M NaCl, 0.5% Triton X-100, pH 8. The column was then washed with 5 vol of 10 mM tris, 0.5% Triton X-100, 0.5 M NaCl, pH 8, followed by 5 vol of 10 mM tris 0.5% Triton X-100, 0.5 M NaCl, pH 9. Elution of CD8 was performed with 50 mM Gly, 0.15 M NaCl, DOC 0.1%, pH 11.5. Fractions (2 ml) were collected in tubes containing enough 10X PBS, pH 5 to bring the pH of eluted samples to a pH of 8–8.5. The CD8-enriched fractions, identified by immunodot (data not shown), were further purified over a Sepharose 4B-wheat germ agglutinin column; the CD8 protein was eluted with 5% N-acetyl-D-glucosamine in PBS-0.2% octyl-β-glucoside (41).

SDS-PAGE analysis of the CD8+ fractions demonstrated a major band at M, 34,000 corresponding to monomeric CD8 along with a minor contaminant at M, 46,000 (Fig. 1). The SDS-PAGE of dot blot-identified CD2+ fractions revealed a broad band at M, 54,000 with small amounts of lower molecular weight proteins (Fig. 1). The band at M, 54,000 and the smaller bands are recognized by anti-CD2 mAb on a Western blot (data not shown).

Preparation of ATCs Expressing Membrane Proteins. The preparation of these vesicles has been described in detail elsewhere (13). Briefly, ATCs are spherical vesicles consisting of a porous nylon matrix surrounding an aqueous compartment containing trapped polymers (protein and Ficoll) that provide colloidal osmotic pressure to keep the vesicles spherical. The resulting spherical ATCs are similar in size and density to intact cells and can be centrifuged and viewed under a microscope. A lipid membrane was constructed by covalent attachment of phosphatidylethanolamine to the surface of the nylon vesicles. This covalently attached lipid serves as a template for the formation of a lipid membrane added subsequently by detergent dialysis. After extensive washing with PBS, ATCs loaded by a lipid membrane were reconstituted with purified proteins as follows. The affinity-purified protein (6 μg HLA, 10 μg CD8, 10 μg LFA-3, or 11 μg CD2) was mixed with PBS containing 107 lipid-sealed ATCs, diluting the detergent to 1 mM final concentration. The mixture was dialyzed against three changes of PBS (1,000 vol each) over a 72-h period to remove the detergent. The ATCs, harvested from the dialysis tubing, were washed three times with PBS by centrifugation at 300 g to remove any protein not associated with the vesicles. ATCs were stored in PBS 0.1% BSA, 0.02% azide, and washed twice with PBS before use. Once associated with the ATC membrane, the reconstituted proteins were functionally stable after repeated washing or storage at 4°C for several months as evidenced by the uniform fluorescence staining pattern visible by fluorescence microscopy and flow cytometry (Fig. 2).

Flow Cytometry. Surface expression of membrane proteins on intact cells and ATCs was analyzed with a cell flow cytometer (Epic V, Coulter Electronics, Inc., Hialeah, FL). Cells or vesicles (105) were incubated in V-bottomed 96-well plates with 50 μl of the indicated mAb plus 50 μg of human IgG (Sigma Chemical Co., St. Louis, MO) for 30 min at 4°C. After two washes with cold PBS containing 2% FCS, 50 μl of a 1:30 dilution of fluorescein-conjugated F(ab')2 goat anti-mouse IgG + IgM (Tago, Burlingame, CA) was added to the cells for an additional 30 min at 4°C. Cells or ATCs were washed twice and fixed with 1% paraformaldehyde in PBS before flow cytometry.

Adhesion Assays. Two different kinds of adhesion assays were done. ATC-cell and ATC-ATC. To discriminate intact cells from ATCs in ATC-cell adhesion assays, tumor cells were fluorescein labeled by a 10-min incubation with fluorescein diacetate (10 μg/ml, Molecular Probes, Eugene, OR) followed by three washes with PBS. When adhesion between two different populations of ATCs was measured, one ATC group was labeled with fluorescein-modified phosphatidylethanolamine (10 μg/ml, Avanti Polar Lipids, Inc., Birmingham, AL) for 15 min at 4°C, followed by three washes with cold PBS. In a typical adhesion assay, ATCs reconstituted with CD8, HLA, LFA-3, CD2, or without added protein were incubated at different ratios with tumor cells or other ATCs. Samples, in 30 μl of 10% FCS RPMI, were centrifuged for 2 min at 40 g, followed by 2–3-h incubation at 4°C in a 0.5-ml microcentrifuge tube. To the appropriate samples, mAbs were added at the beginning of the experiment. At the end of the incubation period, samples were gently
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Resuspended five times with a pipette tip having an internal diameter of 0.5 mm. To quantitate the percent of conjugates, a small aliquot of sample (7 μl) was placed on a glass slide, and a 12-mm coverslip was placed over the sample and held in place with fingernail polish. Observations were made with a light microscope using a fluorescent lamp to locate fluorescein-labeled cells or ATCs and with phase contrast to determine the location of nonfluorescent ATCs. A conjugate was defined as a fluorescent ATC tightly associated with at least two nonfluorescent ATCs or a fluorescent cell associated with two or more ATCs. Samples were analyzed in duplicate, and three or more counts of at least 100 fluorescent cells or ATCs were done for each sample and scored as conjugates or nonconjugates. Results are expressed as the mean value ± SEM of the six counts made for each group. The percent of conjugates was calculated as the total number of conjugates divided by the total number of cells or ATCs observed. For ATC-cell conjugates, background was 3–4%; for ATC-ATC conjugates, nonspecific binding was ∼10% in the CD8/HLA studies and ∼3–5% in the CD2/LFA-3 experiments.

Results

Conjugate Formation between Cells and CD8- or HLA-ATCs. ATCs were reconstituted with affinity-purified HLA class I antigens isolated from JY cells, an EBV-transformed B cell line expressing a high density of MHC-encoded molecules. CD8 was purified from 16.T8-15 cells, a CD8+ hybridoma derived from the infection of the T cell hybridoma Byl55.16 with a defective retrovirus containing a cDNA clone for CD8 (31) (Fig. 1). Flow cytometry of immunofluorescently labeled vesicles indicated that ATCs expressed protein levels that were similar to or greater than the intact cells from which they were purified, except in the case of CD8-ATC/16.T8-15 cells (Fig. 2).

CD8- or HLA-ATCs and fluorescein-labeled JY (HLA+) or 16.T8-15 (CD8+) cells were mixed at a ratio of 1:4, centrifuged at 40 g for 2 min, and incubated for 2 h at 4°C. The percentage of cells bound to ATCs was determined by fluorescence and phases contrast microscopy by counting duplicate samples of at least 100 cells for each group. ATCs reconstituted with CD8 were able to form fivefold more conjugates (22 ± 1%) with JY cells, expressing HLA class I proteins, than did ATCs lacking surface proteins (4 ± 1%) (Fig. 3). Similarly, ATCs reconstituted with HLA class I molecules formed sevenfold more conjugates (29 ± 3%) with the CD8+ 16.T8-15 cells than did ATCs lacking surface protein (4 ± 1%) (Fig. 3). Conjugate formation between CD8-ATCs and JY (HLA+) cells as well as between HLA-ATCs and CD8+ 16.T8-15 cells was specifically inhibited by anti-HLA (11 ± 2%) or anti-CD8 mAbs (6 ± 0.5%) (Fig. 3). Neither anti-CD2 nor anti-LFA-3 mAbs inhibited conjugate formation. The CD8+ (Lyt-2,3) parental murine hybridoma line, Byl55.16, did not form specific conjugates with HLA-ATCs (4 ± 2%) (data not shown in Fig. 3), the percent of conjugates was comparable (4 ± 1%) with that obtained with 16.T8-15 cells and ATCs lacking surface proteins.

Conjugate Formation between MHC Class I and CD8 ATCs. Conjugate formation between ATCs reconstituted with either CD8 or HLA was also evaluated. One population of ATCs was labeled with fluorescein-modified phosphatidylethanolamine as described in Materials and Methods, and conjugate formation was then scored as the number of conjugates formed between fluorescent and nonfluorescent ATCs. When CD8-ATCs were mixed with HLA-ATCs, at a 1:2 ratio, four times more conjugates were formed (42 ± 8%), as compared with the binding between CD8 or HLA-ATCs and ATCs lacking surface protein (10 ± 3%) or LFA-3-ATCs (10 ± 2%) (Fig. 4). Blocking by specific mAbs supported the conclusion that HLA specifically
FIGURE 1. SDS-PAGE analysis of affinity-purified CD8 and CD2 under reducing conditions. Lane 1, No sample (background of an empty lane); lane 2, CD8 (2 µg); lane 3, no sample; lane 4, CD2 (1 µg) (silver stain, 10% acrylamide gel).

FIGURE 2. Flow cytometric analysis of surface molecules on intact cells and ATCs. (a) Jurkat cells; (b) CD2-ATCs; (c) JY cells; (d) LFA-3-ATCs; (e) JY cells; (f) HLA-ATCs; (g) 16T8-15 cells, (h) CD8-ATCs. Samples were incubated with 50 µl of culture supernatant containing 50 µg human IgG and ~2.5 µg of the following mAbs. a and b were stained with anti-CD2 mAb (TS2/18); c and d were stained with anti-LFA-3 mAb (TS2/9); e and f were stained with anti-HLA mAb (W6/32); and g and h were stained with anti-CD8 mAb (OKT8). The left curve represents the background staining with fluorescein-conjugated goat anti-mouse IgG + IgM only. All samples pairs (ATC-cell) were processed the same day.
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FIGURE 3. Quantitation of CD8/HLA-mediated conjugates in a cell/ATC adhesion assay. Fluorescein-labeled JY (HLA*) or 16.T8-15 (CD8*) cells were incubated with CD8-ATCs, HLA-ATCs, or control ATCs at a 4:1 cells/ATC ratio for 2 h at 4°C. Results are expressed as the percent binding of JY or 16.T8-15 cells to CD8-ATCs or HLA-ATCs, respectively. As indicated, the experiments were carried out in the absence or presence of mAb (anti-class I, pool of MA2.1 + PA2.1 + ME1 + MB40.2; anti-CD8, OKT8; anti-CD2, TS2/18; and anti-LFA-3, TS2/9). Data represent mean ± SEM ATC-cell conjugates (n = 6).

interacts with the CD8 molecule, i.e., anti-HLA and anti-CD8 mAbs blocked conjugate formation between CD8-ATCs and HLA-ATCs to background levels while mAb to LFA-3 failed to inhibit conjugates (Fig. 4).

Conjugate Formation between CD2- and LFA-3-ATCs. For comparison, and to extend our previous work, we carried out parallel experiments to determine if the interaction between purified CD2 and LFA-3 would mediate ATC-ATC conjugate formation. When incubating CD2-ATCs with LFA-3-ATCs at a 1:1 ratio, we found nine times more conjugates (43 ± 2%) as compared with the binding of CD2-ATCs to HLA-ATCs (4 ± 3%) or ATCs lacking surface protein (5 ± 0.5%). Anti-CD2 mAb reduced conjugate formation to background (4 ± 3%), whereas mAbs against HLA class I antigens did not block these CD2-LFA-3 conjugates (36 ± 5%) (Fig. 4).

When the ratio of CD2 to LFA-3-ATC was increased (from 1:1 to 1:4), most of the conjugates had the configuration of a rosette made of three or more LFA-3-ATCs per CD2-ATC (Fig. 5, C and D). In comparison, increasing the ratio of CD8-ATC

FIGURE 4. Purified CD8 interacts with purified HLA class I protein and mediates ATC-ATC adhesion. CD8-ATCs were mixed with HLA-ATCs at a 1:2 ratio, and vesicle-vesicle conjugates scored in the absence or presence of mAb (see Fig. 3). Conjugate formation between CD2-ATCs and LFA-3-ATCs (at a 1:1 ratio) was assessed. Results are expressed as the percent of CD8-ATCs binding to HLA-ATCs or the percent of LFA-3-ATCs binding to CD2-ATCs. Data represent mean values ± SEM (n = 6).
to HLA-ATC to 1:4 did not result in a change in the conjugate size, most consisting of two to three HLA-ATC per CD8-ATC (Fig. 5, A and B).

**Discussion**

Nonspecific conjugate formation between effector T cells and target cells precedes the recognition of the specific antigen by the TCR. Previous studies relied largely upon mAb inhibition to identify the surface proteins involved in cell-cell adhesion (5-14). The ability of several T cell-reactive mAbs to inhibit T cell-target cell binding suggests that several different receptors, each interacting with its specific ligand, may be required to establish cell-cell adhesion. However, it has also been suggested that mAbs may block function by inhibiting T lymphocyte signal transduction rather than by directly inhibiting a receptor-ligand interaction (24, 25). Therefore, the inhibitory effects of mAbs on intact cells must be interpreted with caution.
The use of artificial target cells is one approach to identify receptor-ligand pairs involved in cell-cell adhesion. ATCs have an outer lipid membrane, are the size and density of intact cells, and can be reconstituted with purified proteins. The ability to incorporate a high surface density of receptor or ligand onto the ATC membrane enables multivalent interactions to occur, making it possible to detect protein-protein interactions that individually may have a low affinity. The adhesion of these vesicles, mediated by purified proteins, provides a direct and unambiguous method to identify receptor-ligand pairs able to mediate cell adhesion. This direct approach eliminates the possibility that other molecules on the cell surface might be interacting with the ligand or, that adhesion is mediated by other receptor-ligand pairs. We report here that purified CD8 molecules or HLA class I antigens incorporated into artificial membranes can mediate adhesion to either HLA+ or CD8+ cells, respectively. Furthermore, we show that artificial target cells, reconstituted with either purified CD8 or class I molecules, will form conjugates when incubated together.

In cell-ATC adhesion experiments the number of conjugates scored between CD8-ATCs and JY cells was fivefold greater than background binding, and sevenfold more conjugates were formed between the mouse T cell hybridoma expressing human CD8, 16.T8-15 cells, and HLA-reconstituted ATCs as compared with background. When CD8-ATCs were mixed with HLA-ATCs, there were four times more conjugates formed as compared with background binding. Failure of all the protein-reconstituted ATCs to form conjugates may depend upon multiple factors, including the density of receptors and ligands, the association and dissociation constants, the avidity of the interaction, the mobility of the various components, and the conformation of the proteins. The conjugates between CD8-ATCs and HLA-ATCs were found to be specifically inhibited by mAbs to CD8 or HLA class I molecules. The specificity of this interaction was further confirmed by the inability of an irrelevant mAb (anti-LFA-3) to block conjugate formation and by the absence of conjugate formation between ATCs exhibiting LFA-3 molecules and CD8-ATCs or HLA-ATCs. Taken together, these results demonstrate that HLA class I molecules are a ligand for the CD8 receptor.

Experimental data regarding CD8 suggest that this molecule may play several roles on the surface of T lymphocytes. CD8, in conjunction with other accessory molecules, such as CD2 and LFA-1, may facilitate the interaction of the TCR with its specific antigen through the adhesion of the T lymphocyte to the target cell. The association of CD8 with the MHC class I molecule might not only mediate adhesion but could also increase the local concentration of class I molecules and favor a proper orientation of the MHC-Ag peptide complex required for triggering through the TCR. In addition to its involvement with conjugate formation, CD8 has been reported to modulate the activation signal of the TCR-CD3 complex. Anti-CD3-mediated T cell activation has been found to be enhanced by the presence of anti-CD8 mAb (42) whereas on the other hand, lectin-mediated T cell lysis can be inhibited by anti-CD8 mAbs (24).

Although some of the molecules involved in cell-cell adhesion have been identified, little is known regarding the molecular mechanism by which adhesion and de-adhesion is regulated. In the case of the CD8-HLA receptor-ligand pair, one possible mechanism is suggested by a recent report indicating that the CD8 molecule is noncovalently associated with class I molecules on the surface of activated T lymphocytes.
Assuming that CD8 interacts with one MHC molecule at a time, the ability of the CD8-HLA class I complex to interact with the target cell HLA would depend upon the relative density of the class I molecules on the target cell. Once formed, the T cell–target cell conjugate might be reversed by re-establishing the intracellular association of CD8 and HLA on the T cell surface. For CD8 and other receptors involved with adhesion, it is possible that intracellular vs. intercellular binding of receptor-ligand pairs regulate adhesion and de-adhesion.

T cell activation can be divided into several steps: antigen-nonspecific adhesion, antigen recognition, and T cell triggering. CD8, as other accessory molecules (LFA1, CD2, and CD4), may provide more than one function: increasing T cell–target cell binding and positively and/or negatively regulating T cell activation. T cell triggering may be controlled by regulating only a few of the multiple interactions involved in the different phases of T cell activation. In this report we demonstrate that the association between CD8 and class I MHC was sufficient to mediate conjugate formation between cells or cell-sized vesicles.

Summary

Adhesion of T lymphocytes is an essential step for antigen recognition and lymphocyte activation. mAbs to T cell surface proteins have been used to define the receptor-ligand proteins that appear to be involved in adhesion. Since most assays measure the effects of mAbs on T lymphocyte function, it is not known whether mAb-mediated blocking is due to a disruption of receptor-ligand interactions or results in inhibition of some aspect of receptor-mediated triggering. It has been suggested that the CD8 molecule augments T cell avidity for the target cells by binding to determinants on target cell MHC class I molecules. In the present report, we demonstrated that purified CD8 molecules incorporated into large lipid vesicles (artificial target cells) mediate the adhesion of these vesicles to cells expressing HLA proteins, while vesicles expressing purified HLA class I antigens bind to CD8– T cells. Furthermore, vesicles bearing CD8 will form conjugates with vesicles expressing HLA class I proteins. These conjugates were found to be specifically inhibited by mAbs to CD8 or HLA class I molecules. We also demonstrate that CD2-reconstituted vesicles can form conjugates with vesicles bearing LFA-3. These experiments provide direct evidence for an interaction of the CD8 molecule with class I MHC proteins as well as between CD2 proteins and LFA-3 proteins, thus supporting the hypothesis that these molecules can mediate cell-cell adhesion.

Note added in proof: Transfection of the human CD8α gene into murine T cell hybridomas does not rescue expression of murine CD8β (Ratnofsky et al., unpublished data).

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