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Molecular Pathways of Adhesion in Spontaneous Rosetting of T-Lymphocytes to the Hodgkin's Cell Line L428

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ABSTRACT

Spontaneous rosetting of T-lymphocytes to Reed-Sternberg cells has been observed both *in vitro* and *in vivo* but its molecular mechanism has not been defined. We have investigated such rosetting using the Hodgkin's cell line L428. L428 expresses high levels of LFA-3 and ICAM-1, both of which are ligands for T-cell adhesion. Monoclonal antibody inhibition of spontaneous rosetting indicated that it is not dependent on the T-cell receptor complex but is largely mediated by interaction of T-cell CD2 (T11/E-rosette receptor) with its ligand LFA-3 on L428 cells. Studies using an alternate assay of adhesion (conjugate formation) confirm the roles of CD2/LFA-3 and also implicate a second mode of binding via LFA-1 on T-cells to ICAM-1 on L428. These data explain the previously reported finding of T-cell rosetting with Reed-Sternberg cells as an exaggeration of normal antigen-independent T-cell adhesion.

INTRODUCTION

RS[2] cells are a malignant cell type of uncertain origin that are found in diseased lymphoid tissues of patients with Hodgkin's disease. A unique characteristic of RS cells is that they spontaneously form rosettes with T-lymphocytes (1–3). Such rosettes form rapidly *in vitro* with autologous lymphocytes in single cell suspensions of fresh splenic tissue or lymph nodes from patients with Hodgkin's disease; rosettes also form with allogeneic lymphocytes (4). *In vivo* adhesion of lymphocytes to RS cells is evidenced by the frequent finding of similar clusters of lymphocytes around RS cells in Giemsa-stained imprints of Hodgkin's tissue sections (2), as well as electron microscopic studies of Hodgkin's tissues showing tight apposition and uropod formation between lymphocytes and RS cells (5, 6).

The pathophysiological relevance of such lymphocytes surrounding RS cells in tissue sections to tumor immunity has remained unclear. One study suggested that the finding of large numbers of autologous lymphocytes adherent to RS cells in tissue suspension was a favorable prognostic indicator (7). Furthermore, one electron microscopic study (6) noted ultrastructural changes in the RS cells suggesting a cytotoxic effect of the surrounding lymphocytes. However, other studies have demonstrated that autologous lymphocytes may adhere to RS cells in culture for up to several wk without leading to cytolysis (1, 2).

Progress has been made in elucidating the molecular mechanisms involved in other models of T-cell adhesion. Studies have shown that cytotoxic T-lymphocytes may adhere to other cells via antigen-independent mechanisms (8, 9). Since such adhesion does not involve the specific antigen receptor, cytolysis does not occur. Two different molecular pathways of antigen-independent adhesion have been identified (9). The CD2 molecule (also known as T11, LFA-2, or the sheep erythrocyte receptor) on T-lymphocytes mediates adhesion via binding to lymphocyte function-associated antigen 3 (LFA-3), a ubiquitously distributed cell surface glycoprotein (10, 11). CD2 binding to LFA-3 is the mechanism of adhesion in the phenomenon of rosette formation of T-lymphocytes with human erythrocytes (12, 13) and in human thymocyte adhesion to thymic epithelium (14). The other molecular pathway of T-cell adhesion involves interaction of T-cell surface LFA-1 with one or more target cell surface ligands. Functional evidence indicates that intercellular adhesion molecule 1 (ICAM-1) is one ligand for LFA-1; in some experimental systems, LFA-1-dependent adhesion of B-cells and T-cells can be inhibited by anti-ICAM-1[3] (15).

L428 is a Hodgkin's cell line which has morphological characteristics and cell surface marker patterns identical to those of freshly obtained RS cells and shows the typical RS cell phenomenon of spontaneous rosette formation with T-lymphocytes (4, 16, 17). In the present study we demonstrate that the observed spontaneous adhesion of T-lymphocytes to the Hodgkin's cell line L428 occurs primarily via binding of T-lymphocyte CD2 to L428 cell LFA-3, with some lesser contribution to this adhesion by T-cell LFA-1 and L428 cell ICAM-1.

MATERIALS AND METHODS

Cells and Reagents. L428 was kindly provided by Dr. V. Diehl, University of Cologne, Federal Republic of Germany, and was grown in RPMI 1640 with 10% fetal calf serum. For rosette assays human T-lymphocytes were purified from peripheral blood mononuclear cells of normal donors by rosetting with 2-aminoethylisothiouronium bromide hydrobromide-treated sheep erythrocytes. Conjugate assays were performed using a cytotoxic T-lymphocyte clone 8.2 (specific for DPw2 and noncytolytic for L428) (18). Monoclonal antibody TS2/9 to LFA-3 and RR1/1 to ICAM-1 were kindly provided by Dr. T. Springer (Dana Farber Cancer Institute, Boston, MA), (15, 19). MAB 84H10 to ICAM-1 was kindly provided by Dr. P. Mannoni (Institute J. Paoli-I. Calmette, Marseille, France). MAB MHM23, specific for the β chain of LFA-1, was kindly provided by Dr. J. Hildreth (The Johns Hopkins University, Baltimore, MD). Anti-CD2 MAB 95-5-49 was kindly provided by Dr. R. Quinones and Dr. R. Gress (National Cancer Institute, NIH, Bethesda, MD). MAB W6/32, to a framework epitope of human class I HLA, was kindly provided by Dr. S. Jacobson (National Institute of Neurological and Communicative Disorders and Stroke, NIH, Bethesda, MD). The CD3 MAB OKT3 was kindly provided by Dr. G. Goldstein (Ortho Pharmaceuticals, Raritan, NJ). All MABs were used as purified immunoglobulin. Fab fragments for MAB to LFA-1 and LFA-3 were prepared by papain digestion.

Flow Microfluorometry. One $\times 10^6$ cells per sample were incubated with saturating amounts of relevant antibody or culture supernatant for 30 min at 4°C. After two washes, cells were further incubated with a saturating amount of goat anti-mouse IgG F(ab')$_2$ for 30 min at 4°C. Following two final washes, 50,000 cells were analyzed on a Becton Dickinson FACS II flow cytometer and results expressed as linear unit millivolts.

Rosette Assay. One $\times 10^6$ L428 cells and 10×10^6 T-lymphocytes were added to test tubes containing 1.0 ml of RPMI 1640 with 10% fetal calf serum and 10 μg of a purified MAB. Tubes were centrifuged

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[2] The abbreviations used are: RS, Reed-Sternberg; MAB, monoclonal antibody.

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for 30 sec in a table-top centrifuge and placed at 37°C for 1 h. Cells were gently resuspended and rosettes were counted on a wet mount slide. Rosettes, defined as three or more lymphocytes adhering to an L428 cell, were scored by an observer blinded to the antibody treatment; 300 cells per condition were counted.

Conjugate Assay. Conjugate formation of L428 with a human T-lymphocyte clone was assayed as previously described (9, 20). Briefly, L428 cells were stained red with an intracellular dye, hydroethidine, and T-cell clone 8.2 cells were stained green with another intracellular dye, sulfofluorescein diacetate. L428 and T-cells were mixed at a 1:4 ratio in the presence of 100 μg/ml appropriate antibody or 300 μg/ml Fab fragment of αLFA-1 and αLFA-3, allowed to settle for 1 h at 4°C, then incubated for 6 min at 37°C, and dispersed in cold buffer. Cells were analyzed on a Becton Dickinson FACS analyzer and particles fluorescing both red and green were interpreted as conjugates. Data are reported as the percentage of total targets present as conjugates.

RESULTS

Expression of Adhesion Molecules. Flow microfluorometry was used to measure surface expression of adhesion molecules on L428 and T-cells. L428 cells expressed high amounts of two ligands for T-cell adhesion, LFA-3 and ICAM-1, but did not express either the CD2 or LFA-1 molecules implicated as receptors for these ligands (Table 1). In contrast peripheral blood T-lymphocytes express both the CD2 and LFA-1 receptors but low amounts of LFA-3 and ICAM-1. T-cell clone 8.2 expressed higher amounts of all four adhesion molecules compared to unstimulated peripheral blood T-cells but still expressed relatively modest amounts of LFA-3 and ICAM-1 compared to L428 cells.

Inhibition of L428/T-Cell Rosettes by Antibody to Adhesion Molecules. Monoclonal antibody inhibition of rosette formation was used to identify molecules involved in rosette formation. Results of a representative experiment (Fig. 1) show that antibodies against Class I HLA and against CD3 resulted in marginal inhibition of rosette formation. Monoclonal antibodies to either CD2 or to LFA-3 profoundly inhibited rosette formation, but some rosettes remained. Antibody to LFA-1 inhibited rosette formation by about half when present by itself, but in combination with anti-LFA-3 completely inhibited rosette formation (no rosettes in 300 L428 cells counted). These data indicate that CD2 and LFA-3 are primary contributors to T-cell rosetting with L428 but also indicate a role for LFA-1.

Inhibition of T-Cell Clone/L428 Conjugates by Antibody to Adhesion Molecules. Conjugate formation is another system in which we have extensively characterized adhesion of T-cell clones to a variety of target cells (21). Antigen-independent conjugate formation between L428 and a T-cell clone provided another model system in which to study T-cell adhesion to L428 (Fig. 2). A very high frequency of conjugates was observed between the T-cell clone and L428, and in many conjugates there was more than one T-cell per L428 (inferred from higher mean green fluorescence for the conjugates than for individual T-cells; data not shown). Antibody to Class I HLA or to CD3 did not inhibit conjugate formation. As seen in the rosetting assay, (*a*) anti-CD2, anti-LFA-3, or anti-LFA-1 alone each inhibit partially; (*b*) anti-CD2 or anti-LFA3 inhibit more than anti-LFA-1; and (*c*) the mix of anti-LFA-1 and anti-LFA-3 inhibits completely (0% conjugates). Antibody to ICAM-1 alone only marginally inhibited conjugate formation. However, in the presence of anti-LFA-3, anti-ICAM-1 showed synergistic inhibition of conjugate formation (from 67% inhibition with anti-LFA-3 alone to 93% inhibition with anti-LFA-3 and anti-ICAM-1). This inhibition is almost as complete as that with anti-LFA-1 and anti-LFA-3.

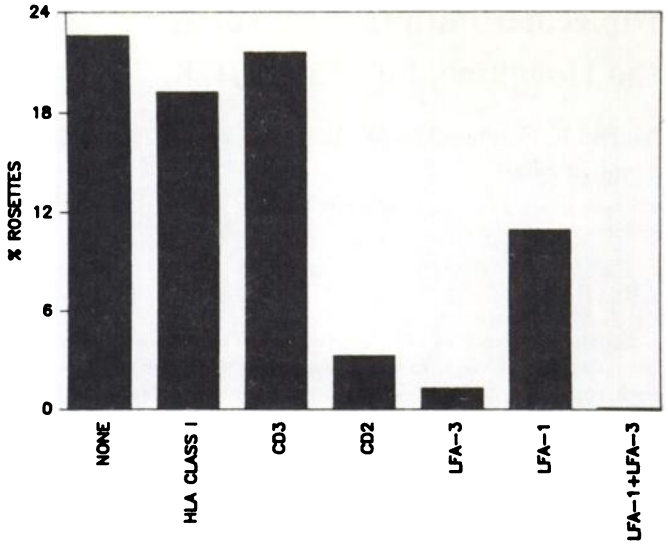

Fig. 1. Monoclonal antibody inhibition of T-cell rosettes with L428. Rosettes were prepared and scored as described in "Materials and Methods." Monoclonal antibodies were continuously present at 10 μg/ml. 300 L428 cells were counted per condition. MAB to HLA Class I or CD3 marginally inhibited rosettes. MAB to CD2, LFA-3, or LFA-1 each partially inhibited and the combination of MAB to LFA-1 and LFA-3 completely inhibited rosettes.

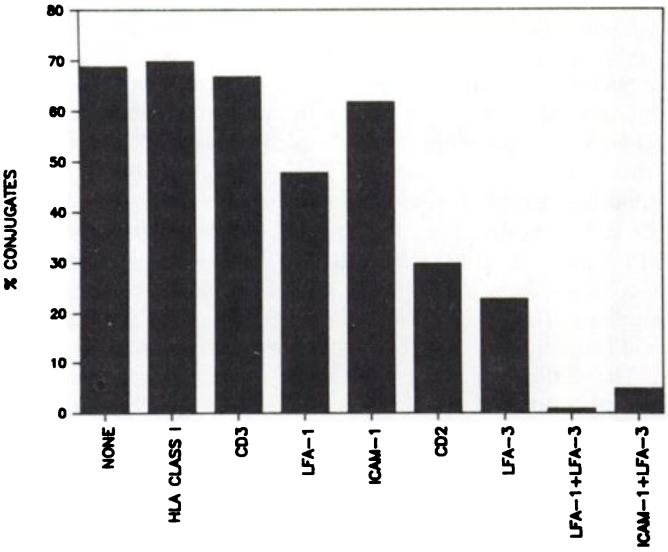

Fig. 2. Monoclonal antibody inhibition of conjugate formation of T-cell clone 8.2 with L428. Conjugates were assayed as described in "Materials and Methods." Monoclonal antibodies (or Fab fragments for anti-LFA-1 and anti-LFA-3) were continuously present at 100 μg/ml (or 300 μg/ml for Fab fragments). MAB to HLA Class I or CD3 did not inhibit conjugates. MAB to LFA-1 showed inhibition by about ⅓ and MAB to ICAM-1 showed minimal inhibition. MAB to CD2 or LFA-3 each inhibited about 50%, while the combination of MAB to LFA-1 and LFA-3 completely inhibited. The combination of MAB to ICAM-1 and LFA-3 also nearly completely inhibited conjugate formation.

Table 1 *Expression of adhesion molecules on T-cells and L428*

Expression of adhesion molecules on peripheral blood T-lymphocytes, T-cell clone 8.2, and the Hodgkin's cell line L428 as determined by flow microfluorometry. Data are expressed in linear units millivolts.

	Molecule			
Cell line	CD2	LFA-1	LFA-3	ICAM-1
T-cells	72	135	12	4
T-cell clone	492	484	62	224
L428	1	0	923	2441

DISCUSSION

We have shown using two different systems that the spontaneous adhesion of T-lymphocytes with the Hodgkin's cell line L428 is mediated primarily through two previously described pathways of antigen-independent T-cell adhesion. In both assays, antibody to either CD2 or its known ligand LFA-3 markedly inhibited adhesion. Since CD2 is expressed only on the T-cells and not on L428, its effect must be on the T-cell side of this interaction. Although there are modest amounts of LFA-3 expressed on T-lymphocytes, the inhibitory effect of anti-LFA-3 on adhesion must be on the L428 side of the interaction since no CD2 receptor for LFA-3 is expressed on the L428. In addition the amount of LFA-3 expressed on L428 is approximately 75 times the amount expressed on T-lymphocytes. Also studies of LFA-3 in adhesion of cytotoxic T-lymphocytes have consistently shown its effect to be on the target cell side (21, 22).

Involvement of the LFA-1 pathway of adhesion in rosette formation is demonstrated by inhibition of the phenomenon by antibody to LFA-1. This inhibition must be due to an effect on the T-cell side since L428 cells do not express LFA-1. In both assays anti-LFA-1 inhibits less than antibody to CD2 or LFA-3. This result is consistent with our finding that various target cell types display a predominance of use of one pathway over the other in antigen-independent conjugate formation with cytotoxic T-cell clones (21). However, complete abrogation of rosetting by the combination of antibodies to LFA-3 and LFA-1 indicates that both pathways are involved and that the contribution of any other surface molecules to this adhesion phenomenon is insufficient to overcome inhibition of both the LFA-1 and CD2 pathways.

Antibody to ICAM-1, a putative ligand for LFA-1[3] (15) which is highly expressed on L428, did not inhibit conjugate formation by itself. However, the combination of antibody to ICAM-1 with antibody to LFA-3 synergistically inhibited conjugate formation in an amount greater than would be predicted by the addition of the inhibitions by each monoclonal separately. Since the LFA-1 and CD2 pathways appear to be the only two involved in adhesion of T-lymphocytes to L428, and since the inhibition seen is synergistic with antibody to LFA-3 (which leaves the LFA-1 pathway unblocked), it is likely that ICAM-1 on the L428 serves as a ligand for T-cell LFA-1 in this model of adhesion. Our data do not exclude the presence of another ligand for LFA-1 on L428. The failure of anti-ICAM-1 alone to block conjugate formation may be due to the presence of another ligand for LFA-1 on the L428 cells. Alterntively, anti-ICAM-1 may bind to the ICAM-1 molecule on an epitope where it is not fully effective in blocking utilization of ICAM-1 in adhesion.

Interaction between CD2 and LFA-3 seems to be the major pathway of adhesion involved in rosette formation between T-lymphocytes and Hodgkin's cells. This is similar to adhesion in the phenomenon of rosetting of human erythrocytes to human T-lymphocytes, where erythrocytes rosette around T cells via binding of erythrocyte LFA-3 by CD2 on the T-cells (12, 13). Unlike erythrocyte rosetting, which involves only one adhesion pathway, adhesion of lymphocytes to Hodgkin's cells involves a second adhesion pathway utilizing LFA-1 on the T-cell and ICAM-1 on the Hodgkin's cell. Expression of LFA-3 and ICAM-1 is widely distributed in normal tissues. Since few cell types spontaneously rosette with T-lymphocytes, there must be regulatory mechanisms on a molecular level that dictate whether spontaneous adhesion occurs. Our previous work has amply demonstrated transient antigen-independent adhesion between T-lymphocytes and a wide variety of cell types (9, 21). However, the Hodgkin's cell line L428 forms conjugates with both resting or activated T-lymphocytes better than 15 other cell lines we have tested. We believe that transient antigen-independent adhesion is a physiological phenomenon that allows T-lymphocytes to "examine" target cells for specific antigen that would lead to T-cell triggering via the antigen receptor. The spontaneous rosetting of L428 cells (and by inference fresh RS cells) to lymphocytes represents an exaggeration of this physiological phenomenon. The underlying basis of this exaggeration may relate in part to the unusually high expression of two adhesion ligands, LFA-3 and ICAM-1, on Hodgkin's cells. In addition, it is possible that structural differences between forms of LFA-3 and ICAM-1 lead to variations in the affinity of LFA-3 for CD2 or LFA-1 for ICAM-1 (9). Thus, unidentified alteration in cell surface proteins associated with malignant transformation of Hodgkin's cells could lead to expression of a high affinity form of LFA-3 and/or ICAM-1 and subsequent enhanced adhesion.

The previously noted phenomenon of adherence of lymphocytes to cultured Hodgkin's cells without ensuing cell-mediated cytolysis can now be explained by the antigen-independent nature of this adhesion phenomenon. Antigen-specific recognition does not appear to be critical to the *in vitro* adhesion since Hodgkin's cells will rosette with unprimed allogeneic lymphocytes and anti-CD3 does not inhibit the interaction. Our findings do not exclude *in vivo* adhesion to and subsequent cell-mediated lysis of RS cells by antigen-specific T-lymphocytes. But a reasonable inference is that the previously reported tight apposition of lymphocytes to RS cells in tissue sections may be in large part due to antigen-independent adhesion.

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