T cell hybrids that express a VH idiotope-related determinant on a glycoprotein distinct from H-2, Thy-1, and Lyt-1 molecules

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T CELL HYBRIDS THAT EXPRESS A Vᵢ
IDIOTOPE-RELATED DETERMINANT ON
A GLYCOPROTEIN DISTINCT
FROM H-2, THY-1, AND LYT-1 MOLECULES*

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Almost 20 years have passed since the recognition that thymus-derived lymphocytes belong to a separate differentiation pathway and do not produce immunoglobulins (1, 2). However, the molecular structure of the antigen-specific receptor on T cells remains an elusive goal necessary for understanding antigen-induced interactions among T cells, B cells, and antigen-presenting cells in the immune response. There is substantial evidence in support of similarity between immunoglobulin (Ig) idiotype (Id) and determinants expressed by antigen-specific T cells. Anti-Id antibodies have been used to either stimulate or inhibit various T cell functions (3–5), and to demonstrate Ig-like Id determinants on antigen-specific T cells (6–9) and the soluble factors they produce (10, 11). The definition of the T cell receptor is complicated by the existence of functionally distinct subsets of T cells, each capable of expressing distinct antigen-specific molecules (12). A number of laboratories have established stable interleukin 2-dependent T lymphocyte clones (13–15) and T cell hybrids derived from fusion between immune T lymphocytes and cells of thymic lymphoma origin (11, 16–18). This approach should result in an increase in the yield of homogenous antigen-binding materials produced by T cells and thereby facilitate the elucidation of the molecular structure of the antigen-specific T cell receptor.

We have developed two mouse monoclonal anti-Id antibodies specific for chicken antibodies to N-acetylglucosamine (NAGA)¹ and p-amino benzoic acid (PABA). The anti-Id antibodies, termed CId-1 and CId-2, respectively, were found to react with non-antigen-binding Vᵢ determinants (19). The CId-1 antibody reacted by indirect immunofluorescence with a limited number of clones of both chicken B and T cells, whereas the CId-2 antibody reacted...

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Abbreviations used in this paper: Con A, concanavalin A; FCS, fetal calf serum; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; LL, lentil lectin; LPS, lipopolysaccharide; NAGA, N-acetylglucosamine; PABA, p-amino benzoic acid; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PWM, pokeweed mitogen; RITC, rhodamine isothiocyanate; Strep A, Streptococcus pyogenes group A strain J17A4; WGA, wheat germ agglutinin.
exclusively with Ig expressed by B cells. We have since found that the Cld-1 antibody recognizes a conserved determinant expressed by a small subset of BALB/c mouse splenic T cells. Encouraged by this observation, we fused enriched Cld-1 T lymphocytes obtained from Streptococcus A-immune BALB/c mice with the AKR BW 5147 cell line. Among the resulting 72 hybrids were two clones that reacted by indirect immunofluorescence with the Cld-1 monoclonal antibody. In this paper, we describe the generation of these T cell hybrids and an initial characterization of their Cld-1 determinants.

Materials and Methods

Antisera. The preparation and characterization of the monoclonal Cld-1 and Cld-2 anti-Id antibodies (IgMx) have been described (19). The rat monoclonal antibodies to mouse Lyt-1 and Lyt-2, the mouse monoclonal antibodies to mouse I-A<sup>d</sup> and I-A<sup>a</sup> and monoclonal fluorescein isothiocyanate (FITC)-conjugated anti-Thy-1.2 antibodies were from Becton, Dickinson & Co., Sunnyvale, CA. The mouse anti-I-J<sup>d</sup> and I-J<sup>k</sup> alloantisera were gifts from Dr. Chella S. David, Mayo Clinic, Rochester, MN. The anti-H-2<sup>a</sup> alloantisera was a gift from Dr. Lori Flaherty, Albany, NY. Affinity-purified goat antibodies specific for mouse Ig isotypes were prepared as described (20).

Immunization. BALB/c mice (H-2<sup>d</sup>) were immunized intraperitoneally three times at 5-d intervals with 10<sup>9</sup> heat-killed Streptococcus group A strain J17A4 (Strep A) organisms.

Enrichment of Cld-1<sup>+</sup> Splenic T Lymphocytes. 3 d after the last immunization, the mice were killed and spleen mononuclear cells were isolated by centrifugation over Ficoll (Pharmacia Fine Chemicals, Piscataway, NY)-Hypaque (Winthrop Laboratories, NY) gradients. To enrich for Cld-1<sup>+</sup> T lymphocytes, the spleen cell suspension was panned twice on culture dishes (Costar, Data Packaging, Cambridge, MA) precoated with 100 μg/ml of affinity-purified goat anti-mouse Ig (21). To enrich further for Cld-1<sup>+</sup> T lymphocytes, the nonadherent cells were treated with 200 μg/ml of the Cld-1 monoclonal antibody for 30 min at 4°C, washed with phosphate-buffered saline (PBS) supplemented with 5% fetal calf serum (FCS), and panned on dishes precoated with 100 μg/ml of affinity-purified goat antibodies to mouse μ chains. After a 90-min incubation at 4°C, the dishes were washed five times with 5% FCS in PBS and 10 ml of complete RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 15% FCS, 2 mM glutamine, 5 × 10<sup>−5</sup> M 2-mercaptoethanol, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of Fungizone (Gibco Laboratories, Grand Island, NY) were added to each plate. After a 1-h incubation at 37°C, the adherent cells were recovered with a sterile rubber policeman and washed before cell fusion.

Cell Fusion and Cloning. After enrichment for Cld-1<sup>+</sup> cells, T cells were fused with the hypoxanthine guanosine phosphoribosyl transferase-resistant AKR (H-2<sup>k</sup>) thymoma line BW 5147 and dispensed into 24-well culture dishes. Hybrid growth was detected 10–14 d after fusion. Screening for Cld-1<sup>+</sup> T cell hybrids was performed using the indirect immunofluorescence assay described below. Hybrid cells were cloned by limiting dilution (22).

Immunofluorescence Screening for Cld-1<sup>+</sup> Hybrids. The surface and cytoplasmic immunofluorescence techniques have been described (19). Capping of the Cld-1<sup>+</sup> molecules was done by incubating Cld-1 stained cells at 37°C for 20 min. Cell surface analysis was performed by fluorescence microscopy and on a fluorescence-activated cell sorter (FACS IV; B-D FACS Systems, Becton, Dickinson & Co.)

Lectin Treatment of Cld-1<sup>+</sup> Hybrid Cells. Cld-1B hybrid cells at 10<sup>6</sup>/ml were cultured overnight at 37°C in the presence of 10–20 μg/ml of concanavalin A (Con A), lentil lectin (LL), wheat germ agglutinin (WGA) (Sigma Chemical Co., St. Louis, MO), pokeweed mitogen (PWM), and lipopolysaccharide (LPS), or with 2% phytohemagglutinin (PHA) (Gibco Laboratories). The surface distribution of the Cld-1 determinant was then analyzed by immunofluorescence.

Treatment of Cld-1<sup>+</sup> Hybrid Cells with Tunicamycin, Pronase, and Trypsin. Cld-1B hybrid
cells at 2 × 10^6/ml were incubated with 0.5 µg/ml of tunicamycin (Calbiochem-Behring Corp., La Jolla, CA) in complete RPMI 1640 overnight at 37°C. For pronase and trypsin treatment, Cld-1B cells were washed in Hank's balanced salt solution (HBSS) at pH 7.2 and cultured at 2 × 10^6/ml with 50–100 µg/ml of pronase (Calbiochem-Behring Corp.) or 25–50 µg/ml of trypsin (Gibco Laboratories) in HBSS for 30 min at 37°C. The Cld-1 expression was then analyzed by the FACS IV.

**Results**

*The monoclonal Cld-1 Antibody Cross-reacts with BALB/c Splenic T Lymphocytes.* BALB/c spleen cell suspensions were stained by indirect immunofluorescence with the Cld-1 anti-Id antibody, followed by rhodamine isothiocyanate (RITC)-conjugated goat antibodies to mouse µ chains, and counterstained with FITC-conjugated anti-Thy-1.2 or rat monoclonal anti-Lyt-1 or Lyt-2, followed by FITC-conjugated goat antibodies to rat IgG. Approximately 0.2% of Thy-1.2+ BALB/c spleen cells co-stained with the monoclonal Cld-1 antibody (Fig. 1). Cld-1+ BALB/c spleen cells were equally distributed between the Lyt-1+ and the Lyt-2+ T cell subsets. When the Cld-1 antibody was replaced with a monoclonal anti-chicken Ia antibody (23) as a control IgM antibody in the staining procedure, no doubly stained cells were found.

*Generation of Cld-1+ T Cell Hybrids.* BALB/c splenic T cells, enriched for Cld-1+ cells (see Materials and Methods), were fused with the AKR BW 5147 cell line and dispensed into 216 wells. Among the resulting 72 wells with hybrid growth, two hybrids (Cld-1A and Cld-1B) reacted by indirect immunofluorescence with the monoclonal Cld-1 anti-Id antibody and not with the Cld-2 antibody. None of the remaining 70 T cell hybrids were reactive with either Cld-1 or Cld-2. Essentially all Cld-1B hybrid cells stained with the Cld-1 antibody in a ringlike pattern of discrete mini-patches on the cell surface (Fig. 2). The faint staining was confirmed by the FACS profiles of the Cld-1A and Cld-1B cells (Fig. 3). The fluorescence intensity exhibited by both the Cld-1A and Cld-1B hybrids was clearly above background, but ~10–20-fold less than that of BALB/c splenic B lymphocytes stained with goat anti-mouse µ-chain antibodies (data not shown). The Cld-1 surface staining of the Cld-1B hybrids was completely inhibited by preincubating the antibody with 20 µg of affinity-purified chicken anti-NAGA but not with 80 µg of anti-PABA antibodies.
To determine the intracellular distribution of Clid-1 determinants, we examined fixed Clid-1A and Clid-1B cells. Diffuse patchy immunofluorescence with Clid-1, but not with Clid-2, antibody could be visualized in the cytoplasm of the Clid-1A and B hybrid cells (Fig. 4).

Cell Surface Analysis of Clid-1A and Clid-1B Hybrids and the Parental AKR BW 5147 Line. The cell surface phenotypes were analyzed with a fluorescence microscope and the FACS (Table I). Both Clid-1A and Clid-B hybrid cells lacked Clid-2 and mouse Ig heavy- and light-chain determinants (Fig. 5). They stained with the anti-H-2^d^ alloantisemur and with the monoclonal FITC-conjugated anti-Thy-1.2 antibody (Fig. 6A) and expressed the Lyt-1 antigen faintly (Fig. 6B) but lacked the Lyt-2 antigen. They expressed neither the parental I-A^d^ (BALB/c)
FIGURE 4. Cld-1 cytoplasmic immunofluorescence staining of the Cld-1B hybrid cells: (A) phase contrast and (B) Cld-1 cytoplasmic staining. A similar staining pattern was observed with the Cld-1A hybrid cells.

(Fig. 6C) nor the 1-A<sup>k</sup> (AKR) alleles but were positive for both the parental 1-J<sup>d</sup> and 1-J<sup>k</sup> alleles (Fig. 6D). The staining patterns of the Cld-1B hybrids visualized by immunofluorescence with anti-Thy-1.2, anti-Lyt-1, anti-H-2<sup>d</sup>, anti-I-J<sup>d</sup>, and anti-I-J<sup>k</sup> antibodies were all distinct from that seen with the Cld-1 antibody.

The Cld-1 Determinant Is Distinct from the Thy-1.2, Lyt-1, H-2<sup>d</sup>, I-J<sup>d</sup>, and I-J<sup>k</sup> Molecules. To determine whether the Cld-1 determinant was physically associated with Thy-1.2, Lyt-1, H-2<sup>d</sup>, I-J<sup>d</sup>, or I-J<sup>k</sup> molecules on the cell membrane, Cld-1B hybrid cells were incubated with the Cld-1 antibody followed by RITC-
TABLE I
Immunofluorescence Analysis of Cell Surface Antigens on the Cld-1A and Cld-1B Hybrids and the Parental BW 5147 Cell Line

<table>
<thead>
<tr>
<th>Antibody specificities</th>
<th>Cld-1</th>
<th>Cld-2</th>
<th>Ig κ and λ</th>
<th>H-2d</th>
<th>Thy-1,2</th>
<th>Lyt-1</th>
<th>Lyt-2</th>
<th>I-A^d</th>
<th>I-A^k</th>
<th>I-J^d</th>
<th>I-J^k</th>
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<tr>
<td>BW5147</td>
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<td>Cld-1A</td>
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<td>Cld-1B</td>
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Figure 5. Cld-1B hybrid cells lack light chain determinants. Fluorescence profiles of the Cld-1B hybrid cells stained with Cld-2 and affinity-purified goat anti-mouse κ- and λ-chain antibodies. Immunofluorescence reactivity of the hybrid cells was also not seen with antibodies to mouse Ig, μ, γ, δ, ε, and α determinants.

Figure 6. Fluorescence profiles of the Cld-1B hybrid cells stained with (A) anti-Thy-1.2, (B) anti-Lyt-1, (C) anti-I-A^d, and (D) anti-I-J^k and anti-I-J^k antibodies.

Conjugated goat antibodies to mouse μ-chains under capping conditions. The Cld-1B cells were then stained either with Cld-1 antibody followed by FITC-conjugated goat antibodies to mouse μ-chains to verify completeness of Cld-1 capping, or with FITC-conjugated anti-Thy-1.2 antibody. After the Cld-1 marker was capped (Fig. 7), Thy-1.2 molecules were still distributed over the entire surface of the hybrid cells. Similarly, Cld-1 antibody-induced capping of the Cld-1 determinant did not result in co-capping of Lyt-1, H-2d, I-J^d, or I-J^k.

Figure 7. Cld-1 antibody-induced capping of the Cld-1 determinant does not result in redistribution of the Thy-1.2 antigen: (A) phase contrast, (B) Cld-1 staining under capping conditions, and (C) anti-Thy-1.2 staining after capping of the Cld-1 marker. Similar results were obtained with the H-2\(^b\), Lyt-1, I-J\(^a\), and I-J\(^b\) antigens.

Molecules. Reverse capping experiments were then performed with anti-I-J\(^a\) and -I-J\(^b\) alloantibodies. Capping of the I-J\(^a\) and I-J\(^b\) molecules did not result in redistribution of the Cld-1 determinant.

Lectin-induced Modulation of the Cld-1 Determinant. Since most cell surface proteins are glycoproteins, we tested a panel of lectins for their ability to bind to and modulate the Cld-1 determinant on the Cld-1B hybrid cells. Incubation of Cld-1B hybrid cells with 10-20 \(\mu\)g/ml of Con A or LL at 37°C resulted in capping of the Cld-1 marker to one cellular pole (Fig. 8). Incubation of Cld-1B hybrids with PHA, PWM, LPS, or WGA had little or no apparent effect on the
surface distribution of the Cld-1 marker. Con A- and LL-induced capping of Cld-1 determinants did not result in co-capping of Thy-1.2, Lyt-1, H-2d, I-Jd, or I-Jk molecules. These results suggest that the Cld-1 marker is a glycoprotein and further support the idea that the Cld-1 determinant is distinct from the Thy-1.2, Lyt-1, H-2d, I-Jd, and I-Jk antigens.

Effects of Tunicamycin, Pronase, and Trypsin on Expression of the Cld-1 Determinant. Incubation of the Cld-1B hybrids with 0.5 μg/ml of tunicamycin resulted in a shift of fluorescence intensity to near background level (Fig. 9A). In contrast, immunofluorescence analysis of fixed cells revealed that the cytoplasmic expression of Cld-1 determinants was not reduced by the tunicamycin treatment. Incubation of Cld-1B cells with 50–100 μg/ml of pronase or 25–50 μg/ml of trypsin also resulted in a shift of the fluorescence intensity of the treated cells to
Discussion

The idiotope defined by the monoclonal CId-1 anti-Id antibody appeared to be a non-binding-site-associated idiotope on the heavy chain of chicken anti-NAGA antibodies, which suggests a \(V_n\) Id (19). The CId-1 Id was found to be conserved in all outbred and inbred chickens tested, as evidenced by its expression on \(\sim 20-25\%\) of outbred and inbred chicken anti-NAGA antibodies and on \(\sim 1\) and 0.4% of chicken B and T cells, respectively. Furthermore, the monoclonal CId-1 anti-Id antibody was cross-reactive with 0.2% of BALB/c mouse spleen cells that expressed the Thy-1.2 antigen. CId-1* BALB/c splenic T cells were found by indirect immunofluorescence within both the Lyt-1+ and Lyt-2+ T cell subsets. The fact that CId-1 is a mouse IgM antibody precluded testing of its reactivity with mouse B cells by indirect immunofluorescence, but \(\sim 0.5\%\) of human plasma cells expressed CId-1* molecules (unpublished observation). Idiotypic cross-reactivity has been reported within inbred strains of mice (24, 25) and rabbits (26, 27), as well as between different strains of mice (28, 29). Idiotypic cross-reactivity has also been demonstrated between human and mouse, in the case of phosphorylcholine-binding myeloma proteins (30) and antibodies to acetylcholine receptor (31), and between goat and sheep antibodies to sickle cell hemoglobin (32).

Based on the observation that the CId-1 antibody reacted with a limited number of BALB/c mouse T cells, we fused enriched CId-1* BALB/c T cells with the AKR BW 5147 line and generated two of 72 hybrids, termed CId-1A and CId-1B, that reacted with the monoclonal CId-1 anti-Id antibody. Both cell hybrids lacked mouse Ig determinants and detectable Lyt-2 and I-A allelic determinants of both parental cells; each expressed the Thy-1.2, H-2\(^d\), and I-J\(^d\) antigens of BALB/c origin, the I-J\(^k\) antigen of AKR origin and the Lyt-1 antigen. These results suggest that both CId-1A and CId-1B cells were T cell hybrids resulting from fusion events between BALB/c and AKR cells.

The relative immunofluorescence intensity of the CId-1 marker on CId-1A and CId-1B hybrids was \(\sim 10-20\) less intense than that of BALB/c \(\mu\)-bearing splenic B lymphocytes stained with the same preparation of goat antibodies to
mouse μ-chains. Assuming that a mature B lymphocyte expresses \( \sim 10^5 \) surface IgM molecules (33), the Cld-1A and Cld-1B hybrids would appear to express \( \sim 5 \times 10^3 \) Cld-1 + surface molecules per cell. This figure is consistent with the idea that the antigen-binding molecules on T cells may be 10-100-fold less dense than that expressed by B cells, and with the observation that T cell hybrids synthesize extremely small amounts of antigen-binding materials (34). A low density of antigen-binding molecules on the T cell surface could also contribute to the difficulty encountered in demonstrating specific antigen binding by T cells.

The binding of Cld-1 antibody to the Cld-1B hybrids was inhibited by preincubating the antibody with affinity-purified chicken antibodies to NAGA, but not by antibodies to PABA. The lack of binding to the T cell hybrids by the control Cld-2 antibody and other mouse monoclonal antibodies of IgM isotype also strongly argues against the possibility of nonspecificity of the Cld-1 binding to the Cld-1B hybrid cells. Moreover, this possibility would not explain the specific immunofluorescent staining of cytoplasmic constituents in the Cld-1A and -1B hybrid cells after fixation. The latter observation may also be pertinent to future biosynthetic studies of the Cld-1 molecule.

The Cld-1 surface marker could be easily capped by incubating Cld-1B hybrid cells with the Cld-1 monoclonal antibody at 37°C. Cld-1 antibody-induced capping of the Cld-1 determinant did not result in redistribution of other surface structures, including Thy-1.2, Lyt-1, H-2\(^d\), I-J\(^d\), and I-J\(^k\). Similarly, capping of the I-J\(^d\) and I-J\(^k\) determinants did not alter the global distribution of the Cld-1 determinant, which suggests that the Cld-1 marker was not physically linked to these surface molecules.

Con A, in subagglutinating concentrations, has recently been reported to block the function of cytotoxic T cells, presumably by binding to surface structures essential for recognition or lysis of target cells (35). Incubation of Cld-1B hybrids with Con A or LL resulted in the capping of the Cld-1 determinant to one cellular pole, whereas incubation with PWM, PHA, LPS, or WGA did not alter surface distribution of the Cld-1 determinant. The Con A-induced modulation of the Cld-1 determinant did not result in a concomitant modulation of the Thy-1.2, Lyt-1, H-2\(^d\), I-J\(^d\), or I-J\(^k\) molecules, which further suggests that the Cld-1 determinant is a distinctive cell surface component.

Treatment of Cld-1B hybrids with tunicamycin, a compound that selectively prevents protein glycosylation (36), dramatically reduced surface expression of Cld-1 + molecules, but did not affect cytoplasmic expression of the antigen. Treatment of Cld-1B hybrid cells with pronase or trypsin also resulted in a near-complete shift of fluorescence intensity of the Cld-1 surface marker to background level. Taken together, these results suggest that the Cld-1 antigen is on a protein molecule that is glycosylated en route to the cell surface, where it can be modulated by Con A or LL independently of the other surface structures recognized on the Cld-1B hybrid cells.

It should be noted that we have no evidence of antigen binding or other functional activity for the Cld-1 + molecule on the T cell hybrids Cld-1A and Cld-1B. However, the pool size of circulating Cld-1 + T cells in the chicken was selectively increased after injections of either the Cld-1 antibody or Strep A
organisms bearing the NAGA antigen (19). Cld-1+ T cells in mice represent a very small subpopulation (~0.2%) of the T cell pool. Clonal restriction in expression of this \( V_H \) idiotope is further emphasized by its low incidence (~3%) of expression by T cell hybrids that were produced by fusion of T cells, from an NAGA-immune donor, preselected by adherence to a Cld-1 antibody-coated plate. We conclude that these T cell hybrids, which express a surface glycoprotein recognized by the monoclonal Cld-1 antibody with \( V_H \) idiotope specificity, may provide a useful model system for identification and molecular characterization of the T cell antigen receptor.

Summary

Two mouse monoclonal antibodies to chicken immunoglobulin \( V_H \)-associated idiotypes (Id), Cld-1 and Cld-2, were used as probes for Id determinants on mouse T cells. Cld-1, which recognized chicken antibodies to N-acetyl glucosamine (NAGA), and ~0.4% of chicken T lymphocytes also reacted with ~0.2% of BALB/c splenic Thy-1.2+ cells. When enriched Cld-1+ splenic T cells from NAGA-immune BALB/c mice were fused with the AKR thymoma BW 5147 cell line, 2 of 72 resulting hybrids, termed Cld-1A and Cld-1B, were reactive by indirect immunofluorescence with the Cld-1 antibody. Cld-1 determinants were expressed both in the cytoplasm and on the cell surface. Immunofluorescence studies revealed that both Cld-1+ T cell hybrids were phenotypically identical: Cld-2+/Ig+/Lyt-1.2+/Thy-1.2+/H-2d+/I-Jd+/I-Ad+/I-Ak+ in the cytoplasm and on the cell surface. Incubation of Cld-1B hybrid cells with concanavalin A or lentil lectin resulted in capping of the Cld-1 determinant, whereas incubation with pokeweed mitogen, lipopolysaccharide, phytohemagglutinin, and wheat germ agglutinin had no effect on the cell surface distribution of the Cld-1 molecule. Trypsin or pronase treatment resulted in the loss of detectable Cld-1 determinant on the cell surface. Treatment of Cld-1B cells with tunicamycin also reduced the immunofluorescence intensity of the surface Cld-1 determinant, but had no effect on its cytoplasmic expression. Cld-1 antibody-induced capping of the Cld-1 marker did not affect the surface distribution of Lyt-1, Thy-1.2, H-2d, I-Jd, or I-Jk molecules. Conversely, capping of I-Jd and I-Jk determinants did not alter the surface distribution of Cld-1. These results suggest that the Cld-1 determinant is on a glycoprotein that is not physically linked to the Lyt-1, Thy-1.2, H-2d, I-Jd, and I-Jk molecules. The clonal restriction of Cld-1 expression by T cells suggests that the Cld-1+ molecule could be a T cell antigen receptor.

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