T CELL HYBRIDS THAT EXPRESS A V\textsubscript{H} 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)\textsuperscript{1} and p-\textsuperscript{2}amino benzoic acid (PABA). The anti-Id antibodies, termed CId-1 and CId-2, respectively, were found to react with non–antigen-binding V\textsubscript{H} 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...
exclusively with Ig expressed by B cells. We have since found that the Cl I-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 Cl I-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 Cl I-1 monoclonal antibody. In this paper, we describe the generation of these T cell hybrids and an initial characterization of their Cl I-1 determinants.

Materials and Methods

Antisera. The preparation and characterization of the monoclonal Cl I-1 and Cl I-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> alloanitsera were gifts from Dr. Chella S. David, Mayo Clinic, Rochester, MN. The anti-H-2<sup>R</sup> alloanitsera 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 Cl I-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 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 Cl I-1<sup>+</sup> T lymphocytes, the nonadherent cells were treated with 200 μg/ml of the Cl I-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 Cl I-1<sup>+</sup> cells, T cells were fused with the hypoxanthine guanosine phosphoribosyl transferase-resistant AKR (H-2<sup>R</sup>) thymoma line BW 5147 and dispensed into 24-well culture dishes. Hybrid growth was detected 10-14 d after fusion. Screening for Cl I-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 Cl I-1<sup>+</sup> Hybrids. The surface and cytoplasmic immunofluorescence techniques have been described (19). Capping of the Cl I-1<sup>+</sup> molecules was done by incubating Cl I-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 Cl I-1<sup>+</sup> Hybrid Cells. Cl I-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 Cl I determinant was then analyzed by immunofluorescence.

Treatment of Cl I-1<sup>+</sup> Hybrid Cells with Tunicamycin, Pronase, and Trypsin. Cl I-1B hybrid
cells at 2 × 10⁶/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 × 10⁶/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 Cld-1 determinants, we examined fixed Cld-1A and Cld-1B cells. Diffuse patchy immunofluorescence with Cld-1, but not with Cld-2, antibody could be visualized in the cytoplasm of the Cld-1A and B hybrid cells (Fig. 4).

**Cell Surface Analysis of Cld-1A and Cld-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 Cld-1A and Cld-B hybrid cells lacked Cld-2 and mouse Ig heavy- and light-chain determinants (Fig. 5). They stained with the anti-H-2d alloantiserum 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)
Vn IDIOTYPE ON T CELL HYBRIDS

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 I-Ak (AKR) alleles but were positive for both the parental I-Jd and I-Jk alleles (Fig. 6D). The staining patterns of the Cld-1B hybrids visualized by immunofluorescence with anti-Thy-1.2, anti-Lyt-1, anti-H-2d, anti-I-Jd, and anti-I-Jk 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-2d, I-Jd, and I-Jk Molecules. To determine whether the Cld-1 determinant was physically associated with Thy-1.2, Lyt-1, H-2d, I-Jd, or I-Jk 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-IA and Cld-IB Hybrids and the Parental BW 5147 Cell Line

<table>
<thead>
<tr>
<th>Antibody specificities</th>
<th>Cld-IA</th>
<th>Cld-IB</th>
<th>Ig (\kappa) and (\lambda)</th>
<th>H-2(^d)</th>
<th>Thy-1,2</th>
<th>Lyt-1,2</th>
<th>I-A(^a)</th>
<th>I-A(^b)</th>
<th>I-J(^k)</th>
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<tr>
<td>BW5147</td>
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<tr>
<td>Cld-IA</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 \(\kappa\) and \(\lambda\)-chain antibodies. Immunofluorescence reactivity of the hybrid cells was also not seen with antibodies to mouse Ig, \(\mu\), \(\gamma\), \(\delta\), \(\epsilon\), and \(\alpha\) determinants.

Figure 6. Fluorescence profiles of the Cld-I B hybrid cells stained with (A) anti-Thy-1,2, (B) anti-Lyt-1, (C) anti-I-A\(^a\), and (D) anti-I-J\(^k\) and anti-I-J\(^a\) antibodies.

Conjugated goat antibodies to mouse \(\mu\)-chains under capping conditions. The Cld-1B cells were then stained either with Cld-1 antibody followed by FITC-conjugated goat antibodies to mouse \(\mu\)-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-2\(^d\), I-J\(^k\), or I-J\(^a\)
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^k, Lyt-1, I-J^a, and I-J^b antigens.

molecules. Reverse capping experiments were then performed with anti-I-J^d and -I-J^b alloantibodies. Capping of the I-J^d 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 μg/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-2^d, I-J^d, or I-J^k 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-2^d, I-J^d, and I-J^k 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
Vn IDIOTYPE ON T CELL HYBRIDS

The idiotope defined by the monoclonal Cld-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 Cld-1 Id was found to be conserved in all outbred and inbred chickens tested, as evidenced by its expression on ~20–25% of outbred and inbred chicken anti-NAGA antibodies and on ~1 and 0.4% of chicken B and T cells, respectively. Furthermore, the monoclonal Cld-1 anti-Id antibody was cross-reactive with 0.2% of BALB/c mouse spleen cells that expressed the Thy-1.2 antigen. Cld-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 Cld-1 is a mouse IgM antibody precluded testing of its reactivity with mouse B cells by indirect immunofluorescence, but ~0.5% of human plasma cells expressed Cld-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 Cld-1 antibody reacted with a limited number of BALB/c mouse T cells, we fused enriched Cld-1+ BALB/c T cells with the AKR BW 5147 line and generated two of 72 hybrids, termed Cld-1A and Cld-1B, that reacted with the monoclonal Cld-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-2D, and I-Jd antigens of BALB/c origin, the I-Jk antigen of AKR origin and the Lyt-1 antigen. These results suggest that both Cld-1A and Cld-1B cells were T cell hybrids resulting from fusion events between BALB/c and AKR cells.

The relative immunofluorescence intensity of the Cld-1 marker on Cld-1A and Cld-1B hybrids was ~10–20-fold less intense than that of BALB/c $\mu$-bearing splenic B lymphocytes stained with the same preparation of goat antibodies to

![Fluorescence profiles of the Cld-1B hybrid cells before and after treatment with (A) tunicamycin, (B) pronase, and (C) trypsin.](image)

Discussion

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![Fluorescence profiles of the Cld-1B hybrid cells before and after treatment with (A) tunicamycin, (B) pronase, and (C) trypsin.](image)
mouse μ-chains. Assuming that a mature B lymphocyte expresses ~10^5 surface IgM molecules (33), the Clb-1A and Clb-1B hybrids would appear to express ~5 x 10^3 Clb-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 Clb-1 antibody to the Clb-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 Clb-2 antibody and other mouse monoclonal antibodies of IgM isotype also strongly argues against the possibility of nonspecificity of the Clb-1 binding to the Clb-1B hybrid cells. Moreover, this possibility would not explain the specific immunofluorescent staining of cytoplasmic constituents in the Clb-1A and -1B hybrid cells after fixation. The latter observation may also be pertinent to future biosynthetic studies of the Clb-1 molecule.

The Clb-1 surface marker could be easily capped by incubating Clb-1B hybrid cells with the Clb-1 monoclonal antibody at 37°C. Clb-1 antibody-induced capping of the Clb-1 determinant did not result in redistribution of other surface structures, including Thy-1.2, Lyt-1, H-2d, 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 Clb-1 determinant, which suggests that the Clb-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 Clb-1B hybrids with Con A or LL resulted in the capping of the Clb-1 determinant to one cellular pole, whereas incubation with PWM, PHA, LPS, or WGA did not alter surface distribution of the Clb-1 determinant. The Con A-induced modulation of the Clb-1 determinant did not result in a concomitant modulation of the Thy-1.2, Lyt-1, H-2d, I-J^d, or I-J^k molecules, which further suggests that the Clb-1 determinant is a distinctive cell surface component.

Treatment of Clb-1B hybrids with tunicamycin, a compound that selectively prevents protein glycosylation (36), dramatically reduced surface expression of Clb-1^+ molecules, but did not affect cytoplasmic expression of the antigen. Treatment of Clb-1B hybrid cells with pronase or trypsin also resulted in a near-complete shift of fluorescence intensity of the Clb-1 surface marker to background level. Taken together, these results suggest that the Clb-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 Clb-1B hybrid cells.

It should be noted that we have no evidence of antigen binding or other functional activity for the Clb-1^+ molecule on the T cell hybrids Clb-1A and Clb-1B. However, the pool size of circulating Clb-1^+ T cells in the chicken was selectively increased after injections of either the Clb-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\textsubscript{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\textsubscript{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\textsubscript{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 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\textsuperscript{-}/Ig\textsuperscript{-}/Lyt-1.2\textsuperscript{-}/Thy-1.2\textsuperscript{+}/H-2\textsuperscript{d}/I-A\textsuperscript{d}/I-A\textsuperscript{k}/I-J\textsuperscript{d}/I-J\textsuperscript{k}. Incubation of Cld-1B hybrids 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-2\textsuperscript{d}, I-J\textsuperscript{d}, or I-J\textsuperscript{k} molecules. Conversely, capping of I-J\textsuperscript{d} and I-J\textsuperscript{k} 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-2\textsuperscript{d}, I-J\textsuperscript{d}, and I-J\textsuperscript{k} 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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