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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Journal Title: Journal of Experimental Medicine
Volume: Volume 158, Number 2
Publisher: Rockefeller University Press | 1983-01-01, Pages 452-464
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1084/jem.158.2.452
Permanent URL: https://pid.emory.edu/ark:/25593/rqgd9

Final published version: http://dx.doi.org/10.1084/jem.158.2.452

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Accessed May 21, 2017 3:21 PM EDT
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-amino benzoic acid (PABA). The anti-Id antibodies, termed Cld-1 and Cld-2, respectively, were found to react with non–antigen-binding V\textsubscript{H} determinants (19). The Cld-1 antibody reacted by indirect immunofluorescence with a limited number of clones of both chicken B and T cells, whereas the Cld-2 antibody reacted

* Supported by grants CA 16673 and CA 13148 from the National Cancer Institute.

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\textsuperscript{1} 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-acetylgalactosamine; PABA, p-amino benzoic acid; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PWM, pokeweed mitogen; RITC, rhodamine isothiocyanate; Strep A, \textit{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\(^d\) and I-A\(^a\) and monoclonal fluorescein isothiocyanate (FITC)-conjugated anti-Thy-1.2 antibodies were from Becton, Dickinson & Co., Sunnyvale, CA. The mouse anti-I-J\(^d\) and I-J\(^a\) alloantisera were gifts from Dr. Chella S. David, Mayo Clinic, Rochester, MN. The anti-H-2\(^a\) alloantiserum 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\(^d\)) were immunized intraperitoneally three times at 5-d intervals with 10\(^6\) heat-killed Streptococcus group A strain J17A4 (Strep A) organisms.

Enrichment of ClD-1+ 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 \(\mu\)g/ml of affinity-purified goat anti-mouse Ig (21). To enrich further for ClD-1+ T lymphocytes, the nonadherent cells were treated with 200 \(\mu\)g/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 \(\mu\)g/ml of affinity-purified goat antibodies to mouse \(\mu\) 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 \(\times\) 10\(^{-5}\) M 2-mercaptoethanol, 100 U/ml of penicillin, 100 \(\mu\)g/ml of streptomycin, and 0.25 \(\mu\)g/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+ cells, T cells were fused with the hypoxanthine guanosine phosphoribosyl transferase-resistant AKR (H-2\(^a\)) thymoma line BW 5147 and dispensed into 24-well culture dishes. Hybrid growth was detected 10–14 d after fusion. Screening for ClD-1+ 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+ Hybrids. The surface and cytoplasmic immunofluorescence techniques have been described (19). Capping of the ClD-1 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+ Hybrid Cells. ClD-1B hybrid cells at 10\(^6\)/ml were cultured overnight at 37°C in the presence of 10–20 \(\mu\)g/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+ Hybrid Cells with Tunicamycin, Pronase, and Trypsin. ClD-1B hybrid
cells at 2 x 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, CId-1B cells were washed in Hanks' balanced salt solution (HBSS) at pH 7.2 and cultured at 2 x 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 CId-1 expression was then analyzed by the FACS IV.

Results

The monoclonal CId-1 Antibody Cross-reacts with BALB/c Splenic T Lymphocytes. BALB/c spleen cell suspensions were stained by indirect immunofluorescence with the CId-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 CId-1 antibody (Fig. 1). CId-1^+ BALB/c spleen cells were equally distributed between the Lyt-1^+ and the Lyt-2^+ T cell subsets. When the CId-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 CId-1^+ T Cell Hybrids. BALB/c splenic T cells, enriched for CId-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 (CId-IA and CId-IB) reacted by indirect immunofluorescence with the monoclonal CId-1 anti-Id antibody and not with the CId-2 antibody. None of the remaining 70 T cell hybrids were reactive with either CId-1 or CId-2. Essentially all CId-1B hybrid cells stained with the CId-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 CId-IA and CId-1B cells (Fig. 3). The fluorescence intensity exhibited by both the CId-1A and CId-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 CId-1 surface staining of the CId-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 alloantiseraum 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)
(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-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>
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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\(^d\) 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-2\(^d\), I-J\(^d\), or I-J\(^k\)
FIGURE 7. Cld-I 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^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-2d, I-Jk, 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-Jk, 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
V_{H} IDIOTYPE ON T CELL HYBRIDS

Discussion

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_{H} 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-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 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 μ-bearing splenic B lymphocytes stained with the same preparation of goat antibodies to background level (Fig. 9, B and C). These results suggest that the Cld-1 determinant is on a glycoprotein, and that glycosylation is required for normal surface expression of the molecule.

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mouse μ-chains. Assuming that a mature B lymphocyte expresses ~10^5 surface IgM molecules (33), the Clδ-1A and Clδ-1B hybrids would appear to express ~5 × 10^3 Clδ-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 Clδ-1 antibody to the Clδ-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 Clδ-2 antibody and other mouse monoclonal antibodies of IgM isotype also strongly argues against the possibility of nonspecificity of the Clδ-1 binding to the Clδ-1B hybrid cells. Moreover, this possibility would not explain the specific immunofluorescent staining of cytoplasmic constituents in the Clδ-1A and -1B hybrid cells after fixation. The latter observation may also be pertinent to future biosynthetic studies of the Clδ-1 molecule.

The Clδ-1 surface marker could be easily capped by incubating Clδ-1B hybrid cells with the Clδ-1 monoclonal antibody at 37°C. Clδ-1 antibody-induced capping of the Clδ-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 Clδ-1 determinant, which suggests that the Clδ-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 Clδ-1B hybrids with Con A or LL resulted in the capping of the Clδ-1 determinant to one cellular pole, whereas incubation with PWM, PHA, LPS, or WGA did not alter surface distribution of the Clδ-1 determinant. The Con A-induced modulation of the Clδ-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 Clδ-1 determinant is a distinctive cell surface component.

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

It should be noted that we have no evidence of antigen binding or other functional activity for the Clδ-1^+ molecule on the T cell hybrids Clδ-1A and Clδ-1B. However, the pool size of circulating Clδ-1^+ T cells in the chicken was selectively increased after injection of either the Clδ-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_m-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-/Ly-1.2+/H-2d+/I-A^d+/I-K^d+1-J_k+.

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-J^d, or I-J^k molecules. Conversely, capping of I-J^d and I-J^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-2d, I-J^d, and I-J^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.

We thank Larry Gartland for performing the FACS analysis, Darcy Wilson for a helpful suggestion, William Gathings for providing the goat antibodies to mouse immunoglobulin isotypes, and John Kearney, Brian Pollok, Joyce Lehmyer, Jane Calvert, and Chen-lo Chen for reading the manuscript. We also thank Ann Brookshire for her excellent secretarial assistance.

Received for publication 28 February 1983 and in revised form 11 May 1983.

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