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Immunology

# Human lymphocyte antigens: Production of a monoclonal antibody that defines functional thymus-derived lymphocyte subsets

(lymphocyte hybrids/immunoregulation)

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**ABSTRACT** **A monoclonal mouse antibody (3A1) that specifically bound to 65% of human peripheral blood (PB) thymus-derived (T) cells but did not bind to complement receptor-positive PB bone marrow-derived (B) cells, polymorphonuclear leukocytes, or human erythrocytes has been produced. The 3A1 antibody was synthesized by a stable cloned lymphocyte hybrid cell line. This lymphocyte hybrid line (3A1) was derived from fusion of P3 × 63/Ag8 myeloma cells and spleen cells from BALB/c mice immunized with HSB-2 cells, a human T cell line. The 3A1 lymphocyte hybrid line produced mouse ascites fluid containing 3A1 antibody in saturating titers of up to 1:25,600. Purified PB T cells that carried the 3A1 antigen incorporated tritiated thymidine maximally in response to phytohemagglutinin and concanavalin A stimulation, whereas purified PB T cells that lacked the 3A1 antigen responded suboptimally to phytohemagglutinin and minimally to concanavalin A. Thus, the 3A1 antibody can be easily used to study the role of 3A1-positive and negative T cell subsets in the regulation of normal and abnormal human immune responses.**

The Ly series of murine thymus-derived (T) lymphocyte differentiation antigens has been defined by using alloantisera (1). Cytotoxic antisera against this series of antigens have been used to physically separate functional murine immunoregulatory and effector T cell subsets (2–5). In humans, xenoantisera against T cell surface antigens have been described, some of which define functional subsets of T cells (6–8). However, these reagents are not homogeneous, and they often are used to define T cell subsets on the basis of relative intensity of antibody binding as determined by fluorescence (9).

In addition, multiple and laborious absorptions of the xenoantisera must be performed in order to attain specificity for T cells. In turn, absorbed xenoantisera, though relatively T cell specific, frequently perturb T cell function and either are mitogenic (10) or block the incorporation of tritiated thymidine in response to mitogens (11).

The methodology of establishing lymphocyte hybrid cell lines that produce monoclonal antibodies against cell surface antigens has only recently been described (12–14), but it has rapidly become a powerful tool in the production of large quantities of reagents with specificity for various cell surface antigens. For example, lymphocyte hybrid lines secreting monoclonal antibodies against numerous human lymphocyte antigens (13–15), including human HLA antigens and $\beta_2$-microglobulin (13), have been described.

We have used the lymphocyte hybrid technique of Kohler and Milstein (12) coupled with selective screening procedures using autologous T and bone marrow-derived (B) cell lines to identify, characterize, and preserve five hybridoma lines producing antibodies against various human lymphocyte cell surface antigens. The present report describes our lymphocyte hybrid line, 3A1, that produces an antibody specific for a functionally distinct human peripheral blood (PB) T cell subset.

## MATERIALS AND METHODS

**Mice and Cell Lines.** Eight-week-old BALB/c female mice obtained from the National Institutes of Health small animal unit were used throughout the study. CCRF-HSB-2 and CCRF-SB lymphoblastoid cells were obtained from the American Type Culture Collection Cell depository. The CCRF-HSB-2 (hereafter designated HSB-2) cell line was originally derived from a patient with acute lymphoblastic leukemia (16) and has subsequently been shown to possess surface receptors for sheep erythrocytes (E), a marker for human T cells, to lack human B cell markers (17), and to be negative for Epstein–Barr virus nuclear antigen (17). The CCRF-SB cell line (hereafter designated SB) was derived from the same patient (16), and subsequently has been shown to be positive for Epstein–Barr nuclear antigen and to possess human B cell surface markers (17). P3 × 63/Ag8 cells were obtained from John Minna. 3A1 hybrid cells were grown in tissue culture in RPMI 1640 media (Microbiological Associates, Walkersville, MD) supplemented with 20% fetal calf serum (GIBCO), and at present are maintained as an ascites-producing myeloma line by serial intraperitoneal passages into BALB/c mice pretreated with pristane (2,6,10,14-tetramethyl pentadecane, Aldrich).

**Lymphocyte Hybrid Antibody Production.** Female BALB/c mice were immunized both intravenously and intraperitoneally with $5 \times 10^6$ HSB-2 T cells on days 0, 14, and 21. On day 24, the spleen was removed from an immunized mouse, and a single-cell suspension was obtained. Immunized spleen cells were fused with P3 × 63/Ag8 mouse myeloma cells in the presence of 0.8 ml of 50% polyethelylene glycol 1000 (Baker) by the method of Galfre *et al.* (18). Normal nonimmunized BALB/c spleen cells were added to the suspension (5000 cells per well) to serve as a feeder layer. To each well of a Linbro flat-bottomed microtiter plate (Flow Laboratories, McLean, VA) was added 0.25 ml of the fused cell suspension. After 8–10 days, colonies became visible and 0.2 ml of medium was removed and assayed for anti-HSB-2 antibody activity.

**Assays to Measure Cell Surface Antibody Binding.** $^{125}$I-Labeled staphylococcal protein A (SPA; Amersham) or $^{125}$I-labeled affinity-purified F(ab)$_2$ anti-mouse IgG (heavy and light chain specific) were used to detect the presence of antibodies against HSB-2 cells, SB cells, or human PB cells in culture supernatant or ascites (19). Briefly, HSB-2 T cells, SB B cells, or PB lymphocytes were suspended in Dulbecco's phosphate-

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Abbreviations: B cell, bone marrow-derived cell; BRBC, bovine erythrocytes; Con A, concanavalin A; E, sheep erythrocytes; PB, peripheral blood; PHA, phytohemagglutinin; SPA, staphylococcal protein A; T cell, thymus-derived cell; $T_G$, T cells bearing an Fc receptor for IgG.

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buffered saline containing 0.1% gelatin and $5 \times 10^5$ cells were added to 50 $\mu$l of supernatant or diluted ascites fluid in plastic V-bottomed microtiter plates (Dynatech, Alexandria, VA). After incubation at 20°C for 30 min, cells were washed three times with 0.1% gelatin/1.0% albumin in Dulbecco's phosphate-buffered saline, and 50 $\mu$l of either $^{125}$I-labeled F(ab)$_2$ anti-mouse IgG (10,000 cpm) or $^{125}$I-labeled SPA (50,000 cpm) was added. After incubation at 20°C for 30 min, the cells were washed three times and each well was cut from the plate and assayed for radioactivity in a gamma counter. Controls included known positive mouse serum containing anti-HSB-2 antibodies, normal mouse serum, supernatant from P3 × 63/Ag8 cell cultures, and ascites fluid produced by a P3 × 63/Ag8 spleen cell hybrid that contained no specific anti-HSB-2 binding activity.

In some experiments, the ability of the lymphocyte hybrid supernatants or ascites fluids to kill HSB-2 or SB cells in the presence of complement was tested (19, 20). In the present study, the 3A1 antibody used was from mouse ascites fluid diluted in either RPMI 1640 supplemented with 20% fetal calf serum for the binding assays or in RPMI 1640 without fetal calf serum for the cell separation studies. In some experiments, F(ab)$_2$ fragments of 3A1 antibody prepared by pepsin digestion (21) were used.

**Identification of PB Lymphocyte Subpopulations.** Purified mononuclear cell suspensions were obtained from heparinized venous blood of 10 normal adults by standard Ficoll/Hypaque density centrifugation (22). T cells were identified by their ability to form rosettes spontaneously with sheep E (23). B lymphocytes were identified by the presence of a cell surface receptor for complement (24). T cell subsets bearing receptors for the Fc portion of IgG ($T_G$) or IgM ($T_M$) were identified by rosette procedures as described (25). PB cells binding the 3A1 antibody were identified by several methods. Fluorescent labeling of 3A1-positive T cells was performed by incubating PB cells with various dilutions of 3A1 antibody for 30 min at 20°C, washing three times, and then incubating with fluorescein-conjugated F(ab)$_2$ fragment of rabbit anti-mouse IgG (lot 11927, Cappel Laboratories, Cochranville, PA). After incubation for 30 min at 4°C, followed by washing three times in phosphate-buffered saline, the percentage of cells positive for fluorescence was determined on a Zeiss fluorescence microscope. Alternatively, because we found that SPA bound to the 3A1 antibody, bovine erythrocytes (BRBC) were coated with SPA (Pharmacia) by the chromic chloride method (26), and 0.1 ml of 0.5% SPA-coated BRBC was incubated with 0.1 ml of medium containing $10^6$ PB cells sensitized with 3A1 antibody. The mixture was pelleted, incubated at 20°C for 30 min, and gently resuspended, and the percentage of lymphocytes that bound three or more BRBC was measured under phase contrast microscopy.

**Fractionation of PB Mononuclear Cell Suspensions.** Unfractionated mononuclear cell suspensions were separated into E-rosette positive and E-rosette negative fractions by sheep E rosetting of lymphocytes followed by centrifugation of rosetted cells over Hypaque/Ficoll gradients (25, 27, 28). This isolation method yielded E rosette-positive suspensions containing >95% E rosette-positive cells and E rosette-depleted suspensions containing <5% E rosette-positive cells. For the isolation of E rosette-positive cells that bound the 3A1 antibody, E rosette-positive cells (T cells) were incubated with a 1:4000 dilution of 3A1 antibody and washed, and then $40 \times 10^6$ 3A1-coated T cells in 15 ml of RPMI 1640 media were mixed with 15 ml of 0.5% SPA-coated BRBC in RPMI 1640 media. The suspension was pelleted, incubated at 20°C for 30 min, gently resuspended, and then centrifuged through a Hypaque/Ficoll density gradient. The 3A1-positive T cells formed rosettes with the SPA-coated BRBC and pelleted at the bottom of the gradient while the 3A1-negative T cells remained at the interface of the gradient. The SPA-coated BRBC were then removed from the 3A1-positive T cells by hypotonic lysis.

**Blastogenic Responses.** Cultures for the determination of blast transformation by PB lymphocytes as measured by tritiated thymidine incorporation were set up in triplicate in microtiter plates and assayed as described (29).

## RESULTS

**Identification of T Cell Specific Antibodies from Lymphocyte Hybrids.** Of 286 lymphocyte hybrid colonies produced, approximately 60% were actively secreting anti-HSB-2 antibodies. To screen for HSB-2 cell specific antibodies (and therefore for antibodies possibly PB T cell specific), supernatants were assayed by a method that simultaneously screened for binding to HSB-2 and SB cells. We maintained in culture 22 lymphocyte hybrid cell lines whose antibody reacted with HSB-2 cells. Of these, three produced antibodies that did not bind to SB cells. Thus, the monoclonal antibody of one of these lymphocyte hybrid lines, 3A1, is the subject of the present report. In the initial screening assay 14 days after hybridization, antibody produced by the 3A1 hybridoma showed high binding activity to HSB-2 T cells but no binding to SB B cells by the $^{125}$I-labeled F(ab)$_2$ anti-mouse IgG binding assay. This cell line has been cloned once by dilution (0.8 cells per well in multiwell plates) and carried in tissue culture for 25 passages and as a mouse myeloma ascites-producing tumor for 15 passages. The 3A1 line is stable. To date, HSB-2 T cell specific antibody is still being produced in the same titer and specificity by the cultured 3A1 cells and by the cloned 3A1 cells passed as a myeloma ascites-producing tumor.

**Characteristics of the 3A1 Antibody.** Fig. 1 shows that significant binding to HSB-2 T cells occurred at a 1:102,400 dilution of 3A1 mouse ascites fluid, with saturation of 3A1 antigen sites occurring at 1:12,800. In contrast, no significant binding of 3A1 antibody to SB B cells occurred at any titer up through 1:200.

Next, the ability of 3A1 antibody to bind to SPA as well as kill HSB-2 T cells in the presence of complement was determined. The 3A1 antibody bound $^{125}$I-labeled SPA, and in the presence of rabbit complement the antibody had cytotoxic effects on

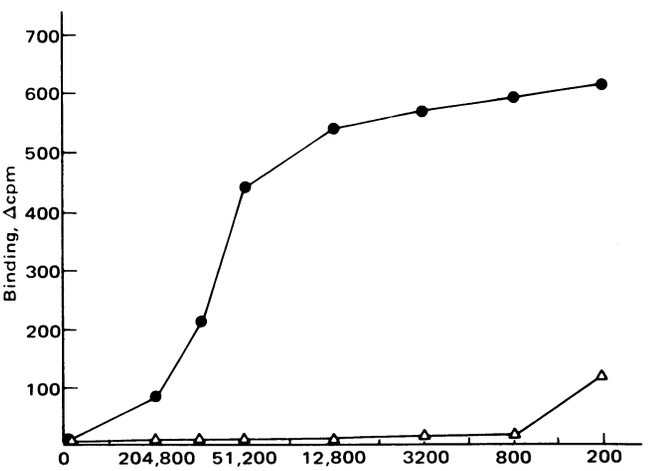


FIG. 1. Binding curves of 3A1 antibody to HSB-2 T cells (●) and SB B cells (△). To $5 \times 10^5$ HSB-2 or SB cells in a microtiter plate were added 1:2 dilutions of 3A1 antibody. Specific 3A1 binding was assayed with $^{125}$I-labeled F(ab)$_2$. Data are expressed as Δcpm as determined by the formula Δcpm = cpm of experimental sample − cpm of control sample. Control samples always bound 1% or less of the total radioactive material added.

HSB-2 T cells in dilutions as high as 1:10,000. Using BRBC coated with IgC, we found no detectable IgG Fc receptors on HSB-2 T cells. However to rule out the possibility that binding of the 3A1 to the HSB-2 T cell was not via IgG Fc receptor attachment, the HSB-2 T cells were treated with trypsin at 5 mg/ml prior to incubation with 3A1 antibody in the $^{125}$I anti-mouse IgG-binding assay. If the binding of 3A1 was via Fc receptors for IgG, then no decrease in binding of 3A1 should occur with trypsin-treated HSB-2 cells because the Fc receptor for IgG on human T cells is resistant to trypsin (30). Fig. 2 shows that the binding of 3A1 to HSB-2 T cells was completely blocked by trypsin treatment of the HSB-2 T cells prior to incubation with 3A1. Thus, the binding of 3A1 was not nonspecific attachment to Fc receptors, but was specific binding to what most likely was a cell surface protein.

**Binding Specificity of 3A1 Antibody to Human PB Cells.** First, PB mononuclear cells were separated into E rosette-positive (T cell) and E rosette-negative (non-T cell) fractions, and the binding of 3A1 antibody to these cell suspensions was then compared with 3A1 binding to unfractionated PB mononuclear cells. Fig. 3 demonstrates that, as the number of PB cells in the binding assay increased, the amount of 3A1 bound (as measured by the incorporation of $^{125}$I-labeled anti-mouse IgG) increased significantly with E rosetting cells (T cell), was lowest with non-E rosetting cells, and was intermediate in amount with unfractionated PB mononuclear cells.

By using fluorescein-conjugated anti-mouse IgG, the percentage of each type of PB mononuclear cell binding 3A1 antibody was determined. 3A1 antibody bound to an average of 38% of unfractionated PB mononuclear cells, 63% of E rosette-positive T cells, and <1% of complement receptor-positive B cells (Table 1). 3A1 also bound to 7% of E rosette-negative PB

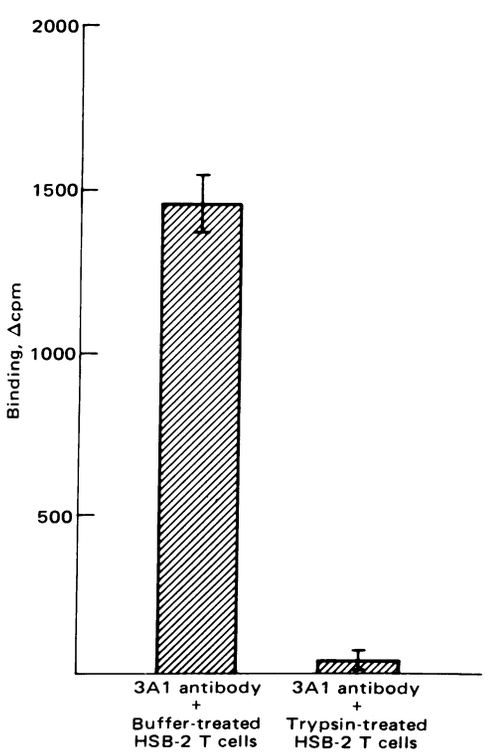


FIG. 2. Effect of trypsin treatment of HSB-2 cells prior to assay of binding activity of 3A1 antibody. Trypsin (5 mg/ml) or phosphate-buffered saline was incubated with 1.5 × $10^6$ HSB-2 cells for 30 min, and the cells were washed in medium three times, coated with 1:400 dilution of 3A1 antibody, and then used in the rabbit $^{125}$I-labeled F(ab)$_2$ anti-mouse IgG binding assay. Error bars indicate SEM for three determinations.

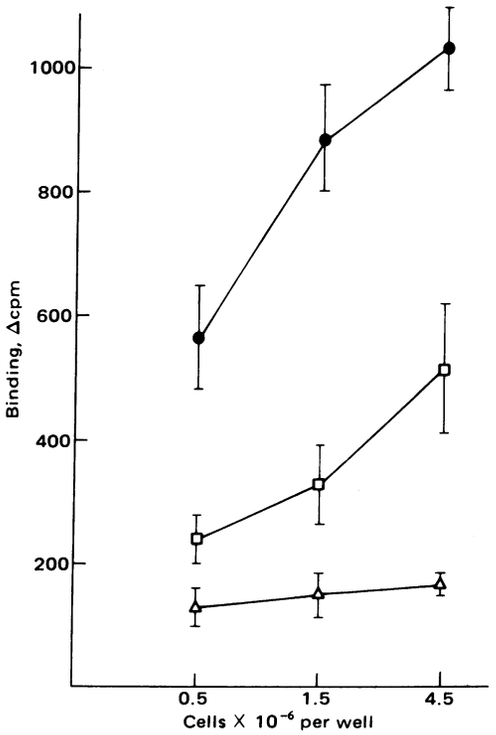


FIG. 3. Binding specificity of 3A1. A saturating amount of 3A1 (1:1000 dilution) or normal mouse serum (1:1000 dilution) was added to increasing numbers of Hypaque/Ficoll-purified unfractionated PB mononuclear cells (□), E rosette-positive T cells (●), or E rosette-negative cells (△). Binding of 3A1 antibody was determined by the rabbit $^{125}$I-labeled F(ab)$_2$ anti-mouse IgG binding assay.

cells (data not shown), although a majority of this binding was IgG Fc-mediated binding to monocytes. In this regard, monocyte binding of 3A1 antibody was blocked when F(ab)$_2$ fragments of 3A1 were used. However, labeling of E rosette-negative PB mononuclear cell suspensions with F(ab)$_2$ fragments of 3A1 antibody still showed that 2–3% of E rosette-negative lymphocytes bound 3A1, although binding to E rosette-positive cells was unchanged with F(ab)$_2$ fragments of 3A1.

Next, we correlated the presence of the 3A1 receptor on E rosette-positive cells with the presence or absence of a T cell Fc receptor for IgG. An average of 29% of E rosette-positive T cells with IgG Fc receptors ($T_G$) bound 3A1 antibody, whereas 74% of E rosette-positive T cells with no IgG Fc receptors ($T_G$-depleted) bound 3A1 (Table 1). Similar relative percentages were seen when fluorescein-conjugated F(ab)$_2$ fragments of 3A1 antibody were used. Furthermore, in four separate experiments, purified PB polymorphonuclear cells and human erythrocytes were found not to bind 3A1 antibody. In separate studies it was demonstrated that binding of 3A1 antibody did not inhibit E rosette formation and did not block the detection of IgG or IgM Fc receptor or complement receptors.

**Effect of 3A1 Antibody on Cell Function.** Purified E rosette-positive T cells were stimulated with wide concentration ranges of phytohemagglutinin (PHA) and concanavalin A (Con A), and the blastogenic responses were compared to those of T cells that had been previously coated with 3A1 antibody. We found that 3A1 antibody did not block the PHA- or Con A-stimulated incorporation of tritiated thymidine by T cells.

Because certain heterologous anti-T cell antisera have been shown to be potent T cell mitogens (10), the ability of 3A1 to stimulate tritiated thymidine incorporation in unfractionated PB mononuclear cells and in E rosette-positive and negative cell suspensions was tested. We found that 3A1 antibody at a dose

Table 1. Immunofluorescent determination of binding of 3A1 antibody to PB lymphocyte subpopulations

| Exp. | 3A1-positive lymphocytes, %* | | | | |
|---|---|---|---|---|---|
| | Unfractionated PB† | E rosette-positive† | Complement receptor-positive‡ | E rosette-positive, IgG Fc receptor-positive§ | E rosette-positive, IgG Fc receptor-negative§ |
| 1 | 45 (3) | 65 (0) | 1 (0) | 30 (0) | 84 (0) |
| 2 | 42 (4) | 63 (0) | 1 (0) | 35 (0) | 65 (0) |
| 3 | 23 (0) | 55 (0) | 1 (0) | 30 (0) | 70 (0) |
| 4 | 40 (0) | 68 (0) | 0 (0) | 23 (0) | 78 (0) |

* Numbers in parentheses represent percent positive with normal mouse serum.

† From each of four subjects, $2 \times 10^6$ cells were incubated at room temperature with a 1:100 dilution of 3A1 antibody or normal mouse serum, washed, and stained with fluorescein-conjugated rabbit $F(ab)_2$ anti-mouse IgG.

‡ Unfractionated or E rosette-negative PB lymphocytes were incubated with a 1:100 dilution of 3A1 antibody or normal mouse serum, washed, and stained with fluorescein-conjugated rabbit $F(ab)_2$ anti-mouse IgG. Bovine erythrocyte–antibody–complement (EAC) reagent was added, and the number of EAC-positive cells that were fluorescein positive was determined.

§ E rosette-positive cells were incubated with 3A1 antibody or normal mouse serum, washed, and stained with fluorescein-conjugated rabbit $F(ab)_2$ anti-mouse IgG. BRBC coated with IgG were added, and the number of IgG Fc receptor-positive and negative cells that were fluorescein positive was determined.

range of 1:2000 to 1:200,000 did not stimulate tritiated thymidine incorporation in any cell suspension tested.

**Stability of the 3A1 Antigen in Culture.** E rosetting T cells placed in culture in Tc199 medium and 20% fetal calf serum (25) were assayed each day for 5 days for the percentage and number of cells binding 3A1 antibody. With fluorescent staining techniques, there was no difference in the percentage of 3A1-positive cells detectable on days 1 through 5 of culture.

**Separation, Morphology, and Function of Subsets of 3A1-Positive and Negative E Rosetting T Cells.** Because 3A1 antibody bound SPA, we were able to use BRBC coupled with SPA as a rosetting marker to identify 3A1-positive cells and to physically separate rosette-positive from rosette-negative cells over Hypaque/Ficoll density gradients.

There was a difference in morphology in the purified T cell populations as shown in Fig. 4. In three separate experiments, purified 3A1-positive cells were composed of 90 ± 0.5% (mean ± SEM) small lymphocytes and 10 ± 0.5% medium to large lymphocytes (Fig. 4*A*). In contrast, 3A1-negative cell suspensions contained 51 ± 6% small lymphocytes and 49 ± 6% medium or large lymphocytes (Fig. 4*B*). Similarly, by using either polyacrylamide beads coated with anti-mouse IgG or BRBC coupled with SPA, the morphology of individually rosetted cells was established and it was found that the great majority of 3A1-positive cells were small lymphocytes (Fig. 4 *C* and *D*).

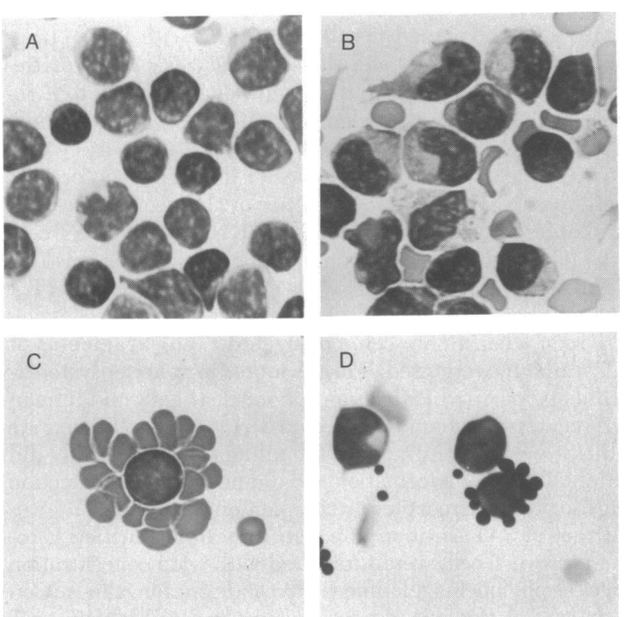


FIG. 4. (*A*) Wright's stained cytopreparation of SPA-coated BRBC rosette-purified 3A1-positive T cells. Note that most of the cells are small lymphocytes. (×400.) (*B*) Wright's stained cytopreparation of SPA-coated BRBC rosette-purified 3A1-negative T cells. Note that nearly half of the cells are moderate to large lymphocytes. (×400.) (*C*) Wright's stained cytopreparation of a 3A1-positive T cell rosetting with SPA-coated BRBC. (×400.) (*D*) Wright's stained cytopreparation of a 3A1-positive T lymphocyte rosetted with anti-mouse IgG-coated polyacrylamide beads (Bio-Rad). (×400.)

Finally, the incorporation of tritiated thymidine of total T cells, 3A1-positive T cells, and 3A1-negative T cells in response to PHA and Con A stimulation was determined. As can be seen from Fig. 5, total T cells and 3A1-positive T cells had similar patterns of tritiated thymidine incorporation in response to PHA (Fig. 5*A*) and Con A (Fig. 5*B*) stimulation. In contrast, 3A1-negative T cells responded submaximally to PHA stimulation (Fig. 5*A*) and extremely poorly to Con A stimulation (Fig. 5*B*).

## DISCUSSION

This study reports the establishment of a stable, cloned murine lymphocyte hybrid line (3A1) that produces a monoclonal antibody highly specific for a trypsin-sensitive antigen on a functionally defined subset of human PB T lymphocytes. The 3A1 antibody did not bind to human erythrocytes, polymorphonuclear cells, monocytes, or B cells. It did not block sheep E rosette formation, nor did it block IgG or IgM Fc or complement surface membrane receptors. 3A1 antibody did not block tritiated thymidine incorporation of PB T cells in response to Con A or PHA and was not mitogenic for the cells to which it bound. It was cytotoxic for HSB-2 T cells in the presence of rabbit complement and it did bind SPA. Approximately 70% of $T_G$ cells were 3A1 negative, whereas only 25% of $T_G$-depleted T cells were 3A1 negative. Purified 3A1-positive T cells were 90% small lymphocytes and 10% medium to large lymphocytes, whereas 50% of purified 3A1-negative T cells were medium to large lymphocytes. Most importantly, the 3A1-positive T cells incorporated tritiated thymidine near maximally after stimulation by PHA or Con A, whereas the 3A1-negative T cells responded submaximally to PHA and only minimally to Con A.

Because the antibody is derived from a cloned cell line, no absorption of the antiserum was necessary, and the specificity and titer of the 3A1 antibody were such that the ascites fluid from a single mouse generated enough T cell-specific antibody for a large number of studies.

Several human T cell subsets have thus far been defined; the subsets may be comparable in number, in morphology, or in

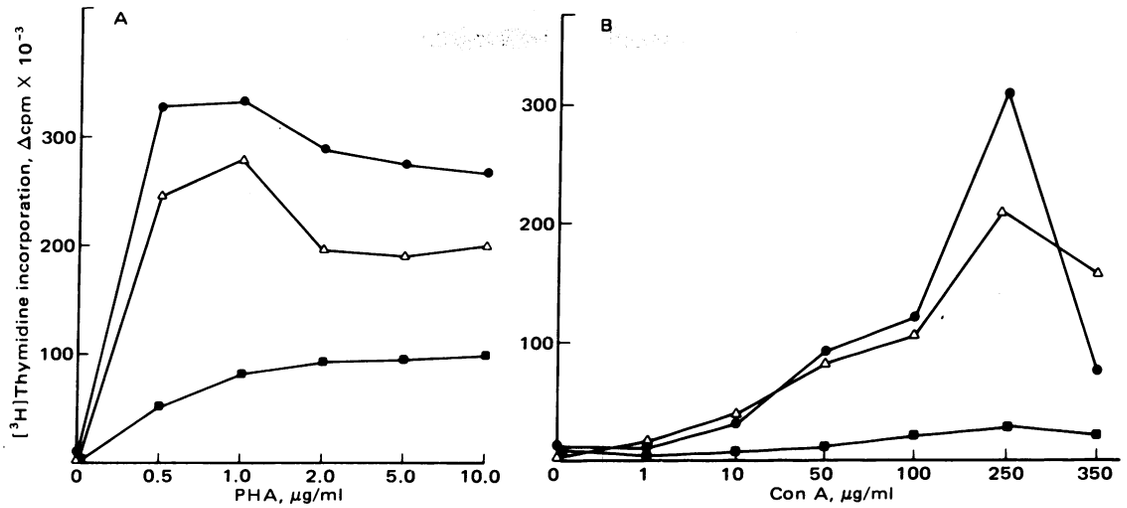


FIG. 5. Tritiated thymidine incorporation of total T cells (●), 3A1-positive (△), and 3A1-negative (■) T cell subsets in response to stimulation by PHA (*A*) and Con A (*B*). Total T cells were obtained by E rosette density centrifugation. 3A1-positive T cells were obtained by SPA-coated BRBC rosette formation with 3A1-coated T cells that were pelleted through a Hypaque/Ficoll density gradient. 3A1-negative T cells remained at the Ficoll/medium interface of the Hypaque/Ficoll gradient. These experiments were repeated on PB T cells from three different individuals with the same results each time.

the pattern of tritiated thymidine incorporation in response to PHA and Con A stimulation. The ratio of 70% 3A1-positive to 30% 3A1-negative T cells in the normal total PB T cell population in the present studies is similar in number to the 60–70% $TH_2$-negative to 20–30% $TH_2$-positive T cell subset ratio described by Evans *et al.* (7). The $T_G$-depleted subset of PB T cells (25, 31) that responds to PHA and Con A is composed primarily of small lymphocytes bearing Fc receptors for IgM ($T_M$ cells) (32). In our study the $T_G$-depleted subset contains approximately 75% 3A1-positive T cells. The $T_G$ human PB T cell subset (25), which makes up 15–20% of total T cells, responds submaximally to PHA and Con A (31) and is made up primarily of medium to large lymphocytes (32). The $T_G$ T cell subset appears from our studies to be made up of approximately 70% of the 3A1-negative T cells.

Thus, using the hybrid technique of Kohler and Milstein (12), and with screening assays using autologous T and B cell lines, we have produced the 3A1 antibody, which defines a major functional human T cell subset and which should be a powerful tool in the study of normal and aberrant human immunophysiology.


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