Null Cell Identification and Characterization With OKT16: An Anti-p40 Monoclonal Antibody

By Mary Anne Talle, Patricia Rao, Mary Makowski, Chris Boselli, Nancy Allegar, and Gideon Goldstein

A murine monoclonal antibody, OKT16, specific for human lymphocytes of T lineage, was isolated by standard immunization and hybridization techniques. The distribution of the antigen defined by OKT16 was similar to the antigen reactive with monoclonal antibodies 3A1 and WT1. This identity of antigen targets was confirmed in an enzyme-linked immunosorbent assay system and by sequential immunoprecipitation. Under reducing conditions, OKT16 reacted with an antigen of 40K daltons; however, under nonreducing conditions this antigen appeared as an 84K-dalton molecule, which suggests that the p40 antigen exists as a disulfide-linked dimer. By indirect immunofluorescence, OKT16 reacted with a greater fraction of nonrosetting, non-B (null) lymphocytes than with antibodies to other T cell-specific proteins. Two-color immunofluorescence demonstrated the coexpression of the T16 antigen and the C3bi receptor on most null cells. The T10 antigen (found on cortical thymocytes and activated peripheral T cells) was restricted to most T16-bearing null cells and expression of the Fc receptor for aggregated IgG (defined by monoclonal antibody 73.1) was restricted to a major subset of T16-bearing null cells. The T cell-specific markers defined by OKT8, OKT11, and OKT17, as well as the monocyte marker defined by OKM5, were expressed by smaller subsets of OKT16-reactive null cells. These studies support by phenotypic analysis the functional heterogeneity ascribed to null cells. The 40K-dalton T16 antigen has the most extensive null cell representation of all the T lineage markers described to date.

NUMEROUS MONOCLONAL ANTIBODIES defining distinct T lymphocyte-lineage restricted proteins have been described within the past few years.7,8 Several of the proteins identified are present on all or most circulating peripheral blood T cells able to form rosettes with sheep red blood cells (SRBCs). Included among these are the 20K-dalton T3 and 67K-dalton T1 antigens expressed by approximately 60% of thymocytes and a fraction of acute lymphoblastic leukemias of T cell origin (T-ALL). They are regarded as mature T cell markers. The 50K-dalton T11 molecule is expressed by all thymocytes and therefore represents an earlier T lineage marker than T1 or T3. It is the receptor for SRBC.5 An early T lineage marker, T6 (45K, 12K daltons), is expressed by cortical thymocytes but is absent from circulating peripheral T cells.4 An additional antigen, defined by the monoclonal antibody OKT17, is expressed by all thymocytes and circulating T cells but is lost from a subset of peripheral T cells after activation with pokeweed mitogen. Loss of the T17 antigen from that subset defines a population of T cells able to provide radiosensitive help for Ig production by B cells.5 The T17 antigen is thus an early T cell marker that may be lost in certain conditions of differentiation.

Each of these T lineage markers is expressed to varying degrees by the minor population of peripheral blood lymphocytes unable to form SRBC rosettes and devoid of B cell phenotype. These “null” cells do not express the mature T cell antigens T1 and T3 or the immature T lineage marker T6. T11 and T17 are each expressed weakly by a subset of null cells. Identification by monoclonal antibody OKT16 of a T cell antigen highly expressed by most, if not all, nonrosetting, non-B peripheral blood lymphocytes enabled us to examine this functionally heterogeneous population carefully for coexpression of T and myeloid lineage markers.

MATERIALS AND METHODS

OKT16 production. The hybridoma that produces OKT16 was derived from a fusion of P3x63Ag8U1 myeloma cells and spleen cells from a CAF, mouse immunized intraperitoneally with human T lymphocytes activated with concanavalin A (Con A) (Vector, Burlingame, Calif). Cells were injected at two-week intervals as previously described.7 Selection of the OKT16-producing hybrid was based on reactivity of culture supernatants with T cells and lack of reactivity with non-T cells. OKT16 was purified from ascitic fluid by affinity chromatography with protein A-Sepharose 4B (Pharmacia, Piscataway, NJ) according to the method of Ey et al.8 The antibody subclass of OKT16 was determined to be IgG2, by indirect immunofluorescence with isotype-specific sera (Meloy Laboratories, Springfield, VA).

Antibodies. The pan T cell-reactive antibodies OKT1 (α-p7), OKT3 (α-p19), OKT11A (α-p45), and OKT17,2 as well as the thymocyte-reactive antibodies OKT6 (α-p49,12) and OKT10 (α-p46,12) and T cell-subset reactive antibody OKT8 (α-p32), have been described.1,5 OKM5 (α-p88) identifies a monocyte surface protein9 and OKM10 recognizes the binding site of the C3bi (CR3) receptor.10 OKB2 recognizes only SmIg+ cells among the nonrosetting lymphocytes10, OKLa2 (α-p29,34) reacts with a monomorphic epitope of the DR molecule.

The 3A1 hybridoma was obtained from the American Type Culture Collection (Bethesda, Md), and an ascites was prepared for use in these studies. WT1, affinity purified by absorption to protein A-Sepharose 4B, was the generous gift of Dr W.J.M. Tax (Sint. Radboudziekenhuis, The Netherlands), and 73.1 was provided by Dr G. Trinchieri (Wistar Institute, Philadelphia).

Isolation of lymphoid cells. Fresh peripheral blood from normal adult volunteers was collected in heparin and spun on Ficoll-Hypaque density gradients (d = 1.077). The interfacial mononuclear cells were depleted of adherent monocytes by incubation on plastic Petri dishes for 45 minutes at 37 °C in RPMI 1640 fortified with 10% heat-inactivated fetal calf serum (GIBCO, Grand Island, NY). Plastic nonadherent mononuclear cells were fractionated into T cells and non-T cells by rosetting with SRBCs as described by Mendes et al.11 More than 95% of the T cells isolated by this method expressed the T11 marker. Nonrosetting lymphocytes were further fractionated into SmIg+ and SmIg- cells by incubation at 4 °C on...
OKT16: NULL CELL CHARACTERIZATION

1125
goat antihuman Ig-coated (Tago, Inc, Burlingame, Calif) plastic Petri dishes according to the method of Wysocki and Sato.12 Less than 10% of the nonadherent cells expressed the T3 antigen and less than 20% expressed SmIg by indirect immunofluorescence. These are referred to, therefore, as null cells.

Single-cell suspensions of thymocytes were prepared from fresh thymus tissue obtained from pediatric patients undergoing cardiac surgery.

Enzyme-linked immunosorbent assay (ELISA). Modification of an ELISA system described by Fuccello et al13 was used to determine whether distinct monoclonal antibodies recognized a common molecule. Flat-bottom wells of a polyvinylchloride plate (Dynatech Labs, Alexandria, Va) were filled with 200 μL of goat antimouse IgG and IgM (Tago) at 5 μg/mL in 0.1 mol/L bicarbonate buffer, pH 9.6. After incubation for 18 hours at 4°C in a humid chamber and subsequent removal of nonadherent antibody, the coated wells were rinsed three times with phosphate-buffered saline (PBS), pH 7.2, containing 0.05% Tween 20 (Sigma, St Louis), and three times with glass-distilled water and then dried at 37 °C for one hour. Wells were filled with the murine monoclonal antibody to be tested (either undiluted culture supernatant, ascites fluid diluted 1:1,000, or purified antibody at 1 μg/mL) for one hour at room temperature in a humid atmosphere and unbound antibody was removed by washing three times with PBS-0.05% Tween 20. Nonidet P-40 (NP40) extract (see below) of sheep erythrocyte rosetting (E+) lymphocytes or HSB-2 (T-ALL) cells containing the T16 antigen was added to the antibody-coated wells and allowed to bind for one hour at room temperature in a humid chamber. Wells were cleared of unbound cellular extract by three washes with PBS-0.05% Tween 20. Mouse immunoglobulin (10 μg/mL) was added to each well for 30 minutes to bind to any free goat antimouse Ig and then removed by the rinsing procedure described earlier. Horseradish peroxidase-conjugated OKT16 prepared as described previously14 was diluted in PBS containing 5% (vol/vol) isotype-matched murine monoclonal antibody ascites of an unrelated specificity and was allowed to bind for one hour. Unbound antibody was removed by three washes with PBS-0.05% Tween 20 and 200 μL of o-phenylenediamine (OPD) substrate was added per well. Color was permitted to develop for 20 minutes at room temperature in the dark, and the reaction was stopped by acidification. Optical density was measured at 492 nm using a Titertek Multiskan plate reader (Flow Laboratories, McLean, Va).

Cell surface iodination and extraction. E+ lymphocytes or HSB-2 cells were suspended in PBS at 106 cells per 200 μL and iodinated with 1 μCi Na125I (New England Nuclear, Boston) using iodogen (Pierce Chemical Co, Rockford, Il). Iodinated cells, freed of unincorporated iodine by three washes of PBS with 5 mmol/L NaI, were adjusted to a density of 2.5 × 107 cells per milliliter, and were then extracted for 15 minutes in ice on 10 minutes in 3% Triton X-100 solution. Cell surface iodination and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cell extracts were cleared of nonspecifically adsorbing material with (a) aggregates of goat a-mouse IgG and mouse IgG (Cooper Biomedical, Inc, Malvern, Pa) (b) fixed Staphylococcus aureus, Cowan I strain (The Enzyme Center, Walden, Mass); and (c) mouse IgG bound to Sepharose 4B beads (Pharmacia, Piscataway, NJ). Purified OKT16 or 3A1 were coupled to CNBr-activated Sepharose 4B beads at 2 μg of antibody per milliliter of packed beads. Antigen was precipitated from up to 200 μL of labeled extracts using 25 μL of packed beads. Nonspecifically adsorbed material was removed as described previously.10 Immunoprecipitates were dissociated by boiling before being loaded onto 12% SDS-polyacrylamide gels as described by Laemmli.11 Reduced samples were dissolved in buffer containing 10% 2-mercaptoethanol and nonreduced samples were dissolved in buffer containing 20 mmol/L iodoacetamide to prevent artificial disulfide associations. Gels were stained, dried, and subjected to autoradiography at −70°C with Kodak XAR film (Eastman Kodak, Rochester, NY) and Cronex lightning plus enhancing screens12 (Dupont, Wilmington, Del).

Immunofluorescence analysis. The percentages of cells expressing a given antigen were assessed by indirect immunofluorescence. In brief, saturating amounts of monoclonal antibodies (ascites diluted 1:100 to 1:1,000) were reacted with 106 cells for 30 minutes at 4°C in medium containing 15% heat-aggregated human plasma. Cells were washed free of unbound antibody and incubated for 30 minutes at 4°C with fluorescein-conjugated goat antimouse IgG (Meloy Laboratories, Springfield, Va) diluted in the same medium. After two additional washes, cells (defined as lymphocytes by light scattering properties)13 were analyzed for fluorescence on a Cytofluorograf FC 200/4800A (Ortho Diagnostic Systems, Westwood, Mass). Cells displaying fluorescence intensity above that of control cells stained with P3X63Ag8 ascites (a myeloma producing light and heavy chains of undetermined specificity) were considered positive for antigen expression.

In some experiments, two-color immunofluorescence was used for analysis of cell surface antigen expression. Null cells (106) were first reacted with predetermined saturating amounts of the appropriate monoclonal antibody. This and each subsequent incubation was for 30 minutes at 4°C in the presence of 15% heat-aggregated human plasma. After two washes to remove unbound antibody, cells were reacted with 100 μL of biotinylated horse antimouse IgG (Vector) diluted 1:20 in RPMI with 25 mmol/L HEPES and 5% fetal calf serum. Excess antiserum was washed from the cells, and the cells were suspended in 100 μL of rhodaminow-avidin D diluted 1:20 (Vector) in PBS. Cells were washed free of unbound avidin, and 50 μg of mouse IgG (Pel-Freeze Biologicals, Rogers, Ark) were added to occupy any unbound sites on the horse antimouse IgG. Finally, the cells were reacted with fluorescein-conjugated monoclonal antibody of the appropriate specificity and dilution. Two-color fluorescence analysis was accomplished on a system 50H Cytofluorograf linked to a 2150 computer system (Ortho).

RESULTS
Isolation and reactivity patterns of the OKT16 monoclonal antibody. OKT16 was isolated on the basis of its reactivity with all SRBC-rosetting (E+) lymphocytes from peripheral blood. Unlike previously described T lymphocyte markers, however, the T16 antigen was detected on a major portion of nonrosetting (E-) lymphocytes. Further fractionation of the E+ population revealed that the T16 antigen was expressed by 74% ± 11% of SmIg-negative or null cells by indirect immunofluorescence (Table 1). Only 40% ± 11% of null cells expressed the T11 (p50; SRBC receptor) antigen, and the density of T11 antigen on these cells as judged by fluorescence intensity was much lower than that expressed by the E+ population (data not shown). Neither the mature T cell markers, T1 (p67) and T3 (p20), nor the immature thymocyte marker, T6 (p45, 12), were expressed by null cells.

Single-cell suspensions of thymocytes showed a broad range of reactivity with OKT16 (35% to 75%) (Table 1).
Peripheral blood B cells, monocytes, granulocytes, and platelets were consistently nonreactive with OKT16 by indirect immunofluorescence (data not shown). Transformed cell lines of T lineage (HSB-2, JM, HPB-ALL, CEM, MOLT-3, JURKAT, MOLT-4, 8402) were reactive with OKT16, whereas transformed cell lines of B (EB3, RAJI, DAUDI, U266), myeloid (HL60), or erythroid (K562) lineage did not bind OKT16.

**Immunoprecipitation of the T16 antigen.** Immunoprecipitation of an extract from 125I-labeled E+ lymphocytes by OKT16 covalently coupled to Sepharose 4B beads, followed by electrophoresis on 12% SDS-polyacrylamide gels, resulted in identification of a 40K-dalton polypeptide in both the presence (Fig 1B, lane 2) and the absence (Fig 1A, lane 1) of 2-mercaptoethanol. Under nonreducing conditions there was precipitated, in addition to the predominant 40K-dalton species, an 84K-dalton molecule. Neither the 40K-dalton nor the 84K-dalton polypeptide was observed if the antibody used for precipitation was normal mouse Ig (Fig 1A, lane 2, and B, lane 1). Reduced precipitates using OKT16 or mouse IgG contained a nonspecifically precipitated molecule of 91K daltons; this species was not present in nonreduced precipitates. The 84K-dalton species precipitated by OKT16 appeared even though 50 mmol/L iodoacetamide was present throughout the cellular extraction procedure and nonreduced samples for SDS-PAGE analysis contained 20 mmol/L iodoacetamide.

**Multiple antibodies detect the T16 (p40) antigen.** Several monoclonal antibodies have been reported that identify a 40K-dalton polypeptide found on peripheral blood T cells. 3A1, a monoclonal antibody originally reported to define a subset of peripheral T cells, precipitates a 40K-dalton polypeptide from several T cell lines, and WT1, a monoclonal antibody that reacts with most prethymic acute lymphoblastic leukemias (ALL), precipitates a 40K-dalton polypeptide from the HSB-2 T-ALL cell line. We developed an ELISA system to determine whether OKT16 recognized the same antigen as 3A1 and WT1. The monoclonal antibody to be tested was bound to a polyvinylchloride microtiter plate that had been precoated with goat antimouse IgG serum, and cellular extract containing antigen was permitted to react with the monoclonal antibody. Free goat antimouse binding sites were filled with excess mouse Ig before the addition of horseradish peroxidase (HRP)-conjugated OKT16.

As shown in Table 2, when either OKT16, 3A1, or WT1 was the monoclonal antibody coating the plate, the levels of HRP–OKT16 bound were significantly higher than those bound by plates coated with either OKT1, OKT3, or OKT11. The surprising finding that the OKT16–T16 antigen complex was able to find HRP–OKT16 suggests that the 40K-dalton polypeptide identified by immunoprecipitation (Fig 1A) is in fact a dimer of the 40K-dalton antigen. The same method of cellular extraction was used in both procedures. The alternative, although less likely, explanation is that the 40K-dalton protein has two identical epitopes.

**Sequential immunoprecipitation with OKT16 and 3A1.** Additional evidence that OKT16 and 3A1 bind to the same molecule was provided by sequential immunoprecipitation. E+ cellular extracts containing antigen were cleared of most nonspecifically absorbing material by immunoprecipitation with mouse IgG coupled to Sepharose 4B beads, and the dissociated immunoprecipitated was subjected to electrophoresis on a 12% SDS-polyacrylamide gel under reducing conditions; detection was by radioautography (Fig 1B, lane 1). A portion of the cleared cellular extract was reacted with
Null cells represent only 10% to 12% of peripheral blood lymphocytes and yet are responsible for a greater percentage of null cells than most other T lineage markers. It was of interest, therefore, to examine highly enriched null cells for coexpression of the T16 antigen with other T cell markers, as well as with non-T cell markers present on but not restricted to null cells.

We first analyzed peripheral blood mononuclear cells from which only the SRBC-rosetting cells had been removed to minimize nonselective loss of E cells. This population was composed of approximately 50% lymphocytes and 50% monocytes by forward-angle v right-angle light-scattering properties and represented about 35% of the total mononuclear cells. Double-marker immunofluorescent analysis of the cells scattering in the lymphocyte region used a three-step staining method to label cells with monoclonal antibody and rhodamine and a single-step method to label cells with fluorescein-conjugated monoclonal antibodies (see Materials and Methods). This lymphocyte population contained less than 5% mature T cells (OKT3 positive) and 65% B cells (OKB2 positive) (Fig 2). OKB2 is expressed by all SmIg+ cells in the peripheral blood. The T16 antigen was expressed by 23% of these E lymphocytes, but more important, less than 2% of the cells shared the T16 and B2 antigens. The sum of lymphocytes bearing the T16 and B2 antigens in this experiment was 88%, and in four additional experiments the sum was greater than 85%. The ratio of T16+B2 cells varied from 1:3 to 2:1. We concluded that the T16 antigen was expressed by the majority of T3 negative,

Table 2. ELISA Assay for Detection of Antibodies

<table>
<thead>
<tr>
<th>MoAb on Plate</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>OKT16</td>
<td>0.244 ± 0.015</td>
<td>0.917 ± 0.144</td>
</tr>
<tr>
<td>OKT1</td>
<td>0.062 ± 0.020</td>
<td>0.195 ± 0.106</td>
</tr>
<tr>
<td>OKT3</td>
<td>0.057 ± 0.001</td>
<td>0.195 ± 0.097</td>
</tr>
<tr>
<td>OKT11</td>
<td>0.062 ± 0.001</td>
<td>0.144 ± 0.028</td>
</tr>
<tr>
<td>3A1</td>
<td>0.544 ± 0.026</td>
<td>1.344 ± 0.098</td>
</tr>
<tr>
<td>WT1</td>
<td>0.129 ± 0.012</td>
<td>0.427 ± 0.105</td>
</tr>
</tbody>
</table>

* Optical density at 492 nm; mean of three wells ± SD.

3A1, and a 40K-dalton (reduced) polypeptide was specifically immunoprecipitated (Fig 1B, lane 5). An identical pattern was obtained if OKT16 was reacted with a separate portion of the extract (Fig 1B, lane 2). After three sequential immunoprecipitations with OKT16, no additional material capable of binding OKT16 or 3A1 remained in the extract (Fig 1B, lanes 3 and 4). This result indicates that OKT16 and 3A1 recognize the same polypeptide.

Characterization of null cells by two-color immunofluorescence and OKT16. Null cells represent only 10% to 15% of peripheral blood lymphocytes and yet are responsible for a variety of functions, including natural killer activity, antibody-dependent cell-mediated cytotoxicity, lectin-dependent cellular cytotoxicity, as well as precursors to other functional cell types. We found the T16 antigen expressed on a far greater percentage of null cells than most other T lineage markers. It was of interest, therefore, to examine highly enriched null cells for coexpression of the T16 antigen with other T cell markers, as well as with non-T cell markers present on but not restricted to null cells.

Fig 2. OKT16 and OKB2 define distinct subsets of E- lymphocytes by two-color immunofluorescence. E- mononuclear cells were stained with (a) monoclonal antibody, biotinylated horse antimouse Ig, and rhodamine-avidin and (b) fluorescein-conjugated monoclonal antibody as described in Materials and Methods. Cells with forward-angle v right-angle light-scattering properties of lymphocytes were analyzed for fluorescence on an Ortho System 50-H Cytofluorograf coupled to a 2150 computer. The percentages of cells stained positive for green but not red fluorescence above an FITC-OKT6 negative control are indicated along the left margin. The percentages of cells stained positive for red but not green fluorescence above a P3x63Ag8 ascites (murine IgG monoclonal antibody of undetermined specificity) negative control are indicated along the bottom margin. Percentages of cells bearing both antigens are reported in the upper right corner.
non-B cells and represented a useful null cell marker. All
cells bearing the B2 antigen coexpressed Ia, whereas Ia was
absent from T16-positive cells (Fig 2).

We exploited the T16 antigen to study coexpression of
several T lineage and non-T lineage markers on highly
enriched null cells. Mononuclear cells were depleted of
monocytes by adherence to plastic. This procedure fre-
quently results in the loss of a significant number of lympho-
cytes, but these adherent lymphocytes accounted for less
than 5% of the total null cells by phenotypic analysis (data
not shown). Lymphocyte-enriched mononuclear cells were
depleted of T cells by rosetting with SRBC. The E+ cells
were 97% OKT3 positive. E+ null cells were separated from
B cells by incubation on antihuman Ig-coated polystyrene
dishes. Nonadherent null cells were collected for double-
marker analyses and less than 10% OKT16-positive null cells
were lost to the adherent B cell population.

As shown in Fig 3, more than 80% of null cells expressed
the T16 antigen and less than 10% expressed the T3 antigen.
B cell contamination was less than 5% in this experiment
(data not shown), and results from three additional experi-
ments indicate that only 10% to 15% of null cells may not
express the T16 antigen. OKM10, an antibody directed
toward the C3bi receptor expressed by monocytes and granu-
locytes,89 reacted with all OKT16-positive null cells. The
T10 antigen, associated with cortical thymocytes and activ-
ted T cells,7 was present on a major subset of T16-positive
null cells, whereas the Fc receptor for aggregated Ig of large
granular lymphocytes defined by antibody 73.123 was
expressed by a smaller subset.

Cells bearing T8, T11, T17, or M5 markers (Table 3) were
restricted to the T16-positive population. The densities of
these antigens on null cells was low (Fig 3), and increased
fluorescence sensitivity might in fact reveal wider distribu-

Fig 3. Surface marker analysis of null cells by two-color
immunofluorescence with OKT16. Null cells were stained
and analyzed as described in Fig 2. The percentages of cells
stained positive for red but not green fluorescence are
indicated along the bottom margin; the percentages of cells
stained positive for green but not red fluorescence are
indicated along the left margin. Percentages of cells bearing
both antigens are reported in the upper right corner.
Null cell analysis with subset markers. The presence of several of these antigens on only a fraction of null cells prompted us to study them by double-marker analysis. As shown in Fig 4, only T8+ and T17+ cells constituted a fully overlapping population. T11 and T8 were expressed by largely nonoverlapping populations, as were T11 and T17. The total number of T8-positive cells was reduced by 50% when costained with OKT17, suggesting a steric inhibition by OKT17 (OKT17 was added to the cells before OKT8). In most experiments the total percentage of T8-positive cells was only a fraction of the T17+ population.

M5, an antigen associated with monocytes, platelets, and some null cells, was coexpressed by only a small percentage of T8-, T11-, or T17-positive cells. The fact that most null
null cell subsets. The results shown are derived from several experiments and are based on populations containing 10% mature T cells and 10% B cells. The precise relationship of 73.1-positive and OKT10-positive cells to cells bearing markers other than Ti6 is unknown; in the figure these populations have been indicated with a hatched bar. Designation of T8-positive cells is by a dashed line to show the donor variation of this population.

**DISCUSSION**

The antigen defined by monoclonal antibody OKT16 is a T lineage marker expressed by most peripheral blood T rosette-positive cells and most null cells (ie, lymphocytes that do not form E rosettes and have neither the mature T cell gut. No staining was observed in heart, muscle, testis, thymocytes. Thymic epithelium did not stain with OKT6. Positive for OKT16, whereas B cells, plasma cells, monocytes, granulocytes, or platelets.

Histologic analysis of a variety of lymphoid and nonlymphoid tissues supports the T lineage restriction of the Ti6 antigen. T lymphocytes in the lymph node and spleen were positive for OKT6, whereas B cells, plasma cells, macrophages, and vascular endothelium were negative. Thymocytes expressed the Ti6 antigen; the antigen density was lower, however, on cortical thymocytes than on medullary thymocytes. Thymic epithelium did not stain with OKT6. Among the nonlymphoid organs studied with OKT6, reactivity was restricted to the T lymphocytes within the skin and gut. No staining was observed in heart, muscle, testis, pancreas, kidney, or brain (G. Janossy, unpublished observations).

Immunoprecipitation of the Ti6 antigen from either peripheral blood T cells or the T-ALL established cell line HSB-2 revealed a 40K-dalton polypeptide under reducing conditions. Two previously described monoclonal antibodies, WT1 and 3A1, bind to a common p40 antigen with a tissue distribution similar to that of the Ti6 antigen. We used an ELISA to show that OKT16 binds to the same molecule as do WT1 and 3A1. Sequential immunoprecipitation with OKT16, followed by 3A1, confirmed this conclusion.

If the antigen immunoprecipitated by OKT16 was analyzed under nonreducing conditions, we detected an 84K-dalton polypeptide in addition to the 40K-dalton protein observed under reducing conditions (Fig 1a). A similar observation was made by Sutherland et al if WT1 was used to precipitate antigen from cell extracts lacking iodoacetamide. However, when these experiments were repeated with a cell extract containing as little as 2 mmol/L iodoacetamide the larger band did not appear, suggesting that the larger protein was an artifactual disulfide-linked dimeric aggregate of the WT1 40K-dalton antigen. The Ti6 antigen was precipitated from extracts containing 50 mmol/L iodoacetamide and was loaded onto polyacrylamide gels under nonreducing conditions in the presence of iodoacetamide, yet the 44K-dalton protein was consistently observed. Recently, a monoclonal antibody termed LAU-A1, described by Carrel et al, was shown to compete for binding with 3A1. Both LAU-A1 and 3A1 precipitated antigens of 74K daltons under nonreducing conditions and 40K daltons under reducing conditions. It is possible, therefore, that the p40 antigen exists in the membrane as both a monomer and a dimer and that OKT6 recognizes both configurations, whereas WT1 recognizes primarily the monomeric form.

Cell lysates containing 50 mmol/L iodoacetamide were also the source of Ti6 antigen for the ELISA assay, in which HRP-OKT16 was able to bind to antigen that had been bound by either 3A1, WT1, or OKT6. If the antigen in the lysate possessed a single OKT16-reactive epitope, then no binding of HRP-OKT16 should have occurred when OKT16 was the antibody coating the well due to competition for the epitope. Because HRP-OKT16/OKT6 produced a positive ELISA reaction, the Ti6 antigen probably exists as a dimer in cell extracts. Our extracts contained more iodoacetamide than previously found necessary to prevent disulfide aggregation but we cannot exclude the possibility of an experimentally induced association of the Ti6 monomer. The ELISA may provide evidence for a difference in the antigenic specificity of WT1 and OKT6. When WT1 coated the well, the amount of HRP-OKT6 bound was less than that observed with coatings of 3A1 or OKT6. Because HRP-OKT6 reacted with antigen bound by OKT6, at least some of the antigen must have been present as a dimer, providing two Ti6 epitopes per molecule. A similar result is obtained if OKT9, an antibody directed to the homodimeric transferrin receptor, is both the antibody coating the plate and the antibody conjugated to HRP. WT1 was able to compete weakly with FITC-OKT16 on intact cells (data not shown).

Fig 5. Relationships of various phenotypic cell sets within the null cell population. Residual mature T cells (OKT3') and B cells (OKB2') each constitute 10% of this population. M10 and T10 density on mature B cells is low (. . . .), T8 representation varies widely between different donors (-----), and the precise relationship of 73.1 and T10 representation to cells bearing antigens other than T16 is unknown (----).
and previous studies have shown that antibodies that are able to compete partially for antigen will exhibit a weak ELISA if the antibodies recognize only a single epitope per molecule. Fully competing antibodies result in a negative ELISA. Our extracts clearly contained forms of the antigen capable of binding two OKT16 antibodies. A plausible explanation for the weak ELISA reactivity of WT1 would be that it binds only the monomeric form of the antigen (Sutherland et al.), and because OKT16 and WT1 compete for antigen binding, a weakly positive ELISA would result on monomeric antigens. Alternatively, a weak ELISA could result if the binding affinity of WT1 for the antigen were reduced by the conditions of the assay, or if the dimeric antigen contained only one WT1 epitope formed by the folding of the two chains.

Expression of the T16 antigen by a greater percentage of null cells than any other T lineage marker described to date prompted us to use this marker to analyze the null cell population for constituting a variety of monoclonal antibodies. Peripheral blood mononuclear cells depleted only of E rosette-forming cells were studied by two-color immunofluorescence. This demonstrated the absence of the p40 antigen on B cells and its presence on most T3+, non-B lymphocytes under conditions in which nonselective cell losses were minimized (Fig 2). In fact, the summation of OKB2+ (or OKIa2+) cells and OKT16+ cells within the nonrosetting lymphocyte population always accounted for more than 80%; the ratio of OKB2+ cells to OKT16+ cells, however, varied considerably among individuals. Cells bearing the DR antigen, as defined by OKIa2, were almost completely restricted to B cells.

If B cells and adherent monocytes were removed from the nonrosetting population, the residual population of null cells was 80% to 90% reactive with OKT16. Two-color immunofluorescence analysis with OKT16 revealed that all OKT16-reactive null cells bear the C3bi receptor as defined by OKM10. The majority of OKT16-positive null cells also bear the T10 antigen, a marker shared by cortical thymocytes and activated (but not unstimulated) mature T cells. Null cells expressing the Fc receptor for aggregated Ig, identified by monoclonal antibody 73.1, were exclusively within the OKT16-positive population and represented about 80% of that population. Other T lineage markers identified by OKT11, OKT8, and OKT17 were detected on smaller subsets of OKT16-positive null cells, as was the myeloid lineage marker defined by OKM5.

Most null cells bearing T11 did not coexpress the T8, T17, or M5 antigens, and most OKM5-positive null cells did not express T8 or T17, but none of these populations was nonoverlapping (Fig 4). All OKT8+ cells, however, were contained within the OKT17+ population. The phenotypic diversity demonstrated by these monoclonal antibodies directed toward antigens usually associated with either T lymphocyte or monocyte lineage cells is in accordance with the functional heterogeneity within this population. Whereas all NK activity resides within the 73.1+ population, the function of the T16+ T3+ 73.1+ cells remains to be established. Perhaps these surface markers will permit more precise definition of cells capable of lectin-dependent cellular cytotoxicity.

Approximately 70% to 80% of the T3+, T16+, non-B cells that display lymphocyte light-scattering characteristics express the Fc receptor for aggregated Ig. Abo et al. have shown that 98% of cells bearing this receptor, as defined by Leu-11, are large granular lymphocytes and possess all the natural killer activity of peripheral blood mononuclear cells. Similarly, Perussia and Trinchieri have demonstrated that all natural killer, as well as killer cell, activity related to antibody-dependent cell-mediated cytotoxicity is restricted to cells reactive with 73.1 and possessing large granular lymphocyte morphology. It is reasonable to conclude, therefore, that large granular lymphocytes represent a large fraction of the OKT16+ null cells defined by lack of reactivity with OKT3 and OKB2.

Large granular lymphocytes bearing receptors for the Fc region of Ig have been reported to lack MHC-class II (DR, DS) molecules and to express the 32K-dalton molecule recognized by OKT8 at a lower density than OKT3+ mature T cells. We have obtained similar results with the broader null cell population. However, it is possible that null cells express la under certain activation conditions. Previous reports concluded that only some Fc receptor-positive cells or natural killer cells bear the 33K or 32K-dalton receptors detected by the monoclonal antibody OKM1. Using an antibody directed toward the C3bi receptor binding site (OKM10), we observe that all OKT16+ cells bear this complement receptor. The epitope of the C3bi receptor reactive with OKM1, however, is either masked or missing on some Fc receptor-positive cells.

The 40K-dalton molecule recognized by OKT16 has been shown by several investigators to be an important diagnostic marker for leukemia classification. A study by Haynes et al demonstrated that 3A1 was able to distinguish peripheral blood T cells in cutaneous T cell lymphoma (3A1+) from those in acute lymphoblastic leukemia (3A1-). Vodinelich et al. reported that WT1 was reactive with 100% of 80 cases of thymic acute lymphoblastic leukemia (ALL) and 18 of 24 cases of presumptive prethymic ALL. Six other T lineage markers were generally not expressed by these 24 cases of presumptive T-ALL. Similarly, Link et al. found 4H9, also directed toward the p40 antigen, to be superior to other antibodies specific for T lineage markers in identifying acute leukemias and non-Hodgkin's lymphomas of T cell origin.

Vodinelich et al. have suggested a therapeutic potential in bone marrow transplantation for antibodies reactive with the p40 antigen. They reported the p40 antigen (as detected by WT1) to be absent from BFU-E, CFU-GM, and multipotential progenitors in the bone marrow. Similar results were obtained using OKT16 (L. Filipovich, unpublished observations). OKT16 is, therefore, a potential candidate for removing normal or malignant T cells from donor bone marrow before bone marrow transplantation.

ACKNOWLEDGMENT

We are grateful to Nancy Lawery for typing the manuscript.
REFERENCES
11. Mendes NF, Tolnai MEA, Silveira NPA, Gilbertsen RB, Metzgar RS: Technical aspects of the rosette tests used to detect human complement receptor (B) and sheep erythrocyte-binding (T) lymphocytes. J Immunol 111:860, 1973
27. Bradley TP, Bonavida B: Natural killer cells are distinct from lectin-dependent effector cells in man as determined by the two-target conjugate single cell assay, in Herberman R (ed): NK Cells and Other Natural Effector Cells. Orlando, Fla, Academic Press, 1982, p 145

From www.bloodjournal.org by guest on April 18, 2017. For personal use only.
Null cell identification and characterization with OKT16: an anti-p40 monoclonal antibody

MA Talle, P Rao, M Makowski, C Boselli, N Allegar and G Goldstein