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IDENTIFICATION AND QUANTITATION OF THYMUS-DERIVED LYMPHOCYTES IN HUMAN PERIPHERAL BLOOD[1]

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A highly specific anti-T cell antiserum (ATCS) was developed by extensively absorbing a goat anti-human thymocyte antiserum with human red cells and buffy coat cells from a patient with chronic lymphocytic leukemia. The ATCS was used in an in vitro cytotoxicity assay for detecting human thymus-derived (T) peripheral blood lymphocytes. A fluorescent cell sorter provided separated populations of normal immunoglobulin (Ig)-bearing and non-Ig-bearing lymphocytes on which to directly test the sensitivity and specificity of the ATCS. The antiserum killed almost 100% of non-Ig-bearing lymphocytes but less than 10% of Ig-bearing (B) lymphocytes and monocytes. Moreover, ATCS was able to abrogate the response to phytohemagglutinin stimulation, a T cell response.

In further studies, Ficoll-purified peripheral blood cells from 12 normal donors were examined for the per cent Ig-bearing cells, monocytes and cells killed by ATCS. A mean of 21% of these cells were B lymphocytes, 17% were monocytes, and 65% were T lymphocytes, thus accounting for 100% of the cells.

The lymphocyte population in laboratory animals has been shown to be functionally and developmentally heterogeneous (1). Lymphocytes which are active in humoral immunity and which give rise to the antibody-forming cells are bursa-equivalent (B)[4] lymphocytes (2, 3). These cells are derived from the bone marrow and develop independent of the thymus (4). B lymphocytes are characterized by distinct surface markers, not present on the remaining lymphocyte population. Some of the more extensively studied markers include cell surface immunoglobulins (Ig) of high density (4, 5), and receptors for complement (6) and antigen-antibody complexes (7). Lymphocytes which participate in cell-mediated immunity, and those which cooperate with B lymphocytes to augment the formation of antibody to certain antigens, are thymus-dependent (T) lymphocytes (8, 9). These cells are also derived from bone marrow progeny, but, in addition, are processed by the thymus. In laboratory animals, thymus-dependent lymphocytes share surface antigens with thymus cells (i.e., θ). These antigens are not discernible on B lymphocytes or on bone marrow cells (10).

Detection of distinctive surface markers offers a way of identifying T and B lymphocytes in man. Human B lymphocytes have been identified by immunofluorescent staining of surface Ig (11, 12), and by the binding of complement *in vitro* (13). Human T lymphocytes have been identified by spontaneous rosette formation with sheep red blood cells (14–16) and more recently by the use of heterologous anti-T cell antisera (17–19). Considerable differences in the

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[4] Abbreviations used in this paper: B, bursa-equivalent; Ig, immunoglobulin; T, thymus dependent; PBS, phosphate-buffered saline; DPBS-FCS, Dulbecco's phosphate-buffered saline with 5% fetal calf serum; M199-FCS, culture medium 199 with 5% fetal calf serum; ATCS, anti-T cell antiserum; CLL, chronic lymphocytic leukemia; ATS, anti-thymocyte antiserum; NGS, normal goat serum; PHA, phytohemagglutinin-P.

reported per cent of T lymphocytes in normal peripheral blood (14–19) may reflect variability in the sensitivity and specificity of these assays.

The present paper describes an *in vitro* cytotoxicity assay using a heterologous antiserum for the detection of human T cells. Direct tests of the sensitivity and specificity of this assay were made on purified populations of normal Ig- and non-Ig-bearing peripheral blood lymphocytes obtained from a fluorescent cell sorter. The experimental findings indicate that the antiserum reacts with almost all (>95%) thymocytes and non-Ig-bearing peripheral blood lymphocytes, but reacts with few (<10%) Ig-bearing cells.

MATERIALS AND METHODS

Purification of peripheral blood lymphocytes. Lymphocytes were separated from human peripheral blood on a Ficoll-Hypaque gradient (20). Fifteen parts of a 25% solution of Hypaque-M 75% (Winthrop Laboratories, New York, N. Y.) were added to 24 parts of a 9% Ficoll solution (Pharmacia Fine Chemicals, Uppsala, Sweden) and stirred for 1 hr at room temperature. Ten milliliters of the mixture were placed in a 40-ml conical centrifuge tube and carefully overlaid with 20 ml of fresh heparinized whole blood diluted 1:1 with phosphate-buffered saline (PBS), pH 7.2. Centrifugation was carried out at 20°C for 35 min at 400 × G. Cells were aspirated from the lymphocyte-rich middle layer, and washed once at 4°C with Dulbecco's phosphate-buffered saline (DPBS). After two further washes with DPBS containing 5% fetal calf serum (FCS), heat inactivated at 56°C for 30 min, the cells were suspended in either culture medium 199 with 5% FCS (M199-FCS) at 5×10^6 cells/ml, or in DPBS-FCS at 2×10^6 cells/ml.

Contamination of Ficoll-purified lymphocytes with monocytes. The monocyte contamination of Ficoll-purified cell suspensions was determined by staining purified cells from normal individuals with α-naphthol acetate according to the method of Yam *et al.* (21). α-Naphthol acetate stains nonspecific esterase present in the cytoplasm of monocytes, but does not stain lymphoid or myeloid cells (21). The mean per cent of monocytes present in cell suspensions from 12 donors was 17% with a range of 8 to 32%. Cells identified morphologically as small lymphocytes were never stained. The large majority (~ 90%) of cells greater than 10 μ in diameter stained for nonspecific esterase activity. Approximately 1 to 2% of Ficoll-purified cells were identified as granulocytes by morphologic criteria.

Preparation of thymus cells for cytotoxicity assay. Portions of normal thymus glands, surgically excised from pediatric patients, were carefully minced with scissors, teased, and passed through wire gauze by a modification of the technique of Billingham (22). The cells were washed three times in DPBS-FCS and then resuspended in M199-FCS at 5×10^6 cells/ml.

Preparation of bone marrow cells. Bone marrow was aspirated aseptically into a heparinized syringe, and transferred to 40-ml conical centrifuge tubes. Particles were broken up by repeated aspirations with a Pasteur pipette. The marrow was mixed with 5% dextran (m.w., 250,000, National Biochemicals Corporation, Cleveland, Ohio) in PBS in a ratio of 4:1 and allowed to sediment by gravity for 30 min at 37°C. The marrow cell rich upper layer was aspirated, then spun at 400 × G for 5 min and the supernatant discarded. The cells were washed three times in DPBS-FCS then resuspended in M199-FCS at 5×10^6 cells/ml.

Staining of Ig-bearing lymphocytes. Rabbit-anti-human Cohn II antiserum (Antibodies, Inc., Davis, Calif.) was conjugated with fluorescein at a fluorescein-protein ratio of 2.8 by the method of McDevitt and Coons (23). One to 2×10^6 Ficoll-purified peripheral blood cells were incubated in 100 μl of a 2 mg/ml solution of the conjugate in DPBS-FCS for 15 min at 5°C. Cells were washed by layering the incubation mixture on 2 ml FCS in a 10- x 75-mm culture tube, and spinning at 300 × G for 5 min. The pellet was resuspended in a drop of DPBS and smeared onto a glass slide. After drying in air, the smears were fixed in 95% ethanol for 20 min. Slides were immersed in PBS, and air dried prior to mounting with 1:9 PBS and glycerine.

Cells were examined at ×1500 magnification using a Zeiss microscope with a tungsten light source and an FITC excitation filter. Each field was scored for the number of fluorescent small lymphocytes (6 to 10 μ), and for the total number of small lymphocytes observed by phase-contrast microscopy. The per cent of Ig-bearing cells was determined after counting

200 cells. Only small cells were counted, since these cells are free of monocyte contamination as judged by histochemical staining. Approximately equal percentages of cells were stained when the concentration of the fluorescein conjugate was varied from 0.5 mg/ml to 4 mg/ml.

The purity of the rabbit anti-Cohn II preparation was tested by immunoelectrophoresis. A single precipitin arc with IgG was observed when the reagent was run against whole human serum. The specificity of the fluorescein conjugate was investigated by passing the material through a column containing human γ-globulin (Cohn II, Hyland, Inc., Costa Mesa, Calif.) covalently bound to Sepharose by a modification of the method of Cuatrecasus *et al.* (24). Immunofluorescent staining of Ficoll-purified cells with the starting material and the effluent at 2 mg/ml showed that the fluorescein conjugate lost all staining activity after passage through the column.

Preparation of anti-T cell serum (ATCS). Thymus cells were obtained from portions of thymus glands removed at the time of surgery to facilitate repair of congenital heart defects of 1- to 6-year-old children. Approximately 5×10^9 fresh thymocytes were emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and injected subcutaneously into an adult male goat at eight different sites. Subcutaneous booster injections with an equal number of cells in saline were administered 14 and 21 days later. Blood was collected in sterile heparinized plastic bags (Fenwall Laboratories, Morton Grove, Ill.) 7 days after the last booster injection. Plasma was separated from the heparinized blood by centrifugation at 700 × G for 15 min and stored at −40°C.

Aliquots of plasma were inactivated at 56°C for 30 min and absorbed six times at room temperature with equal volumes of packed red blood cells from normal donors. Red blood cells were washed three times in DPBS with 5 units/ml heparin prior to use. Each absorption was allowed to proceed for 20 min. A series of similar absorptions were carried out with buffy coat cells from a patient with chronic lymphocytic leukemia (CLL). The patient's white blood cell count was $64 \times 10^3/mm^3$. Approximately 88% of the lymphocytes were Ig-bearing cells. Four milliliters of antithymocyte antiserum (ATS) were absorbed with a total of 28×10^9 leukemic lymphocytes.

In vitro cytotoxicity assay. Duplicate assays were performed in 4- x 50-mm round bottom glass tubes. All incubations were carried out at room temperature. M199-FCS was used for dilutions. Twenty-five microliters of a suspension of target cells (5×10^6 cells/ml) were added to 50 μl of ATCS or normal goat serum (NGS) diluted 1:4. Cells were allowed to incubate for 15 min, after which 50 μl of fresh frozen guinea pig complement diluted 1:4 were added. The reaction mixture was allowed to incubate at room temperature for an additional 45 min. Cells were harvested by centrifugation at 250 × G for 5 min and resuspended in 50 μl of M199. Fifty microliters of a 0.4% trypan blue solution in M199 were added immediately before counting.

Cells were counted in a standard hemocytometer. The per cent of cells killed (cytotoxic index) was calculated using the following ratio.

$$\text{Cytotoxic index (\%)} = \frac{\text{\% experimental cells killed} - \text{\% control cells killed}}{100 - \text{\% control cells killed}}$$

Approximately 300 to 400 viable cells were counted from each NGS control.

Cell analyzer and separator. A fluorescent cell sorter built in the Stanford University Genetics Department (25, 26) was used to separate Ig-bearing from non-Ig-bearing Ficoll-purified peripheral blood lymphocytes. Twenty to 30×10^6 Ficoll-purified cells were incubated with fluorescein-conjugated rabbit-anti-human Cohn II antiserum as described above. The incubation mixture was passaged through two consecutive FCS washes, washed once in DPBS-FCS, and then applied in DPBS-FCS to the cell separator, which was kept at 0°C. Cells applied to the machine are incorporated into a stream of buffer (DPBS) illuminated by two laser beams. Cells pass sequentially through the beams, which almost simultaneously permit detection of cell volume and degree of fluorescence. Scattered and fluorescent light are each converted to electrical signals by two photo detectors. The stream is broken into 40,000 uniform size drops downstream of the lasers. Signals arising from individual cells are used to give appropriate electrostatic charges to drops containing the cells. Drops can then be deflected to appropriate containers as they pass between two charged plates. The system permits the collection of large numbers of viable cells highly enriched for

particular fluorescent and volume types. Ig-bearing (highly fluorescent) and non-Ig-bearing (low or non-fluorescent) cells were collected in the left and right collection tubes containing 0.5 ml of FCS and maintained at 0°C.

Cell cultures. 2.5 × 10^5 Ficoll-purified lymphocytes were cultured in 12- x 75-mm Falcon (Oxnard, Calif.) tissue culture tubes in a volume of 1 ml of M199 supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 20% FCS. Phytohemagglutinin-P (PHA) (Difco Laboratories, Detroit, Mich.) was added to appropriate cultures at a concentration of 2.5 μl/ml. The tubes were incubated in a humidified atmosphere of 5% CO_2, 95% air at 37°C for 72 hr. One microcurie of tritiated thymidine (^{3}H-TdR, 2 Ci/mM, New England Nuclear, Boston, Mass.) was added for the last 16 hr of culture. The cells were then washed three times with cold PBS and precipitated with cold trichloroacetic acid (10%). The precipitate was washed once in cold trichloroacetic acid (5%) and dissolved in 0.5 ml NCS tissue solubilizer (Nuclear Chicago Corp., Chicago, Ill.). After addition of 12 ml of PPO-POPOP in Toluene, the radioactivity of each tube was detected in a Beckman LS-230, liquid scintillation spectrometer (27). The arithmetic mean and standard deviation were determined and the results expressed as the difference in counts per minute between experimental and control cultures (Δcpm). Cells preincubated with ATCS or NGS were washed two times in culture medium and then reconstituted to the original concentration in 1 ml of complete culture medium.

RESULTS

Effect of ATS and ATCS on lymphoid target cells. In order to determine whether absorption of ATS with CLL cells appreciably altered its cytotoxic activity, the effects of absorbed (ATCS) and unabsorbed (ATS) antisera were compared on various target cells. ATS killed more than 90% of Ficoll-purified peripheral blood cells (lymphocytes and monocytes), in the presence of complement, up to a dilution of 1:90 (Fig. 1). In contrast approximately 75% of blood cells from the same donor were killed by ATCS up to a dilution of 1:10. A sharp decline in cytotoxicity was observed thereafter. When thymocytes were used, ATCS killed 90 to 100% of the target cells (Fig. 1).

The effect of the antisera on bone marrow cells was also examined. Figure 2 shows that ATS killed 80 to 90% of normal bone marrow cells. ATCS, in contrast, killed a maximum of 10 to 20% of marrow cells from the same donor. Approximately 17% of the cells in the marrow preparation were identified morphologically as small lymphocytes.

Effect of ATCS on CLL cells. The majority of circulating lymphocytes in patients with chronic lymphocytic leukemia are believed to be B lymphocytes (28). A sizeable proportion of these cells bear surface immunoglobulin (28) and many have receptors for complement (13,

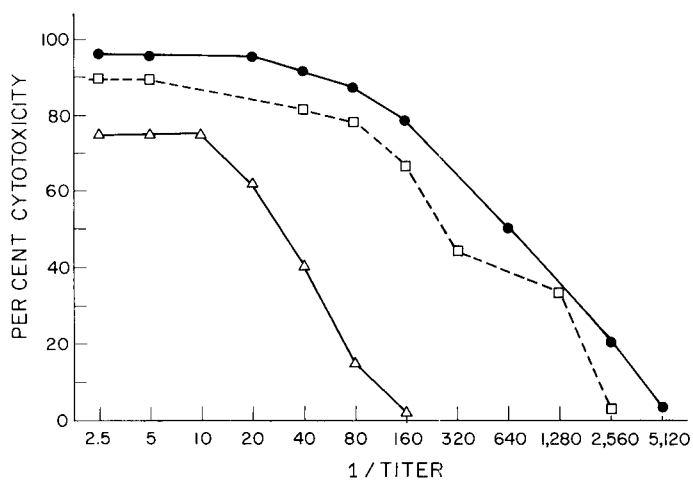

Figure 1. Cytotoxic action of anti-thymocyte (ATS) and anti-T cell (ATCS) antisera on human thymocytes and peripheral blood cells. ●——●, ATS reacted with Ficoll-purified peripheral blood cells; □-----□, ATCS reacted with thymocytes; Δ——Δ, ATCS reacted with Ficoll-purified peripheral blood cells.

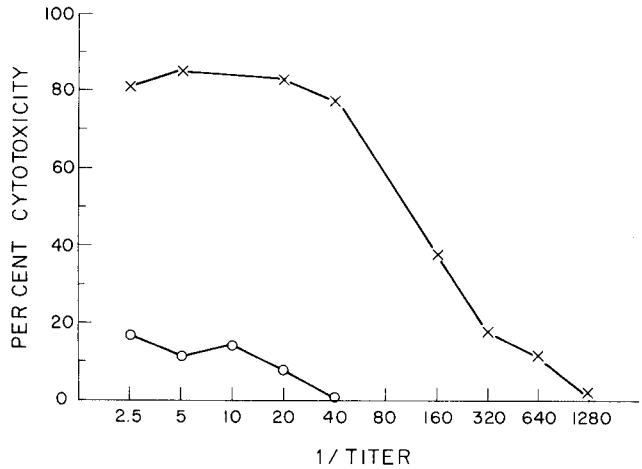

Figure 2. Cytotoxic action of ATS and ATCS on human bone marrow cells. ×——×, ATS reacted with bone marrow cells; O——O, ATCS run against the same cells.

29). The effect of ATCS on such cells was, therefore, examined. Cell suspensions from three donors, none of whose cells were used to absorb ATS, were prepared by the Ficoll-Hypaque technique. Table I shows the results from the three experiments: although, ATS killed 100% of these cells, ATCS killed no more than 10 to 14% of CLL cells.

Sensitivity and specificity of ATCS. The cytotoxic activity of ATCS on target cells fractionated by the cell separator was studied in several experiments. Ficoll-purified peripheral blood cells from four normal donors were incubated with fluorescein-conjugated anti-human Cohn II antiserum, washed, and then applied to the separator. Two cell fractions were collected; one programmed to contain the brightest 15 to 20% of cells passing through the sorter, and the other programmed to contain the dimmest 50 to 60%. Table II, part A, lists the per cent cytotoxicity, per cent Ig-bearing lymphocytes, and per cent monocytes in the Ficoll-purified cell suspensions prior to separation. The post-separation data for non-Ig-bearing and Ig-bearing lymphocyte fractions are listed in Table II, parts B and C, respectively. Table II, part B, shows that ATCS killed 97 to 99% of the dimly fluorescent fraction. This fraction was highly enriched for non-Ig-bearing lymphocytes as judged by fluorescence microscopy.

Table II, part C, shows that the highly fluorescent fraction contained between 84 to 95% Ig-bearing cells as judged by fluorescence microscopy. ATCS killed from 2 to 25 per cent of cells from this fraction (containing lymphocytes and monocytes). The per cent cytotoxicity was inversely proportional to the degree of enrichment for Ig-bearing cells, and was never more than 10% greater than the per cent of non-Ig-bearing cells present in the fraction. In contrast to ATCS, ATS killed a high percentage of the Ig-bearing cells. This removed the possibility that these cells were protected from the cytotoxic effect of the ATCS because of prior fluorescent staining.

TABLE I

Effect of ATCS on peripheral blood lymphocytes from patients with chronic lymphocytic leukemia

Donor	White Blood Cell	% Ig-Bearing Cells	% Cytotoxicity
	counts/mm³		
Ter	34,000	90	13
Mil	42,000	60	14
Bow	22,000	88	10

Range of values in normal donors. The per cent T cells, B cells, and monocytes in 12 normal donors was studied. As shown in Table III, ATCS killed a mean of 65% of Ficoll-purified cells with a range of 56 to 75%. The average monocyte contamination as judged by α-naphthol acetate staining was 17%. The per cent Ig-bearing (B) lymphocytes varied between 10 to 30% with a mean of 22%. Only cells approximately 6 to 10 μ in diameter were considered for counting during fluorescence microscopy, to diminish the inclusion of larger, nonspecifically

TABLE II

Effect of ATCS on Ig- and non-Ig-bearing Ficoll-purified blood cells fractionated by the cell separator

% Cytotoxicity	% Ig-Bearing Cells	% Monocytes
A. Pre-separation		
57	22	15
70	24	19
60	18	9
56	30	32
B. Post-separation—Dim Fraction		
99	0.05	0.05
97	2.5	5
98	0.5	0
97	0.5	1.5
C. Post-separation—Bright Fraction[a]		
ATCS / ATS[b]		
2 / 98	95	34
15 / 84	90	30
19 / 92	88	17
25 / 87	84	24

[a] Cells stained with fluorescent anti-immunoglobulin.

[b] Percentage of cytotoxicity with crude antithymocyte serum absorbed with RBCS only.

fluorescent monocytes (Bobrove, A. M. and Strober, S., unpublished observations).

PHA response after ATCS. PHA responsiveness is considered to be a function of thymus-dependent lymphocytes (30). The effect of ATCS on the *in vitro* response of Ficoll-purified blood cells to PHA stimulation was studied to determine if ATCS would alter this response. Figure 3 shows the ^{3}H-TdR incorporation of stimulated *vs* control cultures expressed as Δcpm. The cpm of control cultures were less than 0.4×10^3 (not shown). The response of normal lymphocytes was completely abrogated by prior treatment with ATCS plus complement, whereas no significant reduction in the PHA response was noted after ATCS treatment alone.

Figure 4 shows the Δcpm of PHA stimulated cultures pretreated with ATCS at various dilutions, plus complement. At each dilution, the per cent cytotoxicity was determined and the cultures reconstituted to the original cell number. The response to PHA stimulation was inversely proportional to the per cent cytotoxicity, or directly proportional to the per cent of viable T lymphocytes. Thus, cells capable of responding to PHA and left viable after pretreatment with ATCS plus complement retain their ability to respond to PHA.

DISCUSSION

Quantitation of human peripheral blood T lymphocytes by previously reported assays (14–19) has shown a great deal of variability, thus posing questions of specificity and sensitivity. The present study overcomes this uncertainty by directly testing for T cells in physically separated populations of normal Ig-bearing and non-Ig-bearing lymphocytes. T cells were identified in an *in vitro* cytotoxicity assay using a goat anti-human thymocyte antiserum thoroughly absorbed with human red blood cells and buffy coat cells from a patient with CLL.

The absorbed antiserum (ATCS) killed almost 100% of normal human thymocytes, but killed less than 20% of bone marrow cells, and less than 15% of peripheral blood cells from patients with CLL. Approximately 60 to 90% of the latter cells bore surface Ig as judged by staining with fluorescein-conjugated rabbit antihuman Cohn II antiserum.

The cytotoxic action of ATCS was also tested on target cells obtained from a fluorescent cell

TABLE III

Per cent T and B lymphocytes in normal peripheral blood

% Cytotoxicity	% Monocytes	% B Lymphocytes[a]
74	12	13
57	15	22
70	19	24
72	13	14
60	9	18
70	22	29
66	8	33
56	32	30
60	15	18
60	13	25
67	25	10
68	22	21
65 ± 6.6[b]	17 ± 6.9[b]	21 ± 7.2[b]

[a] Per cent Ig-bearing small cells.

[b] Mean ± standard error.

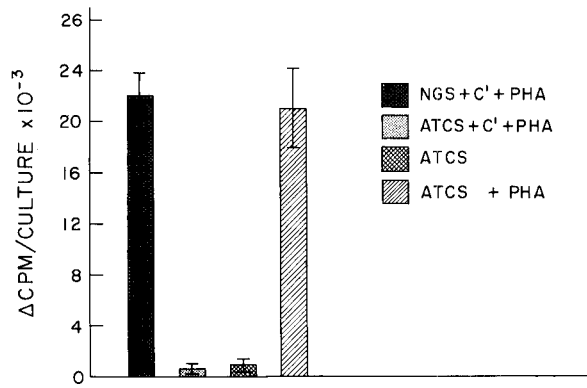

Figure 3. Effect of ATCS on the PHA response of peripheral blood cells. ΔCpm expressed the difference in ^{3}H-TdR incorporation between stimulated and control cultures. Brackets represent standard errors.

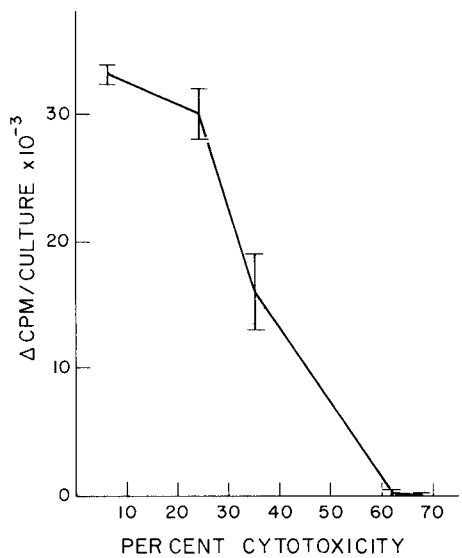

Figure 4. PHA response of peripheral blood cells after cytotoxic action of ATCS at different dilutions. ΔCpm expresses the difference in ^{3}H-TdR incorporation between stimulated and culture controls. Brackets represent standard errors.

separator. Ficoll-purified peripheral blood cells from normal donors were stained with fluorescein-conjugated anti-Cohn II antiserum, and then separated into dimly fluorescent and brightly fluorescent fractions. The latter fraction contained between 17 to 34% monocytes as judged by histochemical staining with α-naphthol acetate. ATCS proved to have a high degree of sensitivity in that it was able to kill almost 100% of the dimly fluorescent (non-Ig-bearing) cells. The specificity of the antiserum was confirmed by the lack of killing ($<10\%$) of brightly fluorescent (Ig-bearing) cells. Although only a small fraction of these cells were killed by ATCS, well over 80% were killed by anti-thymocyte antiserum which was not absorbed with CLL cells. These results strongly suggest that ATCS specifically identifies T lymphocytes in the peripheral blood, since cells killed by ATCS share antigens with human thymocytes, but not with normal Ig-bearing peripheral blood lymphocytes, monocytes, or bone marrow cells.

In further studies, Ficoll-purified peripheral blood cells from 12 normal donors were examined for the per cent Ig-bearing cells, monocytes and cells killed by ATCS (T cells). The experimental results show that a mean of 21% of these cells are B (Ig-bearing) lymphocytes, 17% are monocytes and 65% are T lymphocytes. These findings account for all cells in the Ficoll-purified cell suspensions, and indicate that normal peripheral blood lymphocytes are either non-Ig-bearing, T lymphocytes, or Ig-bearing, B lymphocytes. The ability of ATCS to abolish the response of peripheral blood lymphocytes to PHA in 3-day cultures, an effect directly related to cytotoxicity and not simply to inactivation of still viable lymphocytes, provides additional evidence that ATCS identifies T cells in the peripheral blood. Kreth and Herzenberg (31) have shown that in 3-day cultures PHA responsive cells reside only in the non-Ig-bearing population of human peripheral blood lymphocytes collected from the fluorescent cell sorter. However, recent data suggest that Ig-bearing lymphocytes collected from the fluorescent cell sorter may respond to PHA after 5 days in culture (Epstein, L. B., Kreth, H. W., and

Herzenberg, L. A., personal communication).

Identification of B lymphocytes in the present work rests upon the assumption that human Ig-bearing cells are similar to Ig-bearing cells in laboratory animals, in that they are derived from the bone marrow, independent of thymic influence (4). The per cent of normal peripheral blood cells stained with fluorescein-conjugated anti-Cohn II antiserum described herein is similar to that reported by Siegal *et al.* (12) (21% *vs* 19.8%). The latter investigators showed that the percentage of cells stained with this reagent approximates the sum of the percentages of cells stained with class-specific antisera directed against IgG, A, and M (12). The sum of Ig-bearing cells detected by monospecific antisera directed against IgG, A, and M in members of our donor population (22%) is also similar to these figures (31). It is, therefore, likely that the anti-Cohn II antiserum reacts with all classes of Ig, and identifies all normal B lymphocytes.

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