Human T-Cell Heterogeneity as Delineated with a Specific Human Thymus Lymphocyte Antiserum

IN VITRO EFFECTS ON MITOGEN RESPONSE, MIXED LEUKOCYTE CULTURE, CELL-MEDIATED LYMPHOCYTOTOXICITY, AND LYMPHOKINE PRODUCTION

JAMES N. WOODY, AFTAB AHMED, RICHARD C. KNUDSEN, DOUGLAS M. STRONG, and KENNETH W. SELL

From the Department of Clinical and Experimental Immunology, Naval Medical Research Institute, Bethesda, Maryland 20014

ABSTRACT Human peripheral blood lymphocytes (PBL) were evaluated by their responses to phytohemagglutinin (PHA-P), concanavallin A (con-A), and pokeweed mitogen (PWM), both before and after treatment with an antiserum against human thymic lymphocyte antigens (HTLA) that had been made T-cell-specific by multiple absorptions with immunoglobulin EAC-positive lymphoblast cell lines (B cells). Cells treated with HTLA were examined for their ability to react in a mixed lymphocyte culture (MLC) and to form killer cells in a cell-mediated lymphocytotoxicity (CML) system. Sensitized cells were also examined for their ability to respond to purified protein derivative (PPD) by blastogenesis, migration inhibitory factor release (MIF), and lymphotoxin (LT) production, both before and after treatment with HTLA and complement. The HTLA was in itself highly stimulatory to PBL. However, with the addition of complement and subsequent cell destruction, a marked decrease in its stimulatory response was noted. PBL treated with HTLA and complement exhibited marked inhibition of responsiveness to con-A with little decrease in PHA-P or PWM stimulation except at very high concentrations of HTLA. MLC reaction was inhibited only when responder cells were treated with HTLA + C'. Treatment of stimulator cells with HTLA + C' did not significantly alter the MLC response. The HTLA + C'-treated cells failed to form killer cells in the CML reaction and inhibited PPD-induced blastogenesis from PPD-sensitized individuals; however, treatment of sensitized cells with HTLA + C' had little effect on the release of MIF and LT. It is suggested that subpopulations of T-cells carry surface antigens that bind with this specific antisera, and that the con-A-responsive cells, the responder cells in the MLC, and killer T-cells comprise a separate subset from cells responding to PHA-P or PWM, or the MIF- and LT-producing cells.

INTRODUCTION

Murine thymus-derived lymphocytes (T-cells)[1] have been identified with specific anti-θ serum (1). Antisera directed against other T-cell surface antigens, including thymic leukemia antigen and lymphocyte-defined antigen, have further identified various subpopulations among T-cells (2, 3). The use of these antisera has segregated

[1] *Abbreviations used in this paper:* B cells, bone marrow-derived lymphocytes; C', complement; CML, cell-mediated lymphocytotoxicity; con-A, concanavallin-A; EAC, sheep erythrocyte-antibody-complement complex; E rosette, lymphocyte forming spontaneous rosettes with sheep red blood cells; FCS, fetal calf serum; HTLA, human thymic lymphocyte antiserum; LT, lymphotoxin; MIF, migration inhibitory factor; MLC, mixed leukocyte culture; NRS, normal rabbit serum; PBL, peripheral blood lymphocyte; PHA-P, phytohemagglutinin-purified; PPD, purified protein derivative; PWM, pokeweed mitogen; SRBC, sheep red blood cell; T-cell, thymus-derived lymphocyte; VBS, veronal-buffered saline with calcium and magnesium.

the responses of lymphocyte populations to mitogens (4)[a] and to various T-cell-dependent antigens, such as sheep red blood cells (SRBC) (5).

Such assays in the human systems have been hindered by the lack of specific anti-T-cell antisera, as all preparations are prepared in heterologous species, with the majority of antibodies nonspecific anti-species antibodies and only a small fraction of anti T-cell-specific antibodies present.

Only recently have various authors described the preparation of specific anti-human T-cell antisera (6–10) and examined some of the effects of these antisera on human cells in terms of inhibition of spontaneous SRBC rosette formation, a T-cell marker (11), and effects on mitogen stimulation and mixed leukocyte culture (MLC) responses (12–14). Our studies confirm what has been done previously and further outline functional T-cell subclass heterogeneity, attempting to gain a clear understanding of these subclasses and their role in disease processes.

METHODS

Preparation of human thymic lymphocyte antiserum (HTLA). Rabbit anti-human thymocyte serum was prepared by injecting New Zealand white rabbits with 1×10^9 viable fetal thymocytes on day 1, with complete Freund's adjuvant and footpad injections. The animals were boosted on day 14 with 1×10^9 viable cells given intravenously. On day 21 the animals were bled by cardiac puncture and the serum was collected and stored at $-20°C$. Rabbit anti-human thymocyte serum with a cytotoxicity titer of 1:1,600 or greater, prepared in this manner, has been shown to be consistently immunosuppressive, as tested in the rhesus monkey skin allograft survival assay (15).

A small pool (400 ml) was selected from three rabbits producing highly cytotoxic antisera. The antisera (100 ml) were pooled, heated to 56°C for 30 min and absorbed three times with 30 ml of packed human AB red cells carefully washed free of contaminating leukocytes by repeated removal of the buffy coat. The antisera were then divided into 100-ml aliquots and absorbed 12 times with 10 ml of packed washed human cultured lymphoblasts from three lines known to have surface immunoglobulin and complement receptors. An attempt was made to use cells containing most of the common human lymphocyte antigen types, since the antisera were prepared from thymocytes from more than one donor. The absorptions were done for 1 h at 37°C on a circular rotator with every third absorption performed overnight at $+ 4°C$. After each set of absorptions, the antisera were ultracentrifuged at 18,000 g for 1 h and filtered through a 0.22-nm Millipore filter (Millipore Corp., Bedford, Mass.). The antisera were absorbed until no activity was found against the absorbing lymphoblast cell lines either by cytotoxicity or indirect immunofluorescence. It was found that an additional three absorptions with gluteraldehyde-treated human serum were required to remove antibodies directed primarily against human albumin and IgG. The antisera were stored at $-70°C$ for use in the following assays. When other cell

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[a] Kieselow, P. 1974. Personal communication.

types were used for absorptions, the technique was identical to that outlined above.

Cytotoxicity testing. Peripheral blood lymphocytes were prepared by the method of Böyum (16) and cytotoxicity testing was performed by the method of Terasaki and Mc-Celland (17).

Treatment of peripheral blood lymphocytes (PBL) with HTLA. Ficoll-Hypaque-separated PBL were aliquoted into sterile 10×75-mm (Falcon Plastics, Oxnard, Calif.) test tubes and treated with various dilutions of HTLA at 37°C for 30 min. Rabbit complement (C'), absorbed with lymphoblasts to remove natural antibodies, was added to each tube and incubated further for 1 h at 37°C. These cell suspensions were then relayered onto fresh Ficoll-Hypaque gradients and the viable cells recovered and washed three times with RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.). Cells were counted with an Autocytometer II (Fisher Scientific, Silver Spring, Md.) and the viability was determined by trypan blue dye exclusion. Viability was always $\geq 90\%$. For most assays 5×10^6 cells were pelleted and treated with 0.9 ml of the HTLA dilution and 0.1 ml of rabbit complement (diluted 1:4); this treatment resulted in 50% cytotoxicity. In experiments where HTLA was used as a mitogen, PBL were treated in vitro with various dilutions of HTLA alone, incubated at 37°C for 30 min, washed three times with RPMI-1640, recounted, and dispensed into microtiter plates at $2 \times 10^5/$ culture.

Rosette inhibition studies. The erythrocyte (E) rosette test for spontaneous SRBC T-cell binding was performed with fresh (<2 wk) SRBC in Alsever's solution (Microbiological Associates, Bethesda, Md.). The cells were washed three times in veronal-buffered saline (Ca^{++} and Mg^{++}) (VBS). A 0.5% solution of SRBC was prepared by adding 10 ml of buffer consisting of equal portions of VBS and fetal calf serum (FCS) to 50 μl of packed SRBC. The FCS (10 ml) was absorbed three times with 2 ml packed washed SRBC to remove any antibodies to SRBC, if present. 100 μl of 0.5% SRBC solution was added to 100 μl of PBL containing 2–4×10^6 cells. The mixture was incubated at 37°C for 30 min, centrifuged at 200 g for 5 min, and left at $+ 4°C$ for a minimum of 2 h or overnight. The cells were gently resuspended and a small sample placed on a glass slide with a cover slip. 200 cells were counted under phase and the percent of rosette-forming cells counted. Cells with three or more adherent SRBC were counted as rosettes.

The EAC rosette test was carried out by sensitizing 2 ml of 2% SRBC with an equal volume of 1:64,000 dilution of human 19S anti-Forssmann (compliments of Dr. R. Wistar, Naval Medical Research Institute), incubating the mixture at 37°C for 30 min, and adding fresh mouse serum at a 1:5 dilution in VBS for 30 min at 37°C as a complement source. Reagent made in this manner and stored at $+ 4°C$ could be used for 7–14 days, after which time the coated SRBC failed to exhibit fluorescence with a fluorescent-conjugated anti-C'3 reagent (Meloy Laboratories, Inc., Springfield, Va.). 100 μl of cells and 100 μl of EAC reagent were added and incubated on a rotating platform for 45 min at 37°C; the cells were resuspended and counted as previously described.

Inhibition studies on both types of rosettes were done by adding 100 μl of the HTLA to the lymphocytes, incubating them 1 h at 37°C, and then proceeding with the test as described.

Table I

50% Cytotoxicity Titers of HTLA against Various Cell Types

Cell type	Titer
Thymus cells	1:1,024
PBL	1:64
MOLT-4*	1:64
CLL‡	1:6
Lymphoblast§	<1:2

* MOLT-4, a continuous lymphoblast T-cell line.
‡ Cells from a patient with chronic lymphocytic leukemia.
§ A continuous lymphocytoblast cell known to be a B-cell line.

Mitogen responses. Responses of lymphocytes to the non-specific mitogens phytohemagglutinin-P (PHA-P) (Difco Labs, Detroit, Mich.), concanavallin A (con-A) (Calbiochem, San Diego, Calif.), and pokeweed mitogen (PWM) (Grand Island Biological Co.) were always performed in triplicate as previously described (18). In brief, 100 μl of PBL containing 2×10^6 cell/ml in RPMI-1640, and 100 U penicillin/ml, 100 μg streptomycin/ml, and 2 mM L-glutamine containing 10% heat-inactivated (56°C, 30 min) FCS were plated into a microtiter plate and 50 μl of the appropriate mitogen was added. The cultures were incubated for 48 h at 37°C, 5% CO_2 and 95% air. The cultures were then pulsed with 20 μl of media containing 1 μCi[^{3}H]-thymidine (sp act 1.9 Ci/mmol, Schwarz-Mann Div., Becton, Dickinson & Co., Orangeberg, N. Y.) per well and harvested 18 h later with a multiple automated sample harvester. The triplicate samples were counted for 1 min each in a Packard Tricarb liquid scintillation counter (Packard Instrument Co., Inc., Downer's Grove, Ill.) and the mean, standard error, and percent standard error of triplicate samples calculated with a Wang 700C advance programming calculator (Wang Laboratories, Inc., Tewksbury, Mass.). The percentage of standard error for such experiments rarely exceeded 5%.

MLC. MLC reactivities were measured in triplicate as described in previous publications (19). In brief, stimulator cells were treated with 25 μg/ml mitomycin C (Calbiochem) at 37°C for 30 min to inhibit mitosis. They were washed twice in RPMI-1640, recounted, and used. 2×10^5 cells/well were used as stimulator cells and an equal number as responder cells. Untreated cells and cells treated with either HTLA or HTLA + C' were used as stimulator and responder cells. Cells were incubated for 4 days at 37°C, 5% CO_2 and 95% humidified air, pulsed with 1 μCi of [^{3}H]-thymidine per well for 18 h and harvested with the multiple automated sample harvester.

Assay for purified protein derivation (PPD)-induced blastogenesis. The assay for PPD-induced blastogenesis utilized PPD (Parke, Davis & Co., Detroit, Mich.) at various concentrations, usually 5–20 μg/ml. The assay technique was identical to that of the mitogens, except PPD was added and the culture incubated for 5 days rather than 48 h. When migration inhibitory factor (MIF) or lymphotoxin (LT) was assayed, a 48-h incubation was used.

Assay for MIF. Our assay system for MIF is a modification (20) of the David and David technique (21). In brief, PBL were collected from patients with known sensitivity to tuberculin PPD. 6×10^6 cells were incubated for

48–72 h in a 75×100-mm tube with various concentrations of PPD, before centrifugation and collection of the supernatant fluid. 50 μl of peritoneal exudative cells (50×10^6/ml), obtained by oil induction from guinea pigs, were loaded into plain 75-mm capillary tubes sealed at one end. The capillary tubes were centrifuged at 150 g for 10 min and the capillary cut at the cell-fluid interface. The short piece of capillary tubing containing the exudative cells was affixed to the inside of a migration inhibition chamber (Mini Lab Co., Ville De Laval, P. Q.) with silicone grease, Each chamber contained two capillary tubes and was sealed with a coverslip.

Fluid (0.5 ml) from the lymphocyte cultures were injected into the sealed chambers with a tuberculin syringe and the injection ports were sealed with plastic tape to prevent evaporation. The chambers were incubated at 37°C for 24–48 h. The image of cover slip, capillary, and cell migration was projected with a Bausch and Lomb projecting prism (Bausch & Lomb Inc., Rochester, N. Y.) onto a sheet of paper and the outline of the migrated cells traced. The area of migration was determined by planimetry and the percent migration inhibition was determined by the following formula.

% migration inhibition

$$= 1 - \frac{\text{average area of migration with antigen}}{\text{average area of migration without antigen}} \times 100$$

LT assay. Sensitized lymphoid cells in the presence of the sensitizing antigen, or normal cells when cultured with nonspecific mitogenic agents, cause lymphocyte transformation. The cell-free medium of such activated cultures has been shown to possess a lymphokine termed lymphotoxin (LT) (22). LT activity has been demonstrated by the ability of cell-free supernatant fluids to cause the destruction of mouse L-929 fibroblasts, and the secretion of LT has been shown to be a function of T cells (23). The LT assay was performed in a microassay system (24). In this assay system, the LT activity was measured as a decrease in the uptake of [^{3}H]thymidine by L cells. Briefly, supernatant fluids from sensitized cells stimulated by PPD were added to L cells, and the ID_{50} levels of LT activity were calculated as the dilution that gave 50% of the counts per minute of the media control values.

Cell-mediated lymphocytotoxicity (CML) assay. Responder cells used in this assay were sensitized in vitro by incubation with mitomycin-C-treated allogeneic lymphocytes, as described in the MLC section. The sensitized cells were then incubated with the PHA-P-stimulated, ^{51}Cr-labeled allogeneic target cell type originally used as stimulator cells. The cytotoxic activity of the killer cells was calculated by the percentage of ^{51}Cr release by the following formula:

% ^{51}Cr released

$$= \frac{\begin{array}{l}\text{mean cpm of experimental} \\ - \text{ mean cpm of spontaneous release}\end{array}}{\begin{array}{l}\text{mean cpm of freeze-thaw control} \\ - \text{ mean cpm of spontaneous release}\end{array}} \times 100$$

RESULTS

Specificity of antisera for T-cells. Initial experiments were designed to determine the specificity of our HTLA. We approached the problem by testing the antiserum for cytotoxic activity with complement

against various cell types. The results of this set of experiments is shown in Table I. The 1:1,024 titer observed against human fetal thymocytes was expected, since the sera were selected for their high cytotoxic activity, and fetal thymocytes were used as the immunizing antigen. The titer of activity against PBL, which were 70% T-cells by E-rosette criteria, and MOLT-4 cells, a cultured lymphoblast line with T-cell characteristics (25), is somewhat less than that observed against thymocytes, but suggests that both cells are sensitive to the antisera. Cells from a patient with chronic lymphocytic leukemia, by clinical criteria, were also used in the assay; 85% of these cell exhibited complement receptors and were considered to be B-cells. The HTLA exhibited very low levels of activity against not only these cells but also against the surface immunoglobulin-positive B-cell lymphoblast line used to absorb the antisera.

To identify further the antibodies in HTLA responsible for its cytotoxic activity, a number of absorption experiments were performed, and the absorbed sera tested for cytotoxic activity against PBL in the presence of complement. The PBL in this case were 70% T-cells by E rosette criteria. The results of these experiments are seen in Fig. 1. Absorptions with human thymocytes removed more of the cytotoxic activity toward PBL than any other cell type, while absorptions with B-cell lymphoblasts removed little or none of the activity. Absorptions with MOLT-4 cells, the cultured T-cell line, and with cells from a patient with Sézary syndrome, a T-cell leukemia, with 90% of the cells forming spontaneous E rosettes, both removed significant cytotoxic activity against PBL, but less than observed when thymocytes were utilized. The sham absorption merely diluted the HTLA to the same degree as the actual absorption, acting as a control to assure
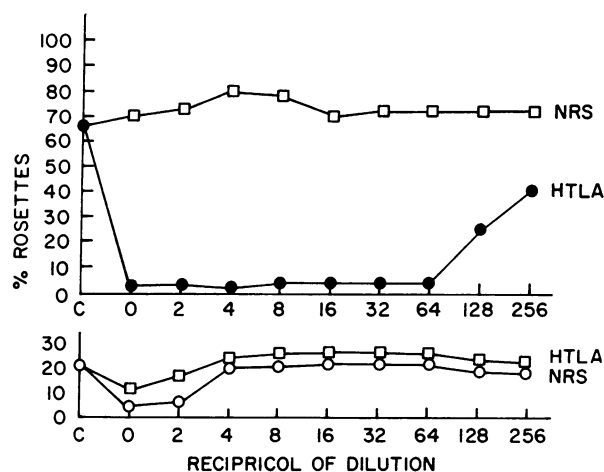
Figure 2 HTLA pretreatment and rosette formation. The effect of HTLA pretreatment on spontaneous SRBC rosette formation (upper) and CRL rosette formation (lower) with PBL.

ourselves that the effect observed was not due to dilution alone.

Additional specificity studies utilized the ability of HTLA to inhibit the formation of either spontaneous E rosettes, a T-cell phenomena, or EAC rosettes, a B-cell phenomena. When PBL were incubated with HTLA, before the addition of SRBC or EAC reagent, it significantly inhibited the formation of spontaneous E rosettes to a dilution of 1:128–1:256, whereas little inhibition of the EAC rosettes was seen except when the antisera were used undiluted (Fig. 2). Normal rabbit serum (NRS) used as a control had little effect on spontaneous rosettes but did inhibit EAC rosettes to a 1:2 dilution.

Stimulation of PBL by HTLA. From our previous experiments we concluded that HTLA exhibited T-cell specificity. To test its functional capacity, we examined the ability of this absorbed antisera to act as a specific T-cell mitogen. In these experiments the PBL were treated with various dilutions of HTLA or HTLA and rabbit complement, as described in the Methods section. The cells were incubated for 48 h and pulsed with [^{3}H]thymidine and harvested as described above. The results are seen in Fig. 3. It was found that HTLA alone was a potent mitogenic agent, with as little as 50 μg of HTLA giving 98,000 cpm while PHA-P, a potent T-cell mitogen, gave a peak response of 90,000 cpm when similar numbers of PBL's were used in the culture. Cells treated with HTLA and complement did not exhibit the response at any concentration, implying that the cells responsible for the activity had been eliminated with the addition of complement to the system.
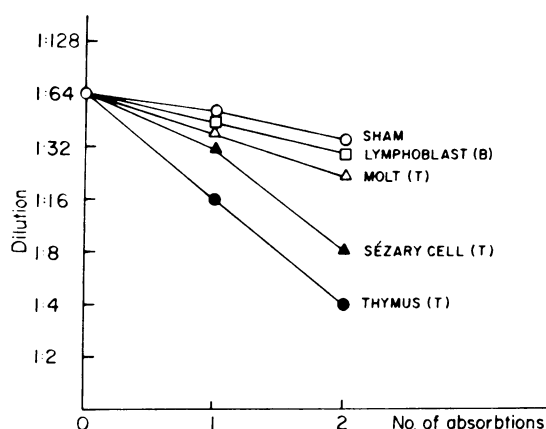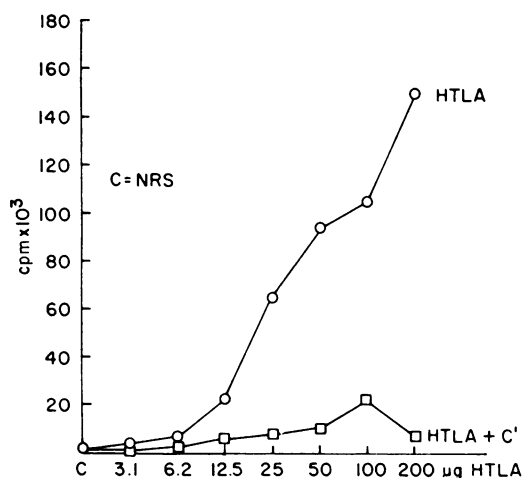

Figure 1 Cytotoxic properties of HTLA after absorptions. The effect of 20 volumes % absorptions with various cell types on 50% cytotoxic titer of HTLA against PBL.

FIGURE 3 Treatment of PBL with HTLA. Uptake of [³H]thymidine by PBL after treatment with various concentrations of HTLA alone or with C'.

Alteration of mitogen responses by HTLA and complement. After the demonstration that HTLA and complement could eliminate the blastogenic response induced by HTLA alone, we undertook studies to determine the effect of HTLA and complement on the ability of cells to respond to various nonspecific mitogens. PBL were treated with HTLA and complement and subsequently incubated with the nonspecific mitogens. As seen in Fig. 4, the response of the cells to PHA-P was not significantly decreased, except at higher concentrations of antisera, while the response of treated cells to PWM was essentially unaltered. The ability of treated cells to respond to con-A was inhibited even

at very low concentrations of antisera, 6.2 μg of HTLA corresponding to a 1:32 dilution of the antisera.

Inhibition of MLC response by HTLA and complement. With the previous data suggesting that HTLA and complement would inhibit the PBL response to con-A at certain dilutions while having little effect on PHA-P responses, we concluded that the PBL could be separated into functional subsets using the antisera at these concentrations. We were interested in looking at other functional parameters thought to be mediated by T-cells and, therefore, began a series of experiments to examine these. One such reaction is the one-way MLC, where cells from one donor (responder cells) are mixed with cells (stimulator cells) from an allogeneic donor that have been blocked with mitomycin C so that they are unable to respond by DNA synthesis, but are capable of stimulating the responder cells. The results of a dose-response assay treating PBL responder cells with various concentrations of HTLA and complement are seen in Fig. 5. Treatment of the responder cells results in a marked decrease in the ability of these cells to react to the stimulator cells, as measured by the uptake of [³H]thymidine. This inhibition occurs even at low concentrations of antibody, similar to the levels noted to inhibit the con-A responses.

The second part of this experiment deals with the treatment of either stimulator or responder cells with HTLA and complement and the ability of these treated cells to function in the MLC reaction. The cells were treated with HTLA at a 1:10 dilution and complement, which corresponds to a level of HTLA between 12.5 and 25 μg; the results are shown in Table II. Control cells did not show any significant responses as mea-
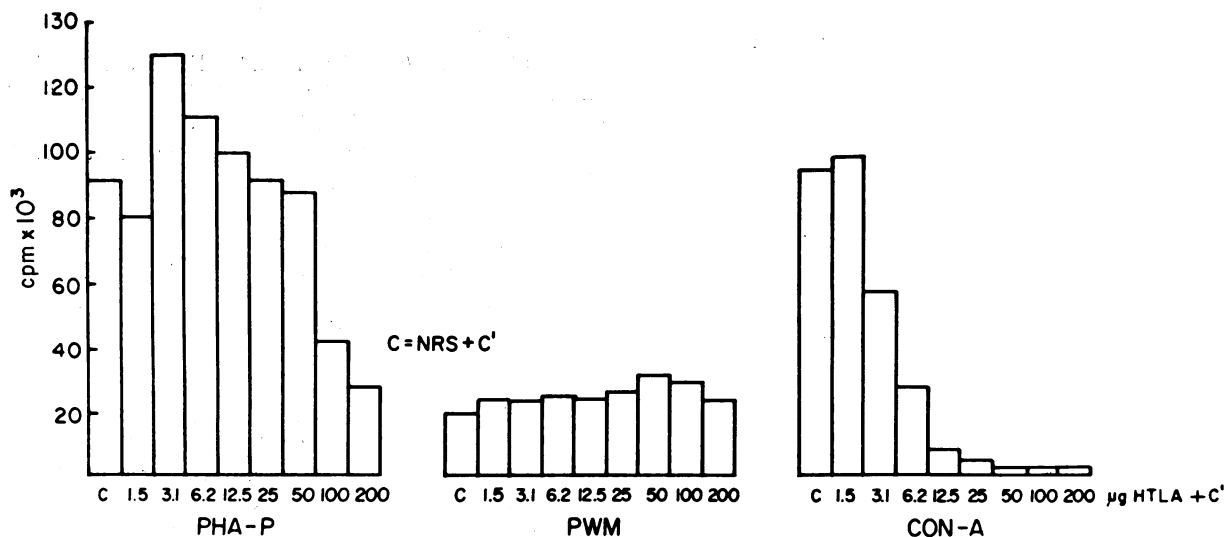

FIGURE 4 HTLA pretreatment and mitogen response. Uptake of [³H]thymidine by PBL after treatment with various amounts of HTLA + C' followed by mitogen stimulation.

sured by the uptake of [^{3}H]thymidine. When untreated responder cells are mixed with allogeneic mitomycin-C-treated stimulator cells, such as with Ax Bm or Bx Am, a normal MLC response is observed. When untreated responder cells are mixed with allogeneic cells treated with HTLA, complement, and mitomycin-C, a moderate decrease in counts is seen as with Ax Bhm and an increased response is noted with Bx Ahm, suggesting that the cells inducing stimulation are only slightly affected by treatment with HTLA and complement. When responder cells are treated with HTLA and complement and mixed with mitomycin-C-treated stimulator cells, such as Ah × Bm and Bh × Am, the MLC response is significantly decreased, indicating that the responder cells are significantly affected by treatment with HTLA and complement, as was also noted in the experiments outlined in Fig. 4.

Inhibition of formation of CML killer cells by HTLA and complement. In the previous experiments we were able to show that treated PBL responder cells were unable to participate in the MLC reaction, whereas treated stimulator cells worked nearly as well as normal cells. Experiments to evaluate the ability of HTLA and complement treatment to inhibit the generation of killer cells and previously activated killer cells in the CML reaction were carried out.

In this series of assays, PBL were used either untreated or treated with HTLA 1:10 and complement, as previously described. Both treated and untreated responder cells were sensitized in vitro as in the MLC studies.

After sensitization, these cells were either directly used in the CML assay or treated with HTLA + C' or NRS + C' and used in the assay. These effector cells were then incubated with ^{51}Cr-labeled target cells (stimulator cells), stimulated with PHA-P, and the percent
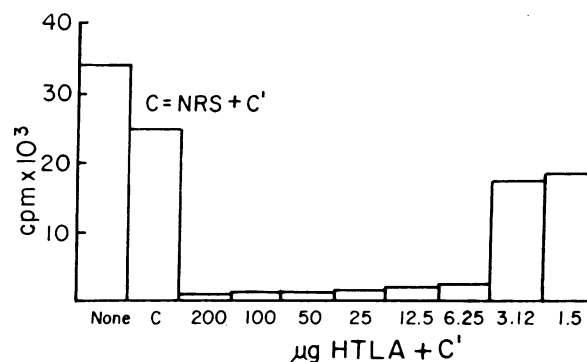

FIGURE 5 HTLA pretreatment of responder cells in MLC. Uptake of [^{3}H]thymidine by responder cells in MLC reaction after pretreatment with various amounts of HTLA + C'.

of chromium release was calculated as an index of target cell lysis by the sensitized cells.

Treatment of responder cells before initiation of MLC (sensitization phase) with HTLA + C' led to no significant activation (Table II) and subsequent generation of killer cells (data not shown). As seen in Table III, untreated in vitro-sensitized responder cells exhibited marked cytotoxic potential, causing 18–49% ^{51}Cr release. Treatment of these same sensitized cells with HTLA and complement nearly eliminated the cytotoxic potential. Treatment of these sensitized cells with NRS and complement had little effect.

In the set of experiments outlined in Table IV, the same protocol was followed except mitomycin-C-treated stimulator cells were incubated with either HTLA and complement or NRS and complement before incubation in the MLC reaction. Treatment of the stimulator cells did not prevent the formation of cytotoxic lymphocytes.

TABLE II
The Effect of Pretreatment of PBL with HTLA 1:10 and Complement on the Ability of These Cells to Act as Stimulator and Responder Cells in the MLC Reaction

Untreated cells			Treated stimulator cells			Treated responder cells		
type	*cpm*	*% SE**	*type*	*cpm*	*% SE**	*type*	*cpm*	*% SE*
A	479	5.4				Ah	257	4.3
B	798	1.7				Bh	399	2.0
Am‡	412	7.7	Ahm§	185	7.7			
Bm	93	5.6	Bhm	134	5.8			
Ax Am	775	3.9	Ax Ahm	639	5.8	Ahx Am	797	6.5
Bx Bm	1,295	6.0	Bx Bhm	1,693	4.0	Bhx Bm	1942	11.6
Ax Bm	18,587	3.7	Ax Bhm	9,076	5.5	Ahx Bm	943	1.8
Bx Am	22,436	5.6	Bx Ahm	29,195	3.5	Bhx Am	2219	6.1

* % SE, percent standard error on triplicate samples.
‡ m, mitomycin-C-treated.
§ h, HTLA and complement-treated.

TABLE III

Effect of Treatment of Sensitized Cells with HTLA + C' on CML-Mediated Chromium Release from Target Cells

Responder cell type		Stimulator cell type	Cr-51 target cell	No treatment	NRS + C'	HTLA + C'
A	X	Bm§	B	49.9*	42.5	−1.8
A	X	Cm	C	33.0	21.6	2.3
B	X	Am	A	29.6	—	0.7
B	X	Cm	C	30.9	25.6	1.2
C	X	Am	A	28.5	22.1	−2.1
C	X	Bm	B	18.6	11.8	1.9
A‡	X	Am	A	2.7	—	—
B‡	X	Bm	B	1.9	—	—
C‡	X	Cm	C	3.4	—	—

* Percent chromium released from target cells.
‡ Untreated controls.
§ Mitomycin-C-treated cells.

TABLE IV

Effect of Pretreatment of Stimulator Cells with HTLA + C' on CML-Mediated Chromium Release from Target Cells

Responder cell type		Stimulator cell type	Cr-51 target cell	No treatment	NRS + C'	HTLA + C'
A	X	Bm‡	B	49.9*	55.0	62.7
A	X	Cm	C	33.7	40.7	45.9
B	X	Am	A	29.6	21.5	38.5
B	X	Cm	C	30.9	22.7	38.9
C	X	Am	A	28.5	32.6	35.9
C	X	Bm	B	18.6	21.9	27.6

* Percent chromium released from target cells.
‡ Mitomycin-C-treated cells.

Effect of HTLA and complement on blastogenesis, MIF, and LT production by sensitized lymphocytes. In the previous experiments we have shown that the con-A-responsive cells and the MLC responder cells are sensitive to treatment with HTLA and complement. To ascertain the effect of HTLA and complement treatment on the responses of sensitized lymphocytes, a number of experiments were undertaken with cells from patients sensitive to PPD by skin test.

In the first of these experiments, cells were treated with either HTLA alone or with HTLA and complement before exposure to various concentrations of PPD. NRS with or without complement served as a control. The blastogenic response to PPD was measured by the uptake of [³H]thymidine. The results are seen in Table V. The HTLA 1:5 alone stimulated the cells, as noted among data for the control samples. PPD induced significant blastogenesis by itself, which was slightly inhibited by NRS and complement, but not by HTLA alone. HTLA and complement at both dilutions tested significantly inhibited the response of the sensitized cells to PPD.

To extend the experiments to evaluate MIF and LT production by sensitized lymphocytes stimulated with PPD, the following studies were performed. Cells from

PPD-sensitive donors were treated with various combinations of sera and complement before exposure to PPD. The supernatant fluid was harvested after 48 h and assayed for MIF and LT production. The results are shown in Table VI. None of the sera used as controls induced the cells to produce significant amounts of MIF or LT, while cells stimulated with PPD alone produced significant amounts of both. Neither NRS, HTLA, or HTLA and complement inhibited the production of MIF or LT in response to PPD stimulation by sensitized cells.

DISCUSSION

The studies reported here provide an initial attempt to classify human T-cells into subpopulations by functional assays. In the murine system, cells have been classified with specific antisera, such as θ, lymphocyte-defined antigens, and thymic leukemia antigen (1–3), both in terms of surface antigens and functional assays (4).

When animals are immunized with a complex antigen, such as a human thymocyte, the heterolgous antisera contain not only specific antithymocyte antibodies but a great deal of nonspecific antibodies, including antibodies directed against other lymphocyte antigens, human species antigens, immunoglobulins, and probably other as yet unknown antigens. This is supported

TABLE V

The Effect of Pretreatment of PPD-Sensitive Cells with HTLA + C' on Blastogenic Response to Various Amounts of PPD as Measured by the Uptake of [H³]Thymidine

	Control	NRS	NRS + C'	HTLA 1:5	HTLA 1:5 + C'	HTLA 1:20 + C'
				cpm		
Control	953	878	683	11,596	221	612
PPD, 20 μg/ml	38,359	27,404	9,999	34,849	624	2,112
PPD, 10 μg/ml	11,797	18,769	12,354	22,709	359	3,889
PPD, 5 μg/ml	7,182	12,560	7,246	19,519	435	1,934

by the fact that many absorptions were required to lend cytotoxic specificity towards T-cells in the HTLA used in this study. The cytotoxic activity seen against chronic lymphocytic leukemia cells suggests that these cells carry antigens not removed from the HTLA by lymphoblast absorptions. The antisera thus prepared are only functionally specific.

In some aspects, HTLA can be compared to θ antisera in the murine system, although it probably detects a group of antigens peculiar to T-cells rather than a specific antigenic site. It would, however, be expected to exhibit more specificity toward cells with thymocyte-surface antigens, as it was prepared against thymocytes. It would also be expected to differentiate types of T-cells expressing more or less thymus-surface antigens. Stobo and Paul (26) and Granger and Kolb (22) have noted that certain T-cells in the murine system contained larger amounts of surface θ antigens, as detected with anti-θ serum. The results obtained by absorbing the HTLA with various cell types tend to support a similar conclusion in human cells. Of particular interest are the MOLT-4 and Sézary cells, considered to be T-cells by their ability to form E rosettes and their lack of surface immunoglobulin. This is exemplified by their inability to remove the cytotoxic activity of the HTLA against PBL as compared to thymocytes. It is possible, however, that HTLA discriminates between different functions of T cells rather than physically being cytotoxic for subpopulations of T cells. The expression of thymus surface antigens may be of clinical significance, since it has been shown by Smith (27) that some types of malignant cells fail to form E rosettes; however, they do react with antisera specific for thymic lymphocyte antigens, suggesting that certain T-cells may modulate or lose their SRBC receptor sites, making clinical recognition difficult when one uses the E rosette as the sole criterion for T-cell identification.

The blastogenic response of cells treated with HTLA alone, noted in our experiments, indicates that T-cells are intensely stimulated by this antisera, more than seen with either PHA-P or con-A. This could prove to be a functional test of T-cells, somewhat more specific than mitogen stimulation, as well as an assay for HTLA, since this response is not seen with antisera raised against B lymphoblasts,[3] and increases as non-specific antibodies are removed from antithymocyte sera by absorption with B lymphoblasts.

The response of cells treated with HTLA and complement to mitogens suggests that the con-A-responsive cells are most sensitive to this antiserum. Only at higher concentrations are the PHA-P-sensitive cells affected, while the PWM-responsive cells are not sig-

[3] Woody, J. N., A. Ahmed, and K. W. Sell. Unpublished observations.

TABLE VI

The Response of Sensitized Cells to Pretreatment with HTLA+C', Followed by Stimulation with PPD, as Measured by the Production of MIF and LT

Treatment of sensitized cells	Migration inhibition	L-cell inhibition
	%	%
Cell control	0	6.9
Cells + NRS	7	10
Cells + C'	3	24
Cells + HTLA	−9	10
Cells + HTLA + C'	−5	6
Cells + NRS + PPD	68	95
Cells + PPD	72	96
Cells + HTLA + PPD	65	95
Cells + HTLA + C' + PPD	75	95

nificantly decreased. Since the original antisera were made against thymocytes, one can speculate that perhaps the same relationship between θ antigen and con-A-responsive cells in the murine system applies to the human system. Granger and Kolb (22) noted that T-cells containing larger amounts of surface θ antigen were more sensitive to anti-θ serum and more responsive to con-A. Cells containing less θ surface antigen were noted to be more responsive to PHA-P. It is entirely possible that the reason that the PHA response required higher amounts of anti-HTLA than the con-A response to be inhibited may be a matter of quantity rather than quality. One would only have to postulate that the PHA conditions were supraoptimal (i.e., reduction of high proportion of reactive cells is not seen until 95% of the cells are eliminated) versus a suboptimal condition with regard to con-A. However, this would seem unlikely since concentration curves of both mitogen and cell concentrations on HTLA + complement-treated PBL's failed to reveal any con-A response.

When the responder cells in the MLC reaction were treated with HTLA and complement, a decrease in activity was observed, suggesting that the MLC responder cell was sensitive to approximately the same concentrations of HTLA as was the con-A-responsive cell. When the stimulator cells were treated with HTLA + C', only slight differences in MLC responses were noted, indicating that large numbers of T-cells are not necessary for stimulation of allogeneic cells. When responder cells were treated with HTLA + C', a marked decrease in the MLC response was noted, indicating that T-cells are necessary for adequate responses.

A similar phenomenon was noted in the generation of killer cells in the CML reaction, where HTLA + C' prevented the development of these cells. The sensitivity of the cells to HTLA would suggest that either the

TABLE VII
Classification of Human T-Cells on Basis of Functional Assays and Sensitivity to HTLA

HTLA-sensitive	HTLA-insensitive
Con-A	PHA-P
PPD, blastogenesis	PWM
Responder cell, MLC	MIF
Killer cell, CML	LT

same cell or a similar cell participating in the MLC is being affected.

Bach and Bach (28) have suggested that the responder cell in the MLC recognizes different antigens from those recognized by the killer cell in the CML reaction. Our data suggest that either both cells are sensitive to HTLA, by detecting a group of thymic antigens on the T-cell surface that might be expressed by both types of cells, or that the responder cell in the MLC transmits information through mediators necessary for the development of the killer cell, hence, without the MLC responder as the afferent limb, the effector cell cannot be generated.

Of interest were the results shown in Table V and VI, where HTLA and complement inhibited the blastogenic response of sensitive cells exposed to PPD, whereas the release of MIF and LT from the same cells was not affected, suggesting that at least two different cell populations were involved, one undergoing blastogenesis and the other releasing mediators. It has been suggested that MIF is preformed and may be released from cells, as cultured B lymphoblasts are known to make MIF (29). Whether LT is preformed or is synthesized is unknown; however, evidently the cells need not undergo blastogenesis for this mediator to be released. Whether LT production is a T- or B-cell function in humans is unknown; however, LT production by PHA or con-A stimulated cells suggests a T-cell function.

From our data, summarized in Table VII, we conclude that the cells responding to con-A, the MLC responder cells, and the CML killer cells, as well as cells responding to PPD in the sensitized individual, express equivalent amounts of thymic lymphocyte antigens as detected by our HTLA. On the other hand, it is unclear at present what types of cells respond to PHP-P or PWM and what types of cells produce MIF and LT. Whether these T-cells exhibit little thymic surface antigens or are B-cells or some intermediate type of cell is at present uncertain.

Recently, a goat antihuman thymocyte globulin (GAHTG) has been described by Owen and Fanger (30). There are some similarities in the results obtained with this absorbed GAHTG and the HTLA re-

ported in this communication: they both inhibit the response of PBL to con-A and allogeneic cells; and they do not affect the response of PWM. However, there are several differences in the experimental procedures and the results obtained: their experiments are performed with continuous incubation of cells with GAHTG, whereas in our case the cells were treated with HTLA and complement, the dead cells removed by gradient separation, and the cells washed before assay; and their antibody is mitogenic to PBL before absorption with chronic lymphocytic leukemia cells, but this mitogenic property is removed after absorption. In the case of HTLA, however, the mitogenic property was increased after absorption with lymphoblasts. These results suggest that (a) the mitogenic principal in GAHTG either resides on B-cells alone or is an antibody against an antigen common to both T and B cells and, therefore, it is not really clear whether it stimulates T cells or B cells, whereas the mitogenic principal in HTLA could not be absorbed out by absorption with lymphoblasts or with cells from patients with chronic lymphocytic leukemia and, therefore, present on T cells and also stimulate T cells; (b) the goat may recognize certain antigens in human thymus cells different from those recognized by rabbits; and (c) both goats and rabbits recognize certain similar antigens, giving rise to antisera that block the con-A response and the MLC response.

The identification of specific antibodies to subsets of T-cells may have a great clinical relevance in light of the description of a naturally-occurring thymocytotoxic antibody found in the sera of old New Zealand black mice (31), thought to play a role in the pathogenesis of the autoimmune disease process (32) related to the loss of a thymic suppressor (33). Such antibodies occur in patients with systemic lupus erythematosus and may be found in other autoimmune diseases. As more is learned about cellular immune dysfunctions in various disease states and the pathophysiologic role of various cell types in immune disorders, the hypothesis given regarding T-cell subpopulations may tend to group certain disorders. One such example is in the case of subacute sclerosing panencephalitis, where cells were shown to liberate LT and MIF on antigenic stimulation, but did not transform (20) to a viral preparation. The cell types involved and the area in the cellular immune system where these factors operate will be most useful in the further delineation of T-cell subsets.

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