

## SPONTANEOUS RELEASE OF THE LEU-2 (T8) MOLECULE FROM HUMAN T CELLS\*

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Unique human T cell surface antigens have been used to distinguish T lymphocytes from other hematopoietic cells. Some of these antigens have been used to analyze and separate human T cells into functional subsets (1–5).

Human T cells can be divided into two major subsets according to the cell surface phenotypes. Thus, the helper/inducer T cells are Leu-1<sup>+</sup>, Leu-2<sup>-</sup>, Leu-3<sup>+</sup>, and Leu-4<sup>+</sup> (T1<sup>+</sup>, T4<sup>+</sup>, T8<sup>-</sup>, and T3<sup>+</sup>), whereas the cytotoxic/suppressor T cells are Leu-1<sup>+</sup>, Leu-2<sup>+</sup>, Leu-3<sup>-</sup>, and Leu-4<sup>+</sup> (T1<sup>+</sup>, T4<sup>-</sup>, T8<sup>+</sup>, and T3<sup>+</sup>) (2–7). Recent studies of cloned T cells have suggested that some of these surface antigens might play a role in T cell function (8–10).

Monoclonal antibodies directed against these antigens are being tested as therapeutic agents for the treatment of T cell malignancies and immunoregulatory diseases (11–15). One problem that can reduce the effectiveness of such treatment is the release of free antigen. In our experience, free Leu-1 antigen appeared in the serum of patients after they received monoclonal antibody infusions (11). These observations suggest that T cell surface antigens can be released into serum under certain circumstances.

In this report we describe the development of sensitive enzyme-linked immunoassays for the detection of soluble Leu-1, -2, and -3 antigens. We also describe the physiological release of the Leu-2 antigen from Leu-2-bearing cells.

### Materials and Methods

*Monoclonal Antibodies.* The production and characterization of mouse monoclonal anti-Leu-1 antibody (clone L17F12) have been previously described (1). Mouse monoclonal anti-Leu-2a, anti-Leu-3a, and anti-Leu-3b have been characterized in detail (2, 16). Purified preparations of these antibodies and their biotinylated derivatives were obtained from Becton, Dickinson & Co., Mountain View, CA.

*Preparation of Rabbit Anti-Leu Sera.* Leu-1 and Leu-2 antigens were purified by absorption onto affinity columns. 1 mg of purified monoclonal antibody was coupled to ~1 ml of packed volume of Sepharose 4B beads (Pharmacia, Uppsala, Sweden) activated by cyanogen bromide (17). A cell lysate from 10<sup>11</sup> human thymocytes in a lysis buffer of 0.5% Nonidet P-40 (NP-40)<sup>1</sup> (BDH Chemicals Ltd., Poole, United Kingdom), 50 mM

\* Supported by grants CA-21223 and CA-32739 from the National Institutes of Health.

<sup>1</sup> *Abbreviations used in this paper:* B-CLL, B cell chronic lymphocytic leukemia; BSA, bovine serum albumin; CTL, cytotoxic T lymphocyte; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HRP, horseradish peroxidase; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; RL-1, RL-2, rabbit anti-Leu-1, -2 serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; T-ALL, T cell acute lymphoblastic leukemia.

Tris, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO) and 1  $\mu$ g pepstatin A (Sigma Chemical Co.), pH 7.6, was applied first to an anti-Leu-1 and subsequently to an anti-Leu-2a column. After washing the columns extensively with 0.5% NP-40 in phosphate-buffered saline (PBS), the beads were mixed with complete Freund's adjuvant and used directly for immunization. 1/8 of the beads were injected in rabbits subcutaneously three times at 2-wk intervals. 1 wk after the last immunization, rabbits were bled and the sera were collected and heat inactivated. Rabbit anti-Leu-1 serum (RL-1) and rabbit anti-Leu-2 serum (RL-2) were passed over their respective mouse monoclonal antibody columns to absorb anti-mouse immunoglobulin activity before their use in immunoassay.

*Enzyme-linked Immunosorbent Assays (ELISA) for the Detection of Soluble Leu-1 and Leu-2 Antigens.* Each well of a 96-well Immulon plate (Dynatech Laboratories, Inc., Alexandria, VA) was coated with 50  $\mu$ l of purified anti-Leu-1 or anti-Leu-2a antibody at a concentration of 10  $\mu$ g/ml in PBS, the plates were incubated overnight at 4°C, and then washed three times with PBS. To block free binding sites, the plates were covered with 1% bovine serum albumin (BSA) (fraction V, Miles Laboratories Inc., Elkhart, IN) and PBS and incubated for 1 h at room temperature. The plates were then washed three times with 0.05% NP-40 and PBS (washing buffer). Then 50  $\mu$ l of sample (cell lysate, culture supernatant, or serum) was added to a well and a twofold dilution was made with 0.1% NP-40, 2% BSA, 0.05% Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>, and PBS (dilution buffer). After incubating for 2 h at 4°C, the plates were washed extensively with washing buffer. Then, 50  $\mu$ l of RL-1 or RL-2 in dilution buffer was put into the appropriate wells. After incubating 2 h at 4°C and washing again extensively, 50  $\mu$ l of 1:1,500 diluted horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Tago Inc., Burlingame, CA) in 0.1% NP-40, 2% BSA, and PBS were added to each well. The plates were incubated for 1 h at room temperature and washed again in the same manner. Finally, 100  $\mu$ l of substrate, 2,2'-azino-di(3-ethylbenzthiazoline sulfonic acid-6) ammonium salt (Sigma Chemical Co.), at a concentration of 150  $\mu$ g/ml in 0.05 M citric acid, pH 4.0, with 0.01% H<sub>2</sub>O<sub>2</sub>, was added to each well. After incubating for 30 min at room temperature in the dark, the absorbance at 405 nm of each well was determined by ELISA reader (Dynatech Laboratories, Inc.).

*Assay for Leu-3 Antigen.* The procedures were similar to Leu-1 and Leu-2 ELISA except that anti-Leu-3b was used to coat the plate and 50  $\mu$ l of 1  $\mu$ g/ml biotinated anti-Leu-3a in dilution buffer was used as the detector. After incubating for 2 h at 4°C, the plate was washed extensively with washing buffer and 50  $\mu$ l of HRP-conjugated avidin-D (Vector Laboratories, Burlingame, CA) diluted 1:1,500 in 0.1% NP-40, 2% BSA, and PBS were added to each well. The plate was incubated for 20 min at room temperature and washed. The final steps were done in the same manner as in Leu-1 and Leu-2 ELISA.

*Cell Culture.* The human cell lines used in this study and their phenotypes are listed in Table I. Cell line 255.88, a gift of Dr. E. Engleman, Department of Pathology, Stanford

TABLE I  
Phenotype of Cell Lines

	Leu-1	Leu-2a	Leu-3a	Leu-4
HPB-ALL	+	+	+	+
JM	+	+	+	+
MOLT-4	+	+	+	+
HSB-2	+	-	-	-
8402	+	-	-	-
255.88	+	-	+	+
Jurkat	+	-	-	+
8866	-	-	-	-
SB	-	-	-	-
Raji	-	-	-	-
UC729/6	-	-	-	-

University, is a T-T hybridoma made with human peripheral T cells and an azaserine-hypoxanthine-sensitive variant derived from the Jurkat cell line (18). All cells were maintained in RPMI 1640 supplemented with 15% fetal calf serum (FCS) and antibiotics at 37°C in a 5% CO<sub>2</sub> atmosphere.

*Preparation of Cell Lysates.* Cultured cells ( $5 \times 10^7$ ) were washed twice with PBS and were lysed for 1 h on ice with 1 ml of lysis buffer. Nuclei were removed by centrifugation at 5,000 g for 10 min. Cell lysates were then centrifuged at 25,000 g for 1 h and they were kept frozen at -20°C until use.

*Preparation of Culture Supernatants.* Culture supernatants of cell lines were collected when cell numbers in culture were  $\sim 1 \times 10^6$ /ml. Sodium azide was added to a final concentration of 0.05%. In a time course experiment, cell numbers were adjusted to  $2.5 \times 10^5$ /ml and the cells were cultured in the presence or absence of FCS at 37°C in a 5% CO<sub>2</sub> atmosphere. Cell numbers and cell viability were determined every 24 h by trypan blue dye exclusion.

*Preparation of Human Sera.* Sera from healthy donors and from leukemia patients, including three cases of T cell acute lymphoblastic leukemia (T-ALL) and two cases of B cell chronic lymphocytic leukemia (B-CLL), were collected and kept frozen at -70°C.

*Immunoprecipitation and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).* HPB-ALL cells,  $1 \times 10^7$ , were labeled with 1 mCi of <sup>125</sup>I by the lactoperoxidase method (19). Cell lysates were prepared in 1 ml of lysis buffer as described above. The affinity-purified Leu-2 antigen ( $1 \times 10^7$  cell equivalents) from culture supernatant of HPB-ALL cells was radioiodinated by the chloramine-T method and radiolabeled protein was separated by Sephadex G25 sizing column (Pharmacia). 10 μl of rabbit serum or 10 μg of mouse monoclonal antibody was incubated with 100 μl of radioactive cell lysate or radioiodinated purified Leu-2 antigen (500,000 cpm), and immune complexes were precipitated with either 150 μl of 10% heat-fixed *Staphylococcus aureus* Cowan I (The Enzyme Center, Boston, MA) for rabbit serum, or 150 μl of goat anti-mouse IgG (Tago Inc.) for mouse monoclonal antibody. Immunoprecipitated materials were analyzed by SDS-PAGE under reducing or nonreducing conditions (20).

## Results

*Characterization of Rabbit Anti-Leu-1 and Anti-Leu-2 Antisera.* The rabbit antisera were compared with monoclonal antibodies in their ability to recognize their homologous cell surface antigens. Immunoprecipitates were made using RL-1 or RL-2 and radioiodinated cell surface materials from HPB-ALL cells. These were analyzed on SDS-PAGE (Fig. 1).

RL-1 precipitated a 67,000 mol wt protein from HPB-ALL cell lysate. Side by side comparison using RL-1 and monoclonal anti-Leu-1 antibody showed that these two reagents detected the same molecular weight protein. Sequential immunoprecipitation resulted in the removal of the same molecule by both antibodies (data not shown).

RL-2 precipitated a single band of 33,000 mol wt protein in a reducing gel from HPB-ALL cell lysate. A similar 33,000 mol wt protein was precipitated with the anti-Leu-2a antibody. Sequential immunoprecipitations demonstrated that RL-2 and anti-Leu-2a detected the same molecule (data not shown). Under nonreducing conditions, the 33,000 mol wt component was replaced by a specific band of 69,000 mol wt in addition to higher molecular weight material in both the RL-2 and anti-Leu-2a immunoprecipitates. Thus, the RL-2 antiserum detected the Leu-2 antigen, which is known to be composed of two or more disulfide-bonded subunits of 33,000 mol wt each (16).

*Specificity and Sensitivity of Leu ELISA.* Soluble Leu antigens were specifically absorbed to the monoclonal antibodies attached to a plate and these antigens

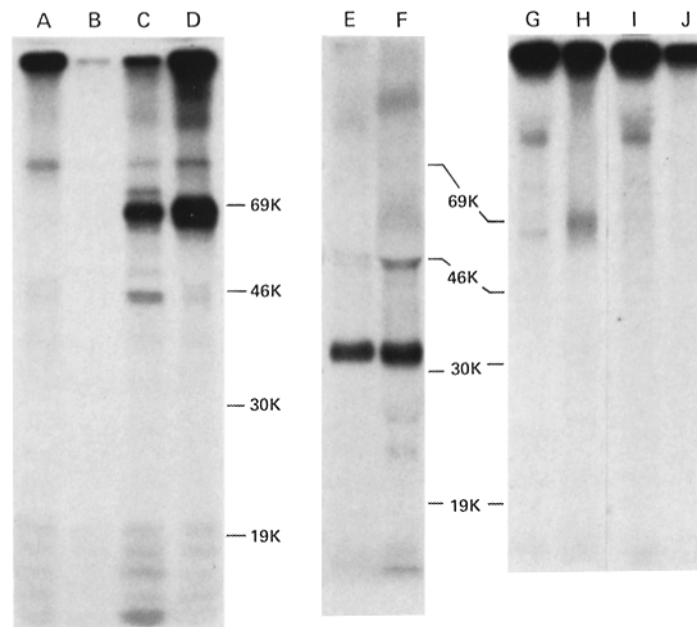


FIGURE 1. SDS-PAGE analysis under reducing conditions of Leu-1 and Leu-2 antigens precipitated from radioiodinated HPB-ALL cell lysate. (A) normal rabbit serum (NRS), (B) monoclonal anti-HLA-DR L227, (C) monoclonal anti-Leu-1, (D) RL-1, (E) RL-2, and (F) monoclonal anti-Leu-2a. Leu-2 antigen was analyzed also under nonreducing conditions: (G) RL-2, (H) anti-Leu-2a, (I) NRS, and (J) L227. The monoclonal anti-HLA-DR framework (L227) is used as a control antibody in this experiment (35).

were then detected by specific antisera (RL-1 or RL-2) or by another monoclonal antibody (detailed in Materials and Methods). As a source of Leu antigens, cell lysates from a variety of cell lines known to vary in their Leu antigen expression were used (Fig. 2).

In the Leu-1 assay, cell lysates from all the T cell lines were reactive, whereas a cell lysate from the B cell line, 8866, was inactive. These results correlated very well with the cell surface expression of Leu-1 antigen. No signal was obtained when the inappropriate rabbit antiserum (i.e., anti-Leu-2) was used as a detector in this assay. This result confirmed the specificity of the rabbit anti-Leu-1 antiserum. Moreover, it indicates that the Leu-1 molecules in the detergent lysate were dissociated rather than part of membrane fragments.

In the Leu-2 ELISA, only cell lysates from T cell lines that expressed Leu-2 antigen on their cell surface were positive. Once again, no signal was obtained when the inappropriate rabbit antiserum (in this case anti-Leu-1) was used as detector. The Leu-2 signal varied among the Leu-2-positive cell lysates. Cell lysates from Leu-2 antigen-negative cell lines and that from a B cell line were completely negative.

For the detection of Leu-3 we used two monoclonal antibodies, anti-Leu-3a and anti-Leu-3b, which recognize different antigenic determinants on the same molecule (2). The avidin-biotin reaction was used to amplify the antigen signal. By using mouse monoclonal antibodies on both sides of the antigen there was no

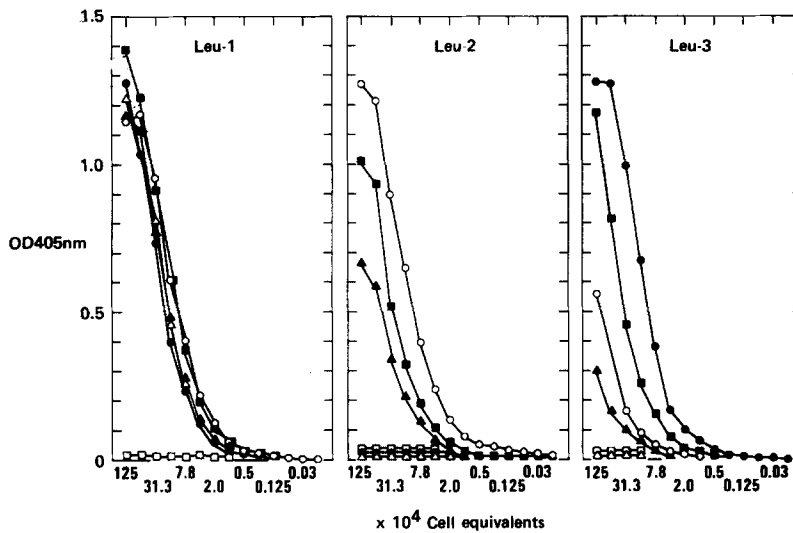


FIGURE 2. Standard curves obtained in each Leu ELISA with cell lysates from HPB-ALL (○), JM (■), MOLT-4 (▲), 255.88 (●), Jurkat (△), and 8866 (□). Antigen amounts are expressed as cell equivalents.

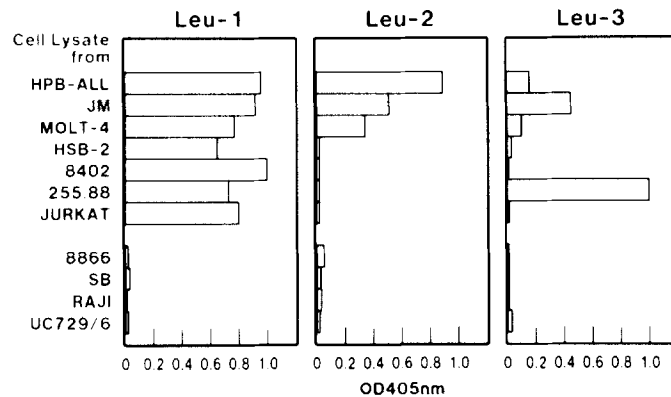


FIGURE 3. Antigen signals obtained in each Leu ELISA with cell lysates equivalent to  $6.25 \times 10^5$  cells.

cross-reactivity of the detecting reagents. Fig. 2 shows the standard curves obtained from various cell lines. Of note are the cell lysates from 255.88 and Jurkat, which demonstrate the specificity of this Leu-3 ELISA. Cell line 255.88 is a hybridoma made between activated human peripheral blood T cells and a drug-marked variant of the Jurkat cell line. Interestingly, Leu-3 antigen, which is not present on parent cell Jurkat, is expressed on the hybrid clone, 255.88 (18). The cell lysate from 255.88 showed the strongest Leu-3 signal among the Leu-3-positive cell lines tested whereas the cell lysate from the Jurkat parent was negative. Cell lysates from other Leu-3-positive cell lines also showed various degrees of positive signals. Once again, the lysate from a B cell line was negative.

Fig. 3 shows the Leu-1, -2, and -3 ELISA results for each of the cell lines listed in Table I. All the B cell lysates were negative and the results from the T cell

lysates were totally concordant with their surface phenotypes. The sensitivity of Leu ELISA assays is shown in Table II. In all three assays, antigen signals from as few as  $5 \times 10^5$  cell equivalents could be reliably detected.

*Leu Antigen Activities in Culture Supernatant from Cell Lines.* To investigate the possibility of Leu antigen release from cells, supernatants of cultured cell lines were examined (Fig. 4). The supernatants were collected when the cell density was  $\sim 10^6$ /ml; a time when cell viabilities remained  $>93\%$ . All the supernatants were negative for Leu-1 and Leu-3. However, some of the supernatants from T cell lines gave positive results in the Leu-2 ELISA. Leu-2 antigen was found only in the supernatants from the cell lines that expressed Leu-2 antigen on the cell surface. The presence of Leu-2 antigen in the supernatant was unlikely to be the result of cell destruction during culture since neither Leu-1 nor Leu-3 was found in the same supernatant. Therefore, the existence of Leu-2 antigen in the supernatants appeared to be the result of spontaneous release from cells.

*Time Course of Leu-2 Accumulation in Culture Supernatant.* The kinetics of Leu-2 antigen release by cultured HPB-ALL cells is shown in Fig. 5. A comparison was made between cells cultured in the presence or absence of FCS. Cell numbers in culture with FCS increased progressively with viability remaining  $>92\%$ . Cell numbers in culture without FCS did not increase beyond one doubling. Cell

TABLE II  
Sensitivity of Leu ELISA Assays

Assay	Number of experiments	Optical density reading with cell lysates from indicated number of cells*		
		$5 \times 10^5$	$0.16 \times 10^5$	P
Leu-1	22	$93.4 \pm 15.2^\ddagger$	$06.5 \pm 04.3$	$< 0.0005^\S$
Leu-2	27	$79.6 \pm 09.9$	$10.7 \pm 05.0$	$< 0.0005$
Leu-3	16	$63.7 \pm 09.8$	$06.9 \pm 04.8$	$< 0.0005$

\* Cell numbers of HPB-ALL cells for Leu-1 and Leu-2 assays and of 255.88 cells for Leu-3 assay.

$^\ddagger$  Mean optical density 405 nm readings  $\pm$  SEM ( $\times 10^{-3}$ ).

$^\S$  Student's *t* statistic.

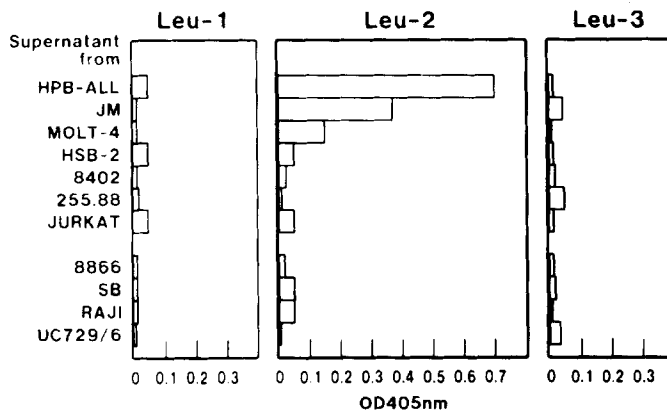


FIGURE 4. Antigen signal obtained in each Leu ELISA with  $50 \mu\text{l}$  of culture supernatants from various cell lines.

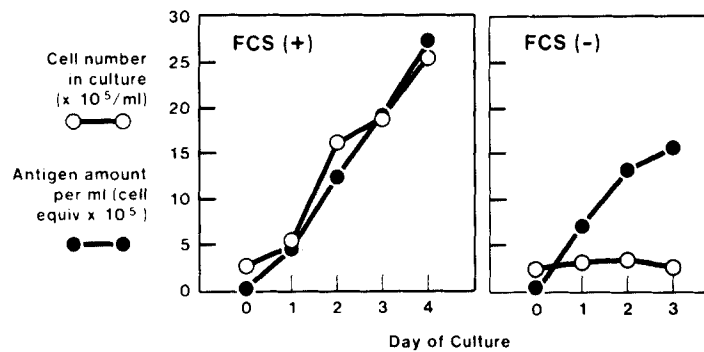


FIGURE 5. Time course of Leu-2 antigen appearance in supernatant. HPB-ALL cells were adjusted to  $2.5 \times 10^5$ /ml and cultured with or without FCS. (○) Cell numbers in culture; (●) Leu-2 ELISA signals obtained from 50  $\mu$ l of culture supernatant. Antigen amount is calculated as cell equivalents per milliliter.

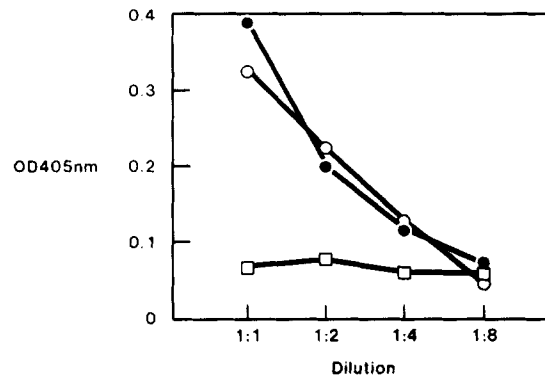


FIGURE 6. Purification of Leu-2 antigen from supernatant of HPB-ALL cells. Leu-2 antigen activity of culture supernatant was measured by ELISA before application on an anti-Leu-2a affinity column (○); effluent fraction from the column (□); and fraction eluted with glycine HCl, pH 2.7 (●).

viability without FCS remained >90% by day two but fell to 54% by day 3. In the culture with FCS, Leu-2 activity in the supernatant was found to correlate well with cell numbers. In culture without FCS, Leu-2 activity still increased although the cell number remained relatively constant. From these results, the following conclusions can be drawn (a) the appearance of Leu-2 in the supernatant does not depend on the presence of FCS; (b) Leu-2 level in the supernatant correlates with the cell number in growing cultures; (c) Leu-2 antigen accumulates in the supernatant of very low density culture; and (d) the amount of Leu-2 antigen accumulated in the supernatant can exceed that contained within the cells by a factor of three.

*Purification of Leu-2 Antigen from HPB-ALL Supernatant with an Anti-Leu-2a Affinity Column.* A 20-ml sample of culture supernatant from HPB-ALL was applied to an anti-Leu-2a affinity column. After dialyzing the eluted fraction against PBS and adjusting the volume, the Leu-2 ELISA was repeated. As shown in Fig. 6, the Leu-2 activity in the supernatant was completely absorbed by an anti-Leu-2 affinity column and was almost completely recovered from the column

by elution with 0.2 M glycine HCl, 0.5 M NaCl, pH 2.7 buffer.

*Leu-2 Antigen in the Supernatant Has Only One Leu-2a Determinant.* The Leu-2 antigen has been shown to be composed of multiple polypeptide chains bound by disulfide bonds. To explore the molecular nature of the Leu-2 antigen in the supernatant we developed another ELISA which used the monoclonal anti-Leu-2a antibody on both sides of Leu-2 antigen (repetitive determinant ELISA). Instead of using RL-2 for the detection of Leu-2 antigen, we used biotinylated anti-Leu-2a followed by HRP-avidin. The sensitivity of the repetitive determinant ELISA was  $\frac{1}{8}$  that of the original assay (Fig. 7A). However, the Leu-2 antigen was readily detected in cell lysates by both assays. This result confirms that Leu-2 antigen in cell extracts has at least two Leu-2a determinants. By contrast, the Leu-2 antigen in culture supernatants failed to give a positive signal in the repetitive determinant assay (Fig. 7B). These results suggest that Leu-2 antigen in the supernatant had only one Leu-2a determinant per molecule.

*Released Leu-2 Antigen is a Single Polypeptide Chain.* Since the results obtained in the repetitive determinant Leu-2 assay suggested that the Leu-2 antigen present in the supernatant might be a single chain, we characterized the structure of the released Leu-2 antigen. The affinity-purified Leu-2 antigen from culture supernatant of HPB-ALL cells was radioiodinated and the radiolabeled antigen was immunoprecipitated and analyzed by SDS-PAGE. As shown in Fig. 8C, the released Leu-2 antigen was a single band of 27,000 mol wt under reducing conditions that was smaller by 5,000 mol wt than the Leu-2 chain present on the HPB-ALL cell surface (Fig. 8, A and B). Furthermore, under nonreducing

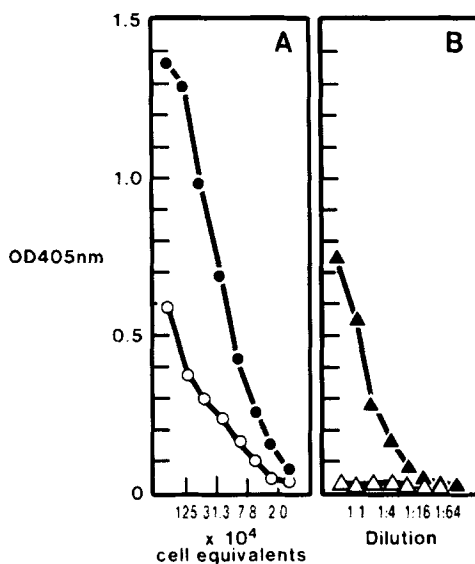


FIGURE 7. Comparison of Leu-2 antigen signals obtained from cell lysate or culture supernatant of HPB-ALL cells using two different ELISA assays. Closed symbols represent the signals obtained by conventional ELISA using anti-Leu-2a and RL-2. Open symbols represent the signals obtained with a repetitive determinant Leu-2 ELISA that uses the same monoclonal anti-Leu-2a antibody on both sides of the antigen. (A) Cell lysates; (B) culture supernatants from HPB-ALL cells.



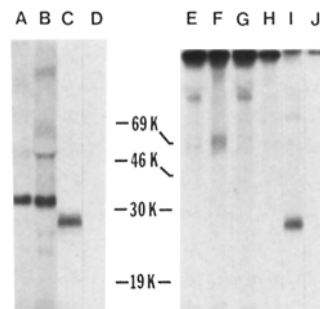


FIGURE 8. Leu-2 antigen was immunoprecipitated from either radioiodinated HPB-ALL cell lysate (A, B, E-H) or from radioiodinated Leu-2 antigen purified from supernatant of HPB-ALL cells (C, D, I, and J) and was analyzed by SDS-PAGE under reducing (A-D) or nonreducing (E-F) conditions. RL2 (A, C, E, and I), anti-Leu-2a (B, F), NRS (D, G, J) and L227 (H).

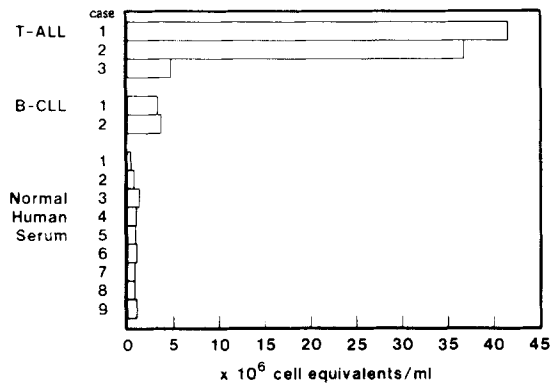


FIGURE 9. Leu-2 antigen ELISA with normal human sera and sera from leukemia patients. Leu-2 antigen amounts are expressed as HPB-ALL cell equivalents per milliliter. Surface phenotypes of leukemia cells: T-ALL (cases 1 and 2), Leu-1<sup>+</sup>, 2<sup>+</sup>, 3<sup>+</sup>, and 4<sup>+</sup>; T-ALL (case 3), Leu-1<sup>+</sup>, 2<sup>-</sup>, 3<sup>-</sup>, and 4<sup>+</sup>; B-CLL (cases 1 and 2), Leu-1<sup>+</sup>, 2<sup>-</sup>, 3<sup>-</sup>, 4<sup>-</sup>, and surface IgM<sup>+</sup>.

conditions, the released Leu-2 antigen (Fig. 8I) showed exactly the same molecular weight found under reducing conditions, whereas the Leu-2 antigen from the cell surface moved to higher molecular weight positions (Fig. 8, E and F). Therefore, it seems very likely that Leu-2 antigen present in the supernatant is a single polypeptide chain which has only one Leu-2a determinant.

*High Levels of Leu-2 Antigen in Serum of Patients with Leu-2<sup>+</sup> Leukemia.* Normal sera were examined for the presence of Leu-2 antigen and almost all of them were found to have a low but significant level (Fig. 9). Sera from five leukemia patients, which included T-ALL (three cases) and B-CLL (two), were also tested in the assay; high amounts of Leu-2 activity were found in sera from Leu-2-positive T-ALL patients. These sera were also checked for Leu-1 and Leu-3 antigen activity and they showed no significant activity (data not shown).

### Discussion

In this report we describe the development of sensitive ELISA assays for the detection of human T cell antigens in soluble form. Leu-1 and Leu-2 antigens

were detected by using a combination of monoclonal antibodies and newly prepared antisera. RL-1 and RL-2 were made in rabbits by immunization with Leu antigens absorbed to monoclonal antibody affinity columns. The advantage of heterologous antisera is their reaction with multiple antigenic determinants on an antigen molecule. On the other hand, monoclonal antibodies, which react with single antigenic determinants, can easily be purified and used to prepare solid-phase affinity matrices. These respective advantages were exploited in the development of the Leu ELISA assays.

For the detection of Leu-3 antigen, two monoclonal antibodies, anti-Leu-3a and anti-Leu-3b, which recognize different determinations on the same molecule, were used (2). The specificity and avidity of the avidin-biotin reaction were used to strengthen this assay (21). The ELISA assays could detect antigen signals from as few as  $5 \times 10^3$  cell equivalents of cells expressing those antigens (Table II). Assuming an antigen content on the order of  $10^5$  molecules per cell and molecular weights of 67,000, 70,000, and 55,000 for Leu-1, -2, and -3, respectively (22), the ELISA assays have a sensitivity in the sub-ng range.

With these Leu ELISA, we found the specific appearance of Leu-2 antigen in the supernatants from Leu-2-positive T cell lines. The presence of Leu-2 in the supernatant did not depend on FCS or on cell proliferation. The Leu-2 antigen level in the supernatant was found to correlate with cell numbers in culture, although in the absence of FCS where cell proliferation was minimized, the antigen accumulated to an amount greater than that present in the existing cells. The Leu-2 molecule in the supernatant could be effectively purified with an anti-Leu-2a antibody affinity column. This technique will clearly be of value for the isolation and structural characterization of the Leu-2 antigen.

The Leu-2, or T8, antigen is considered to be the human homologue of the mouse Lyt-2, Lyt-3 antigens (16, 23, 24). The Leu-2 antigen has a subunit structure of two or more disulfide-bonded polypeptide chains (16). These chains have mol wts of 32,000 and 43,000 when isolated from thymocytes. However, only a 32,000 mol wt chain was isolated on peripheral T lymphocytes (16). The T5 and T8 antigens are apparently the same as the Leu-2 antigen (24, 25). Anti-T5 precipitated a 76,000 mol wt glycoprotein from human thymocytes and mitogen-stimulated human peripheral T lymphocytes. This molecule separated into 30,000 and 32,000 mol wt proteins under reducing conditions (25). Both anti-T5 and anti-T8 precipitated the 33,000 mol wt protein from HPB-ALL cells under reducing conditions (24). The T5 and T8 determinants are apparently on different portions of the molecule. In a functional study, anti-T8 was shown to have an inhibitory effect on cytotoxic T lymphocyte (CTL), whereas anti-T5 did not (24). Our rabbit antiserum RL-2 and anti-Leu-2a detected a single chain which had a mol wt of 33,000 from radioiodinated HPB-ALL cell lysates under reducing conditions. In a nonreducing gel, the 33,000 mol wt chain was no longer detectable. Instead, 69,000 and higher mol wt forms were found.

To study the molecular nature of Leu-2 antigen released from T cells, we have developed another Leu-2 assay which depends on the fact that the intact Leu-2 molecule has repeating antigenic determinants. In this repetitive determinant assay, supernatants from T cell lines were negative while cell lysates from the same cell sources were positive. We concluded from these observations that

Leu-2 antigen in the supernatant had only one Leu-2a determinant, whereas the Leu-2 antigen from cell lysates had at least two Leu-2a determinants. These observations suggested that only a single chain of Leu-2 antigen was released into the supernatant. To confirm this point, we characterized the structure of the released Leu-2 antigen. The affinity-purified Leu-2 antigen from supernatant of HPB-ALL cells was radioiodinated, immunoprecipitated, and analyzed by SDS-PAGE. Since the released Leu-2 antigen showed a single band under both reducing and nonreducing conditions, we concluded that the Leu-2 antigen present in the supernatant of HPB-ALL cells was a single polypeptide chain with only one Leu-2a determinant, whereas the Leu-2 antigen present on HPB-ALL cell surface was composed of identical chains and had at least two Leu-2a determinants per molecule. More interestingly, the released Leu-2 antigen was a protein of 27,000 mol wt that was smaller than the Leu-2 antigen present on the cell surface. The mechanism of Leu-2 release is not yet clear. There are at least two possible mechanisms: shedding from cell surface and secretion by exocytosis. We have not yet detected the Leu-2 antigen in cell lysates from cell lines that have no detectable Leu-2 antigen on their cell surface. Thus, shedding of this antigen may be one possible mechanism. However, the possibility of the secretion cannot be excluded. More precise study, which includes analysis of cell lines or mutant cells that do not express Leu-2 antigen on cell surface, internal labeling, and pulse and chase experiments, may resolve these questions.

Is release of Leu-2 antigen from cells a physiological phenomenon or is it an artifact of *in vitro* cell culture? To answer this question, we examined normal human sera and sera from leukemia patients for the presence of Leu-2 antigen. Normal human serum contained small amounts of Leu-2 antigen but when sera from Leu-2<sup>+</sup> patients were tested, high amounts of Leu-2 antigen were found. Interestingly, sera from a patient with Leu-2-negative T cell leukemia and from patients with B-CLL had low, but significant, levels of Leu-2 antigen.

In mouse, Lyt-2 antigen has been thought to be a general marker for CTL and has been supposed to be involved in CTL function (26, 27). Recent studies have suggested that Lyt-2 antigen is not required in cytotoxic activity but is responsible for the recognition of major histocompatibility complex (MHC) (28, 29). In humans, Leu-2, or T8, antigen is thought to play a role similar to Lyt-2 in cellular interaction. Anti-Leu-2a antibody has shown to block proliferation of Leu-2<sup>+</sup> T cells in allogeneic mixed lymphocyte reaction (MLR) (7). Anti-Leu-2a or anti-T8 also block the killing of CTL generated in allogeneic MLR (2, 24). Clonal analysis suggested that T8<sup>+</sup> CTL recognized class I MHC molecules on the target cell but T8<sup>-</sup>T4<sup>+</sup> CTL recognized class II MHC molecules (9, 10).

T8<sup>+</sup> or Leu-2<sup>+</sup> T cells have been shown to suppress immunoglobulin production by B cells induced by T4<sup>+</sup> or Leu-3<sup>+</sup> T cells (30–32). Further, production of helper factor by T4<sup>+</sup> cells capable of inducing polyclonal activation of B cells was found to be suppressed by T8<sup>+</sup> cells (33). Although Reinherz et al. (24) reported that anti-T8 did not influence suppression of immunoglobulin secretion mediated by T8 cells, the direct participation of T8 or Leu-2 antigen in the suppressive function of T8<sup>+</sup> or Leu-2<sup>+</sup> cells remains unclear. Functional analysis using purified Leu-2 or T8 molecules may clarify these points and, in this regard, the use of released Leu-2 molecule may provide a new approach to study the

mechanism of cellular interaction.

The use of monoclonal antibodies has not been restricted to the analysis of cellular function. Clinical trials are underway to explore the effects of monoclonal antibodies as therapeutic tools for the treatment of malignant and immunoregulatory diseases (11-15, 34). We have found that circulating Leu-1 antigen can be detected in the serum of patients after monoclonal antibody infusion (11). Such release of Leu-1 after anti-Leu-1 antibody administration is not accompanied by the release of Leu-2 or Leu-3 antigens. These results suggest that the release of cell surface antigen after antibody treatment is not the result of cell destruction but is an antigen-specific phenomenon. Further analysis will be necessary to clarify the precise mechanism of antigen release from cells *in vivo*, especially when the release of antigen is induced by antibody. The fact that in one circumstance, Leu-2, there is basal release of antigen into the serum of leukemia patients, and in another case, Leu-1, there is release after antibody administration, underscores the importance of the assays reported here to monitor patients receiving therapy with antibodies and, possibly, during chemotherapy as well.

### Summary

Sensitive enzyme-linked immunosorbent assays (ELISA) for the detection of human T cell antigens in soluble form have been developed. The assays use mouse monoclonal antibodies and specific anti-Leu sera prepared in rabbits by immunizing with Leu antigens absorbed to monoclonal antibody affinity columns. With these assays, Leu-1, -2, and -3 antigen signals from extracts of as few as  $5 \times 10^3$  cells could be detected. When culture supernatants from various cell lines were tested, Leu-2 antigen, but not Leu-1 or Leu-3, was found to be present. Leu-2 antigen was present only in supernatants from T cell lines that expressed Leu-2 on their cell surface. Leu-2 antigen accumulated progressively in the supernatant of low density culture and its presence did not depend on cell proliferation or on fetal calf serum in the culture medium. The Leu-2 antigen in the supernatant was found to have only one Leu-2a determinant, whereas Leu-2 antigen from cell extracts had at least two determinants. The Leu-2 molecule was effectively purified from supernatant with an anti-Leu-2a affinity column. The purified Leu-2 antigen from supernatant of HPB-ALL cells was a single polypeptide chain of 27,000 mol wt, whereas Leu-2 antigen present on HPB-ALL cell surface was composed of two or more identical polypeptide chains of 33,000 mol wt linked by disulfide bonds. Normal human sera and sera from leukemia patients were also examined for the presence of the Leu-2 antigen. Normal human sera contained low levels of Leu-2 antigen but sera from Leu-2-positive leukemia patients had high levels. These results indicate that Leu-2 antigen is released from human T cells under physiological conditions.

We wish to thank Dr. Edgar Engleman for the use of the 255.88 cell line and Dr. Stanford Stewart and Dr. David Maloney for helpful discussions.

*Received for publication 7 February 1982 and in revised form 20 April 1983.*

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