TLiSA1, A HUMAN T LINEAGE–SPECIFIC ACTIVATION ANTIGEN INVOLVED IN THE DIFFERENTIATION OF CYTOTOXIC T LYMPHOCYTES AND ANOMALOUS KILLER CELLS FROM THEIR PRECURSORS

By GORDON F. BURNS, TONY TRIGLIA, JEROME A. WERKMEISTER, C. GLENN BEGLEY,* AND ANDREW W. BOYD‡

From The Lions Clinical Cancer Research Laboratory, Clinical Research Unit, the *Cancer Research Unit of The Walter and Eliza Hall Institute of Medical Research, and The Royal Melbourne Hospital, Post Office, Royal Melbourne Hospital, Victoria 3050, Australia, and the ‡Division of Tumor Immunology, Dana-Farber Cancer Institute and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115

Lymphocyte activation leads to the orderly expression of antigens that were absent or only weakly expressed on the surface membranes of the resting cells. The functions of some of these induced antigens on human cells are now known to include receptors for transferrin and insulin to enable rapid proliferation and increased expression of class II major histocompatibility complex molecules to augment interlymphocyte communication (1–3); the functions of other antigens defined by monoclonal antibodies (mAb)[1] such as 4F2 and T10 await elucidation (2, 4).

Relatively few activation antigens are specific for T cells, and it seems likely that such T lineage–specific activation antigens (TLiSA) play an important role in the activation or regulation of functional T cells. Thus, the receptor for interleukin 2 (IL-2-R), detected by mAb such as anti-Tac (5–8), although not strictly a TLiSA (9), is one example with both proliferative and regulatory functions (5–9), and the T11₃ antigen, which is part of the receptor for sheep erythrocytes, participates in the alternative pathway of T cell activation (10). To date, the functions of the TLiSA of mol wt 160,000 and 210,000, which are subunits of the A-1A5 antibody–defined complex (11), and the Ta₁ of mol wt 105,000 (12) are not known.

In this report, we describe a novel TLiSA that appears to be involved in the differentiation of cytolytic T lymphocytes (CTL), and, more particularly, of anomalous killer (AK) cells (13–16) from their precursors. LeoA1, an mAb

[1] Abbreviations used in this paper: AK, anomalous killer; CTL, cytotoxic T lymphocyte; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HAT, hypoxanthine, aminopterin, thymidine selective medium; IL-2-R, interleukin 2 receptor; mAb, monoclonal antibody; MLC, mixed lymphocyte culture; MNC, mononuclear cell; NK, natural killer; PHA, phytohemagglutinin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLiSA, T lineage–specific activation antigen.

against this antigen, inhibited the generation of these effector cells, but had no effect on proliferation or on effector cell function. The antigen named TLiSA1 was clearly distinct from the IL-2-R, although the kinetics of its expression on activated T cells were similar to the appearance of this receptor and, like Tac, it was absent from a number of leukemic T cell lines. We consider the possibility that the antigen may function as a receptor for a human analogue of the T cell differentiation factor that has been described in murine studies (17–19).

Materials and Methods

Preparation of mAb. BALB/c mice (maintained at The Walter and Eliza Hall Institute) were immunized over a period of 30 d by three intraperitoneal injections of 10^7 human T cells, which had been activated by coculture with irradiated B lymphoblasts, and cultured in medium containing IL-2, and by a further intravenous boost 1 wk later. After 3 d, spleen cells from the immunized mice were fused with NS-1 cells, and the hybrids selected with hypoxanthine, aminopterin, and thymidine (HAT), as described (20). The hybridoma supernatants were screened for selective binding to mixed lymphocyte culture (MLC)-activated T cells by using indirect immunofluorescence and flow cytometry. Cells secreting an antibody of interest were cloned as described (20), and used to prepare ascites fluid in BALB/c mice primed with pristane. A radioimmunometric assay was used to isotype the antibodies, and LeoA1 and LeoA2 were determined to be IgG1κ, and the LeoLFA-1 to be IgMκ. For direct immunofluorescent studies, the LeoA1 antibody was purified by DEAE chromatography and directly coupled to fluorescein isothiocyanate (FITC) by standard techniques. For immunoprecipitation and proliferation studies (see below), the purified antibody was coupled directly to cyanogen bromide–activated Sepharose beads (Pharmacia Fine Chemical, Piscataway, NJ). F(ab')₂ fragments of the antibody were prepared with pepsin at pH 3.5, as described (21).

The anti-Tac antibody used in comparative biological studies was the generous gift of Dr. H. Waldman (National Institutes of Health, Bethesda, MD), and for immunoprecipitation studies, a comparable antibody against the IL-2-R (22) was the generous gift of Dr. E. Reinherz (Dana-Farber Cancer Institute, Boston, MA).

Cell Isolation. Peripheral blood mononuclear cells (MNC) were isolated from the heparinized blood of normal individuals by collecting cells from the interface after centrifugation over lymphoprep (Nygard, Oslo, Norway). The MNC were washed and resuspended in RPMI 1640 medium containing 10% fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, penicillin (100 U/ml) and streptomycin (100 μg/ml) (TC medium). Normal human thymocytes (from two donors) and bone marrow cells (three donors) were obtained from patients undergoing thoracic surgery. The thymocytes were monodispersed and separated from erythrocytes by mechanical disruption, passage through a wire sieve, and centrifugation over lymphoprep; bone marrow cells were washed with Eisen's balanced salt solution and centrifuged over lymphoprep. Interface cells were washed with Eisen's with 10% FCS, counted, and used for FACS analysis.

Cell Lines. Lines of normal human B lymphoblasts were established by infection with Epstein-Barr virus, as described (23). The T leukemic cell lines Molt 3 and Molt 4, and the fibroblast line Flow 2000 were obtained from Flow Laboratories Inc. (Melbourne, Australia), and the gibbon cell line MLA-144 (24) was a gift of Dr. Peter Hersey, Royal Newcastle Hospital, Newcastle, Australia. Other cell lines including K562 (erythroleukemic), HL60 (promyelocytic), U937 (histiocytic), CEM (T cell leukemia), LiBr, and TAM (melanoma) have been maintained in the laboratory for a number of years, and are free of mycoplasma contamination (25). Hybrid T cell lines were made by preparing a HAT-sensitive variant of the CEM line, and then fusing this with MLC-activated T cell blasts using polyethelene glycol. Hybrids were selected using HAT medium, and cloned by limiting dilution.

Proliferation Assays. Proliferation assays were carried out in round-bottomed microtiter trays (76-013-05 Linbro Chemical Co., Hamden, CT), measuring [³H]thymidine

incorporation, as described (26). The effect of different dilutions of LeoA1 and control antibodies was tested on untreated MNC (for mitogenesis), and together with irradiated B lymphoblasts (for the effect on MLC). MNC from at least three different donors were used for each assay, and separate plates were harvested on days 2–8 to assess kinetic effects. The mitogenic assays were also carried out with LeoA1 antibodies coupled to Sepharose beads.

Induction of CTL and AK Cells. Autologous or allogeneic B lymphoblasts were irradiated (4,000 rad) and used to generate CTL and AK cells from MNC of normal subjects in MLC, as given in detail elsewhere (15, 16, 20, 26). Flasks containing the cells (Lux 5375) were maintained upright for 7–10 d in 10% CO_2 in air, and after this time the cells were harvested and counted for testing in cytotoxicity assays. These cultures induced both CTL and AK cells. The CTL were able to kill the stimulating B lymphoblasts and, at the effector phase, they could be inhibited from killing by monoclonal antibodies to T3 and T8 (15, 16, 20). The AK cells were tested by their ability to kill the LiBr melanoma cell line, a cell which is insensitive to lysis by natural killer (NK) cells (16): the AK cells had not been sensitized against the melanoma cells and the killing could not be inhibited by antibodies to T3 or T8.

Limiting-dilution Assays. CTL and AK cells were induced by stimulating limiting numbers of MNC with autologous or allogeneic irradiated B lymphoblasts (15). For this, graded numbers of responder MNC were added to each well of a microtiter tray, together with 2×10^4 irradiated autologous MNC (filler cells) and 10^4 irradiated B lymphoblasts (stimulator cells). The plates were incubated for 3 d, and 10% (vol/vol) MLA-derived supernatant was added as a source of IL-2; thereafter, the IL-2 was supplemented every 3 d. The cells in each well were assayed for cytolytic activity against the two different target cells: B lymphoblasts for CTL function, and LiBr for AK cell function. We added each of the ^{51}Cr-labeled targets directly into the wells of half of each plate, and proceeded as described below for the cytolytic assay. In all experiments, 96 replicate cultures were set up at each responder cell concentration, and of these, 48 replicates were tested against each target cell. Results for each well were considered positive when the release of ^{51}Cr exceeded the mean of the 48 control wells (containing irradiated stimulator cells and filler cells only) by >3 SD. The fraction of negative cultures was plotted against the responder cell number; the data were calculated according to the zero-order term of the Poisson probability distribution based on the least squares method, and the 95% confidence limits were established as described (15). In experiments measuring the effect of antibody LeoA1, the ultracentrifuged antibody was added as a final volume of 10% of hybridoma supernatant, and the parallel series of (control) plates received the same volume of an irrelevant mAb of the same isotype.

Cytotoxicity Assay. We used ^{51}Cr-release assay, as described (26). Target cells were labeled with ^{51}Cr (100 μCi per 10^6 cells) and added to the wells of microtiter plates as 5,000 cells/well. Effector cells were added at different concentrations, the plates centrifuged (400 g for 1 min), and incubated at 37°C for 4 h. The plates were then centrifuged again, and 100 μl of supernatant, containing released radioactivity, was removed for measurement in the gamma counter. In bulk cultures, the results were calculated as the mean percent specific lysis (msl) (26). The spontaneous release of ^{51}Cr by each of the cell lines used was <10%, and the standard deviation of triplicate mean values was always <3% of the response. Significant differences between mean values were calculated by the one-tailed Student's t test.

Cell Staining and Flow Cytometric Analysis. Cultured cells were stained by direct or indirect immunofluorescence and analyzed using a fluorescence-activated cell sorter (FACS) (model II, Becton Dickinson and Co., Sunnyvale, CA) as described (27). The FACS was also equipped with a computer handling system, and three parameters were measured for each cell: forward light scatter (related to cell size), fluorescence from FITC-conjugated antibodies, and red fluorescence from propidium iodide, which was used to identify nonviable cells. In the antigen modulation studies, the antigens TLiSA1 and Tac were removed from MLC-activated T cells, which had been cultured in IL-2, by incubating them overnight at 37°C with LeoA1 or anti-Tac, respectively. The modulated cells were

then stained directly with FITC-labeled LeoA1 or rhodamine-labeled anti-Tac, and analyzed by dual-laser cytofluorimetry.

Immunoprecipitation and Polyacrylamide Gel Electrophoresis (PAGE). Human T cells that had been activated in MLC and cultured for 8 d in medium containing IL-2, and cells from the MLA-144 gibbon cell line were surface labeled with ^{125}I by the Iodogen method (28) and immunoprecipitated with antibody, as described (26, 28). The extracted lysates were precleared with uncoupled Sepharose beads and then Sepharose beads coupled to control (nonreactive) mouse mAb (kindly provided by Dr. A. López, Walter and Eliza Hall Institute) before precipitation with LeoA1 coupled directly to Sepharose beads. Sequential immunoprecipitations were performed in an identical manner, except that the lysates retained after the removal of TLiSA1 were used to precipitate IL-2-R with the anti-IL-2-R antibody coupled to Sepharose beads or, in the reverse procedure, LeoA1 was used to precipitate antigen after the removal of IL-2-R. The immunoprecipitates were washed, and run on 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide vertical slab gels (28).

Competitive Binding Assay and Scatchard Analysis. Purified LeoA1 antibody was labeled with ^{125}I to 10 μCi/μg protein by the chloramine-T method, and saturation-binding experiments were performed with increasing numbers of activated T cells. The T cells used were activated in MLC with irradiated B lymphoblasts for 6 d, then maintained in medium containing IL-2 for two additional days. For the competitive binding assays, 4 × 10^5 cells were treated with decreasing amounts of ^{125}I-labeled LeoA1 in the presence or absence of an excess of unlabeled LeoA1 for 1 h at 4°C (29). A binding curve was constructed to ensure that saturation was obtained, and points on the linear portion of this curve were used to construct a curve of the Scatchard type, as described (29). On the ordinate was plotted r/A−x, which is equal to the bound counts divided by free counts, divided by the concentration of cells expressed in "moles per liter," which is equal to the number of cells per milliliter divided by 6.023 × 10^{20} (29). On the abscissa was plotted r, which is the number of antibody molecules bound per cell; obtained by dividing the number of antibody molecules bound per milliliter by the number of cells per milliliter. The regression line was fitted by the least squares method, with the aid of a computer. From this line, the x-intercept gives directly the maximum number of antibody binding sites per cell, which equals the number of antigens per cell if each antigen molecule binds only one antibody molecule. The slope from the line yields directly the reciprocal molar concentration of the association constant, K_a.

Results

Cellular Distribution of LeoA1 Antibody Reactivity. Peripheral blood cells from 10 normal subjects were tested for reactivity with LeoA1 by indirect immunofluorescence and FACS analysis. Granulocytes, erythrocytes, and platelets did not bind the antibody. MNC from eight subjects showed low or negligible binding with a small proportion of cells that stained with low fluorescent intensity (Fig. 1A), and the resting cells from two individuals had 10–20% of cells displaying weak antibody binding (not shown). Monodispersed thymocytes from the thymuses of two children were analyzed in the same way, and these showed no antibody binding. Bone marrow cells from three adults did not bind LeoA1: this result was confirmed as zero binding by expanding the 5% of cells forming the tail of the fluorescence profile (which was identical to the control profile obtained using a nonreactive mouse mAb) by use of the computer facility on the FACS, and demonstrating that this population of cells had a similar size and 90° light scatter profile to the original sample; morphological examination of these cells showed no enrichment of any particular cell type.

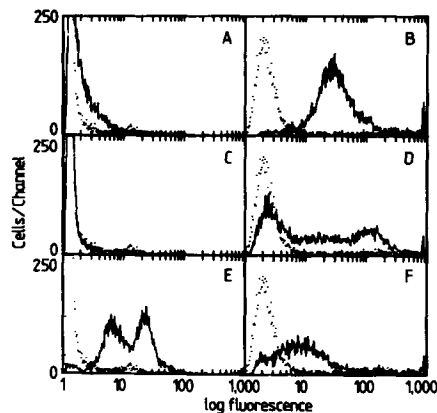LeoA1 did not bind to any of the human cell lines tested. These included the

FIGURE 1. Immunofluorescence profiles of human MNC before (*left*; A, C, E) and after (*right*; B, D, F) activation for 10 d in MLC. The cells were incubated with LeoA1 (*A* and *B*), LeoA2 (*C* and *D*), or LeoLFA-1 (*E* and *F*), and then FITC-labeled goat F(ab')₂ anti–mouse F(ab')₂. The dotted lines show staining in the absence of the first layer mouse mAb.

leukemic T cell lines CEM, Molt 3, and Molt 4; three B lymphoblastoid cell lines, K562, U937, and HL60; a fibroblastoid line; and two melanoma cell lines. Cells from the gibbon cell line MLA-144 displayed moderate antibody binding, and it is of interest that, alone of the cell lines tested, the MLA-144 cells secrete IL-2 and are dependent on this growth factor. Murine CTLL cells (an IL-2-dependent cell line) did not bind LeoA1.

After MLC of MNC with irradiated B lymphoblasts, the resultant T lympho-blasts expressed strong binding with LeoA1 (Fig. 1B). This was not due to expansion of a small subpopulation of LeoA1⁺ cells, since pretreatment of MNC with LeoA1 and complement had no effect on proliferation or on subsequent antigen expression (data not shown). For comparison, Fig. 1, C and D show the binding of an activation-specific antibody, LeoA2, which is not lineage-specific but is closely associated with proliferation, and Fig. 1, E and F show the binding of an antibody, LeoLFA-1, with the same specificity and function as other antibodies to LFA-1 (T. Triglia, unpublished observations). Results with these control antibodies suggest that the binding of LeoA1 is not absolutely dependent on cell proliferation, and show that the increased antibody binding after activation is not the result of a nonspecific increase in surface antigen expression. In MLC experiments with the MNC from eight different normal subjects, the distribution of binding of LeoA1 to the activated T cells was Gaussian on a logarithmic scale (Fig. 1B), and strong binding of LeoA1 by these activated cells was observed over 4 wk of culture. Moreover, after cloning and maintenance of the T cells in IL-2-containing medium for 2–3 m, these cells continued to bind LeoA1.

Kinetics of Expression of LeoA1 Reactivity on Activated T Cells. MNC were stimulated with the lectin phytohemagglutinin (PHA), and monitored for binding of LeoA1 at various time points. One of three such experiments is illustrated in Fig. 2. An increase in antibody binding was seen by 24 h of culture, but the majority of the cell population did not bind the antibody until after 48 h, and
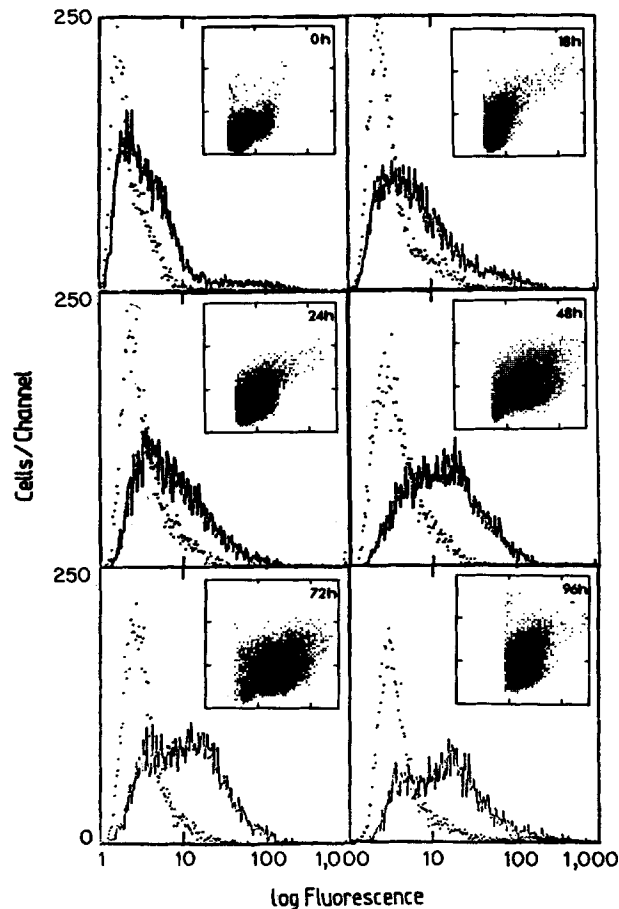
FIGURE 2. Kinetics of the expression of binding of LeoA1 antibody to human MNC after stimulation with PHA. Cells were stimulated with PHA (0.5 μg/ml), then tested for binding of LeoA1 antibody by indirect immunofluorescence at each of the time points indicated in the top right corner of each panel. The main panels illustrate the immunofluorescence profiles, dotted lines show fluorescence in the absence of LeoA1; each inset displays the fluorescence intensity (ordinate) versus the relative cell size (abscissa). Nonviable cells have been gated out.

the number of cells binding LeoA1 continued to increase through 96 h of culture. It is also clear from Fig. 2 (inset) that it is not only the large (presumably dividing) cells that bind LeoA1. These data indicate that the antigen bound by LeoA1 is an "early" activation antigen (1), although the kinetics of its expression and the persistence of expression appear to be slower and longer than that of the IL-2-R on PHA-activated T cells.

Characterization of the TLiSA1 Antigen Recognized by LeoA1. Surface labeling of alloantigen-activated T cells with ^{125}I, and immunoprecipitation and gel analysis of the extract (Fig. 3) showed that the LeoA1 antibody precipitated a single broad band spread over an area representing a range of M_r ~65,000–75,000. When surface-labeled MLA-144 cells were used, a much sharper band of M_r 95,000 was obtained (Fig. 3). The reasons for this discrepancy are not
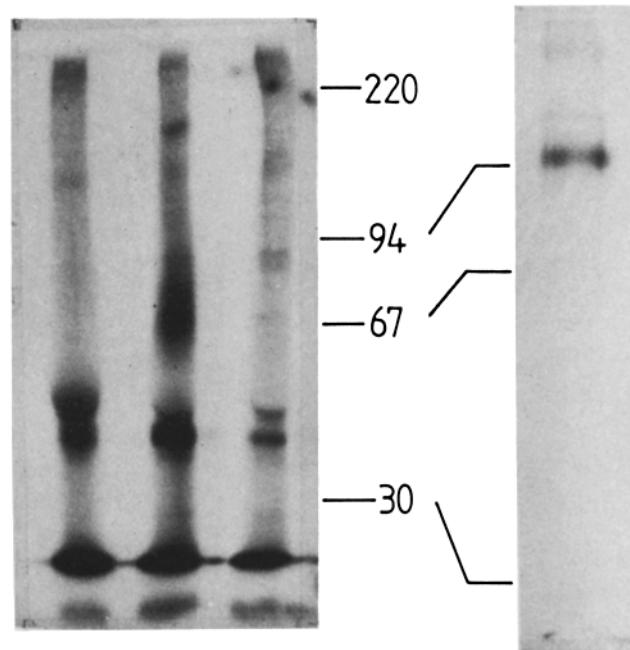
FIGURE 3. Immunoprecipitate of the TLiSA1 antigen from human T cell blasts and from cells of the gibbon MLA-144 cell line. Cells were surface-labelled with ^{125}I by the Iodogen method, immunoprecipitated with LeoA1, and run out on 7.5% SDS-PAGE under reducing conditions. The first track shows the preclear of human T blasts with Sepharose beads coupled to an irrelevant mouse mAb. The second shows the same lysate precipitated with Sepharose-LeoA1, and the third shows the absence of immunoprecipitate when labeled K562 cells were treated with Sepharose-LeoA1. The dark spots at ~220,000 mol wt in 2 and 3 are nonspecific spots on the autoradiograph. The right panel shows the precipitate obtained when MLA-144 cells were immunoprecipitated with LeoA1 under the same conditions. The molecular weight markers are: ferritin subunit (220,000), phosphorylase b (94,000), albumin (67,000), and carbonic anhydrase (30,000).

clear, but immunoprecipitation obtained from the activated T cells of some subjects revealed a weak band in the region of M_r 95,000, in addition to the strong band at M_r 70,000.

Cell Surface Expression of TLiSA1 Antigen. Cold antibody displacement of ^{125}I-labeled LeoA1 was used to determine the number of antibody-binding sites per MLC-activated T cell blast; two independent experiments gave the same result. Analysis of the results (Fig. 4) showed that LeoA1 bound to a maximum of 66,000 sites per cell, with an association constant, K_a, of ~1.1 × 10^9/M.

Function of the LeoA1 Antibody. The LeoA1 antibody was tested for its mitogenic effect on the MNC of six donors at different times. The antibody had no direct mitogenic effect at final dilutions of 1:100–1:5,000 of ascites, nor when coupled to Sepharose beads, when the cells were assayed for proliferation on days 3, 4, 5, and 6 (data not shown). The antibody also had no effect on the proliferation induced either with PHA (data not shown) or with irradiated allogeneic B lymphoblasts (Table I). Because of the effect of LeoA1 on MLC-induced maturation of CTL and AK cells (see below) it was particularly important to demonstrate that the antibody did not alter the magnitude or the kinetics of
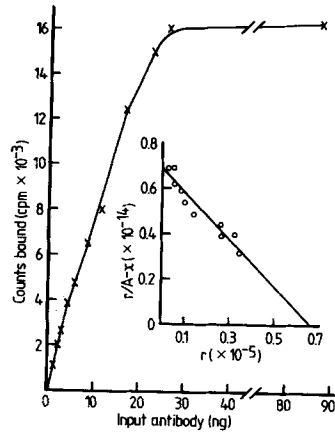
FIGURE 4. ^{125}I-labeled LeoA1 binding to human T cell blasts. The binding of nanograms of added antibody to saturating amounts is shown; the inset shows Scatchard analysis of the data. From this experiment, the maximum number of antibodies bound was 66,000 per cell, with a K_a of 1.1×10^9/M.

TABLE I

Effect of LeoA1 and Anti-Tac Antibodies on the Proliferation of MNC
Induced with Allogeneic B Lymphoblasts

Antibody	Proliferation (cpm ±SD)
Control (25 E11)	56,280 ± 3,186
LeoA1	56,410 ± 4,942
Anti-Tac	18,808 ± 3,049*

The proliferation shown is that obtained with MNC after 6 d of culture in the presence of antibody at a final dilution of 1:5,000 ascites.
* $P < 0.001$ by Student's t test.

the proliferative response. The experiment shown in Table I was repeated on MNC from six different subjects, with antibody present at final dilutions between 1:100 and 1:5,000, and with harvesting of the plates at days 4–8 of the response. On no occasion did the proliferation in the presence of LeoA1 differ significantly from that in the presence of an antibody of irrelevant specificity, whereas the anti-Tac antibody significantly inhibited proliferation even when present at a final dilution of 1:5,000 ascites (Table I).

The ability of LeoA1 to block the effector phase of killing of B lymphoblasts by CTL, and of melanoma cells by AK cells generated in the same MLC was tested by adding antibody to the cultured effector cells immediately before, or 30 min before, the addition to ^{51}Cr-labeled target cells, and then measuring cytotoxic activity. A control antibody that does not bind to T cells (25 E11) was included in every experiment, and the LeoLFA-1 antibody, which inhibits killing by CTL, AK, and natural killer cells (T. Triglia, unpublished data) was included as a positive control. LeoA1 failed to inhibit killing mediated by CTL or AK cells (Table II), with similar data being obtained in seven separate experiments.

In marked contrast, the addition of LeoA1 to MLC at the beginning of culture

TABLE II

Antibody LeoA1 Does Not Inhibit Killing by CTL or AK Cells at the Effector Phase

Exp.	Antibody	Final antibody dilution*	Cytolysis against[‡]	
			B lymphoblasts	Melanoma cells
			%	
1	Control (25 E11)	1:800	58 ± 2.5	61 ± 3.7
	LeoA1	1:800	58 ± 1.8	67 ± 2.4
	LeoLFA-1	1:800	19 ± 3.2[§]	37 ± 2.7[§]
2	Control (25 E11)	1:30	12 ± 0.6	31 ± 2.4
		1:300	13 ± 0.4	30 ± 0.9
	LeoA1	1:30	13 ± 0.5	29 ± 2.4
		1:300	12 ± 0.6	29 ± 1.7
	LeoLFA-1	1:30	7 ± 0.4[§]	17 ± 0.9[§]
		1:300	4 ± 0.1[§]	10 ± 0.5[§]

* The dilution given is the final dilution of ascitic fluid in each well. In each assay the ascitic fluid was diluted and ultracentrifuged before testing for blocking activity.
[‡] CTL and AK cells were generated by coculture of MNC with irradiated B lymphoblasts, as described in Materials and Methods. After culture, the activated T cells were harvested and tested against the stimulating B lymphoblast and melanoma cells. The lysis shown is the percent mean specific lysis ±SD from three replicates at an effector/target ratio of 10:1 from cells harvested after 6 d of culture.
[§] $P < 0.001$ by Student's t test.

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TABLE III

Inhibition of the Generation of CTL and AK Cells by the Presence of LeoA1 or Anti-Tac During MLC

Exp.	Antibody	Isotype or fragment	Cell lysis	
			B lymphoblasts	Melanoma
			LU*	
1	WEM-G11	IgG1	420	900
	LeoA1	IgG1	170	430
	25 E11	IgG2a	450	920
	Anti-Tac	IgG2a	430	910
2	WEM-G11	IgG1	308	615
	WEM-G11	F(ab')₂	308	615
	LeoA1	IgG1	83	66
	LeoA1	F(ab')₂	80	114
	Anti-Tac	IgG2a	25	229

* Cell lysis data are expressed as the lytic units (LU) per 10^7 cells causing 30% lysis, obtained from the effector cells grown in flasks for 6 d with antibodies present at a final concentration of 1:2,000 ascites in exp. 1. The F(ab')₂ results shown in exp. 2 were obtained at a concentration of 150 μg/ml; a range of concentrations was tested, with similar results. The cells were then harvested, counted, and tested for cytotoxicity against B lymphoblasts and melanoma cells over a range of effector/target ratios.

resulted in a great reduction in the activity of induced effector CTL and AK cells (Table III). The antiproliferative effect of anti-Tac was excluded in these experiments, since they were carried out by setting up the MLC in flasks and then testing the CTL and AK effector cells over a range of effector/target ratios.

The results (Table III) indicated that LeoA1 inhibited the generation of both CTL and AK effector cells, and that anti-Tac did not consistently inhibit this process. Since it is known that the Fc fragment of antibodies can exhibit inhibitory effects (30), the experiments were repeated with $F(ab')_2$ fragments of LeoA1. The $F(ab')_2$ fragments consistently inhibited the development of CTL and AK effector cells at a final concentration as low as 30 μg/ml (Table III). Also, the addition of LeoA1 after 1 d of coculture resulted in the same inhibitory effect (Table IV), indicating that the antibody was not interfering with a primary recognition event. The addition of antibody on days 4 (data not shown) or 6 (Table IV) did not lead to any inhibitory effect. The experiments shown in Tables III and IV were repeated six times using MNC from different subjects, all with similar results. We then decided to determine the effect of LeoA1 on the frequency of CTL and AK cells generated under conditions of limiting dilution.

In two experiments, the presence of LeoA1 from the beginning of culture decreased the numbers of CTL developing from their precursors from 1:450 (in the presence of control antibody) to 1:750 (Fig. 5), and from 1:900 to 1:1,750 (not shown); the frequency of AK cells generated in the same experiments was also greatly reduced, from 1:200 to 1:1,550 (Fig. 5), and from 1:400 to 1:1,800 (not shown). The proliferation obtained in these cultures could not be measured directly, but microscopic examination of the wells before testing for cytotoxicity confirmed our previous results that LeoA1 did not inhibit proliferation. Nevertheless, when we analyzed these data as the mean cytotoxicity obtained for all of the wells at each different concentration of responder cells added, we found that the presence of LeoA1 reduced significantly the levels of cytotoxicity obtained at every responder cell concentration, even when all of the wells were positive and therefore excluded from Poisson analysis of the limiting-dilution data. Together, these findings suggest that LeoA1 antibody inhibited the development of CTL and AK cells without affecting the proliferation of their precursors.

Discrimination of the TLiSA1 and Tac Antigens. As shown in Table I, the LeoA1 antibody, unlike anti-Tac, did not inhibit the proliferation of activated T

TABLE IV
Inhibition of the Generation of CTL and AK Cells by the Addition of LeoA1 During MLC

Antibody*	Day anti-body added	Cytolysis against[‡]	
		B lymphoblasts	Melanoma cells
		%	
Control (25 E11)	0	31 ± 1.8	61 ± 2.1
LeoA1		13 ± 0.4[§]	38 ± 1.6[§]
Control	1	31 ± 3.9	57 ± 1.8
LeoA1		11 ± 0.7[§]	30 ± 3.8[§]
Control	6	30 ± 1.1	55 ± 1.6
LeoA1		30 ± 3.0	56 ± 5.1

* Antibody was added to a final dilution of 1:100 ascites.
‡ As in Table II.
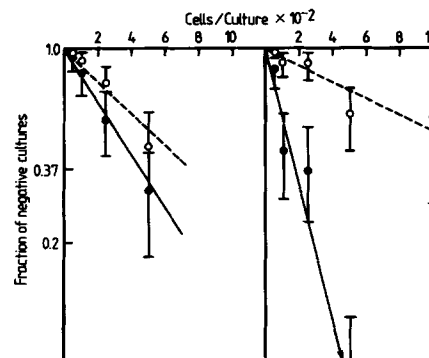§ $P < 0.001$ by Student's t test.

Cells/Culture × 10⁻²

FIGURE 5. Limiting-dilution analysis of cytotoxic precursor cells from blood MNC generated in MLC with irradiated B lymphoblasts in the absence (——) and presence (- - -) of LeoA1 antibody. Control antibody or LeoA1 was added on day 0, and a source of IL-2 was added on days 3, 6, and 9. The cultures were tested on day 10 for cytotoxicity against the stimulating B lymphoblasts for CTL (*left*), and against unrelated melanoma cells for AK cells (*right*). Each data point was determined from 48 cultures, and the lines were fitted by the method of least squares. Error bars are the 95% confidence limits obtained from Geigy's statistical tables.

cells. This does not exclude, however, the possibility that the two antibodies are detecting different epitopes on the same antigen, with only anti-Tac blocking the actual binding site for IL-2. That LeoA1 was not detecting an epitope on the IL-2-R was shown in four ways: (a) LeoA1 was used to strip PHA-induced T cell blasts of all detectable TLiSA1, and the cells were used to absorb IL-2 from the supernatant of MLA-144 cells. After this treatment it was found that the TLiSA1⁻ cells absorbed IL-2 equally as well as untreated control blasts (data not shown). (b) In fluorescent antibody binding studies, removal of TLiSA1 by modulation with LeoA1 overnight at 37°C did not affect the level of staining with the anti-Tac antibody, and modulation in the reverse direction, by first removing Tac, did not alter the expression of TLiSA1. (c) The same results were obtained in sequential immunoprecipitation experiments using the lysates of T cell blasts surface labelled with ¹²⁵I. Preclearing the lysates with anti–IL-2-R did not reduce the TLiSA1 precipitate obtained with LeoA1 precipitation, and conversely, removal of TLiSA1 did not reduce the subsequent amount of IL-2-R precipitated by the anti–IL-2-R antibody (data not shown). (d) A cloned hybrid cell line was constructed between MLC-activated T cell blasts and a HAT-sensitive variant of the CEM cell line. This hybrid line expresses T3 and T4 antigens but does not respond to nor produce IL-2 (data not shown). Using indirect immunofluorescence and FACS analysis, we found that this clone (and several others from different fusions) expressed the TLiSA1 but not Tac (Fig. 6).

Discussion

Activation antigens that are lineage specific appear to be likely candidates in the search for important functional molecules. In this paper, we study an antigen, TLiSA1, which was absent from the surface of a variety of normal tissue cells, transformed cells, and tumor cell lines, but which was expressed on activated human T cells and short-term cultures of these cells, and on a retrovirus-infected
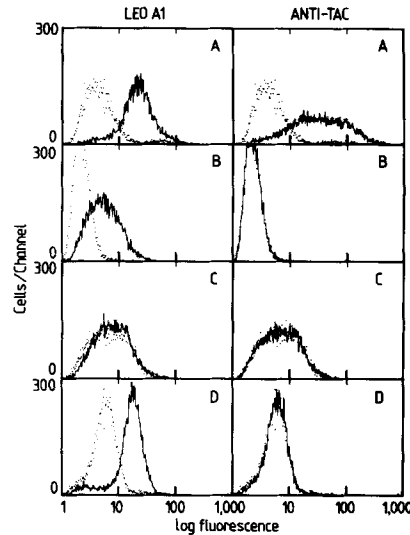
FIGURE 6. Immunofluorescence profiles of various cell lines indicated, treated with LeoA1 or anti-Tac antibody, then FITC-labeled goat F(ab′)₂ anti–mouse F(ab′)₂. The dotted lines show staining in the absence of the first layer mouse mAb. (A) peripheral blood T cells activated in MLC; (B) a hybrid cell line (47C7) between MLC-activated T cells and CEM cells; (C) the CEM cell line; (D) the gibbon cell line MLA-144.

primate T cell line. This distribution pattern distinguishes the antigen from several other known activation antigens that are not lineage specific (1–3, 31), and places it alongside the very few T lineage–specific activation antigens that have been reported (5, 10–12). Size alone is sufficient to distinguish TLiSA1 from the 160 and 210 kilodalton (kD) subunits of the A-1A5–defined complex of Peters et al. (11) and from both the T11$_3$ (10) and Ta$_1$ (12) antigens described previously.

TLiSA1, however, appeared as a broad band after SDS-PAGE, a feature that is also characteristic of Tac (32). Although the Tac antigen is usually described as being of M_r 52,000–57,000 on normal activated T cell blasts (32), estimates of its size vary, and it has been described (9, 12) as a band of 60–65 kD. Also, the numbers of molecules of anti-Tac and LeoA1 antibodies bound by activated lymphoblasts are approximately equivalent (32), as is the kinetics of expression of both antigens after PHA stimulation of resting MNC. It was important, therefore, to distinguish TLiSA1 from Tac. Removal of TLiSA1 from the surface of T blasts did not prevent these cells from absorbing IL-2, and the two antigens did not comodulate or coprecipitate, indicating that they are not linked. Anti-Tac antibody did not bind to the MLA-144 gibbon cell line, which was TLiSA1$^+$, but this is most likely due to absence of the Tac epitope on primate cells, since this line is IL-2-dependent. More pertinent was the existence of a human T × T hybrid cell line that was TLiSA1$^+$, Tac$^-$. This line expressed other T cell antigens and activation antigens (T. Triglia, unpublished observations), and would be expected to express the Tac epitope if the gene was expressed (11). Thus, the results strongly support the independence of TLiSA1 and Tac, and perhaps

suggest that the genes coding for these two proteins are on separate chromosomes.

In experiments using MLC to generate CTL and AK cells, the presence of LeoA1 antibody inhibited the induction of mature effector cells, but did not affect proliferation. It is now being recognized that the appearance of AK cells in both mouse and man is caused by activated T cells that are able to kill NK cell–resistant target cells (13–16, 33–36), and not by residual NK cells. Indeed, the current concept of AK cells is that they arise in culture by the further differentiation of MHC-restricted CTL (16, 33, 37–40). If this is so, the results described herein suggest that LeoA1 antibody is inhibiting the differentiation of the precursors of CTL, and of CTL cells themselves.

The mechanism of this inhibition is speculative. Suppressor cells may be activated by the antibody but, since suppressor mechanisms probably operate by preventing proliferation (41), this seems unlikely. It has been suggested (42) that, in mice, differentiation of T cells can be dissociated from proliferation, and that a differentiation molecule distinct from IL-2 is involved in this process (17–19, 43). It is possible, therefore, that LeoA1 acts by interfering with human cells' uptake of a homologous factor, or by interfering with the function of such a factor. Investigation of this possibility is the subject of current research in our laboratory.

Summary

The characteristics of a novel T lineage–specific activation antigen, termed TLiSA1, are described. The antigen was detected with a mouse monoclonal antibody, LeoA1, that was raised against activated human T cells generated in mixed lymphocyte culture (MLC). The antigen became strongly expressed on T cells 48–72 h after stimulation with phytohemagglutinin, and retained expression on MLC-activated T cells after 10 d of culture. The antigen was absent from a range of human T, B, myeloid, fibroblast, and tumour cell lines, but was present on the surface of the interleukin 2 (IL-2)-dependent gibbon cell line MLA-144. Analysis of the antigen by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of immunoprecipitates obtained from activated human T cells demonstrated a broad band in the region of 70 kD, whereas precipitates obtained from MLA-144 revealed a single narrow band of 95 kD. The molecule was expressed with a maximum density of 66,000 copies per cell on the surface of MLC-activated T cell blasts, as assessed by Scatchard analysis. TLiSA1 was distinguished from the IL-2 receptor bound by the anti-Tac monoclonal antibody by demonstrating that the antigens did not comodulate or coprecipitate, and by constructing an IL-2-independent human T × T hybrid that expressed the TLiSA1 but not the Tac antigen. MLC with B lymphoblasts was used to generate cytotoxic T lymphocytes (CTL) specific for the stimulating cell, and anomalous killer (AK) cells able to kill melanoma target cells. The presence of LeoA1 or F(ab')$_2$ fragments of the antibody from the beginning of coculture did not affect proliferation in these cultures, but did inhibit the induction of both CTL and AK cells from their precursors. This inhibition of differentiation by LeoA1 was confirmed under conditions of limiting dilution, where it was shown that the antibody reduced the frequency of CTL produced, and greatly (fourfold) re-

duced the frequency of AK cells generated from their precursors. We discuss the possibility that human CTL may express a differentiation factor receptor that is distinct from the receptor for IL-2.

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