Increased Density of Ecto 5' Nucleotidase Antigen on Leukemic T Cells From Patients With Cutaneous T-Cell Lymphoma and Adult T-Cell Leukemia/Lymphoma

By Yoshitaka Fukunaga, Sharon S. Evans, Masayo Yamamoto, Yutaka Ueda, Kazuo Tamura, Toshihumi Takakuwa, Douglas Gebhard, Janet Alloppenna, Sandra Demaria, Bayard Clarkson, Linda F. Thompson, Bijan Safai, and Robert L. Evans

Malignant CD4+ T cells in adult T-cell leukemia/lymphoma (ATL) and cutaneous T-cell lymphoma (CTCL) express a number of cell surface molecules that are upregulated on normal T cells activated by foreign antigen. In this report we describe an interesting exception to the parallel phenotypic features of activated T cells and malignant CD4+ T cells. A monoclonal antibody (MoAb; termed 27.2) that was raised to HTLV-1+ , CD4+25+ leukemic T cells stained weakly 25% of peripheral T cells, including approximately 50% of CD8+ T cells and 20% of CD4+ T cells. Flow cytometry analysis indicated that the surface density of the 27.2 antigen was unchanged or diminished when normal T cells were activated by antigen. However, 3/4 Sezary cases and 4/8 cases of ATL had relatively high densities of the 27.2 antigen. Immunoprecipitation and sodium dodecyl-sulfate polyacrylamide gel electrophoresis of the NP-40-solubilized membranes of surface-iodinated ATL cells indicated that MoAb 27.2 reacted with a 75 Kd molecule. The size and distribution of the 27.2 antigen on T cell subsets suggested that it might be the enzyme ecto-5' nucleotidase (NT), a phosphatidylinositol-linked enzyme that catalyzes dephosphorylation of monophosphate nucleotides to their respective nucleosides. This was confirmed by demonstrating that lymphocyte ecto-5'NT activity was blocked partially and inhibited completely by preincubating cells with MoAb 27.2 for 1 hour at 4°C and 24 hours at 37°C, respectively. When used with a second MoAb (27.1) to a novel T cell activation antigen found on all ATL and CTCL leukemias examined, 27.2 was found to discriminate between normal and leukemic T cells in two patients with ATL. These studies suggest that ecto-5'NT has diagnostic value in T cell malignancies and may be aberrantly expressed in some cases of ATL and CTCL.

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From the Department of Pediatrics, Nippon Medical School Hospital, Tokyo, Japan; the Roswell Park Memorial Institute, Buffalo, NY; the Department of Internal Medicine, Miyazaki Prefectural Hospital, Miyazaki, Japan; the Department of Pathology, St Marianna University School of Medicine, Kanagawa, Japan; the Memorial Sloan-Kettering Cancer Center, New York, NY; the Scripps Clinic and Research Foundation, LaJolla, CA.

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Address reprint requests to Robert L. Evans, MD, Department of Molecular Medicine and Immunology, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14263.

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Hoist, Miyazaki, three were seen at St Marianna University Hospital, Kanagawa, and one patient was seen at the Nippon Medical School Hospital, Tokyo. The clinical diagnosis of ATL was based on clinical features, characteristic hematologic findings,3,4 and the presence of serum antibodies to HTLV-I (ATLA).25

**MoAbs.** The MoAbs 27.2 (immunglobulin G2b [IgG2b]) and 27.1 (IgG1) were raised by immunizing an outbred CD-1 mouse (Charles River, Boston, MA) with HTLV-I + leukemic T cells from patient A.W. and fusing the splenocytes of the hyperimmune mouse to the hypoxanthine aminopterin thymidine (HAT)-sensitive mouse myeloma line NS-1.26 Leukemic T cells from A.W. were HTLV-I +, CD25 +, CD34 + and Leu-4 +. The mouse MoAbs to the CD5, defined by anti-Leu-1), CD8, (anti-Leu-2a), CD4 (anti-Leu-3a) and CD3 (anti-Leu-4) antigens were generated in our laboratory and used as culture supernatants for cell staining. A CD25 antibody to the IL-2 receptor was used (Becton-Dickinson Company, Sunnyvale, CA). The P3 myeloma protein (IgG1) which does not bind to human cell surface determinants was used as a control for nonspecific binding.

**Isolation of leukemic subpopulations.** Mononuclear cells were separated from fresh heparinized blood of normal individuals by a two-step process involving passage over nylon wool columns followed by ethyrocyte-rosette formation at 25°C using untreated sheep erythrocytes and density-gradient centrifugation. Monocytes were isolated using plastic adherence. B cells were isolated from tonsillar lymph node specimens by rosetting the cells at 4°C with 2-aminoethylisothiouronium bromide (AEI)-treated sheep red blood cells (SRBC) and separating the ethyrocyte-rosette forming cells (ERF-C), (T cells), from the non-ERF-C, (B cells), by density-gradient centrifugation. Polymorphonuclear leukocytes were isolated from the sediment of whole peripheral blood that had undergone Ficol-Hypaque density-gradient centrifugation by lysing RBCs with NH4Cl. Neutrophil viability was greater than 90% after each of these procedures routinely yields a purity of greater than 95% of the relevant leukocyte subset with the exception of tonsillar B cells, which are greater than 90% pure.

**Isolation of T-cell subsets.** Purified subsets of T cells were obtained by a "panning" technique in which T cells labeled with anti-Leu-2a or anti-Leu-3a were incubated on plastic petri dishes coated with an F(ab)2 goat-anti-mouse reagent (Cappel Laboratories, Cochranville, PA). Gentle removal of the nonadherent population, (Leu-2a or Leu-3a), and flow cytometry analysis revealed that the negatively selected cells were less than 5% positive for the relevant marker.

**Indirect immunofluorescence analysis.** Analysis of all populations was performed by indirect immunofluorescence with the F(ab)2 fragment of affinity-purified, fluorescein-conjugated goat-anti-mouse IgG (goat anti-mouse FITC; Cappel Laboratories, Cochranville, PA), in a fluorescence-activated cell sorter (FACS IV; Becton-Dickinson Co., Sunnyvale, CA). In brief, 1 x 10^6 cells were incubated at 4°C with an appropriately diluted culture supernatant containing a MoAb for 1 hour. The cells were then washed and mixed with 0.1 mL of goat-anti-mouse fluorescein isothiocyanate (FITC) at 1/40 dilution for 1 hour, washed two times, and fixed in 1% paraformaldehyde-phosphate buffered saline (PBS) before analysis.24

**Iodination, immunoprecipitation, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.** Surface iodination of normal and leukemic T cells was performed by labeling approximately 1 x 10^6 viable cells with 4 mCi ^125I (Amersham; Oakville, Ontario, Canada). After addition of 400 μg lactoperoxidase to a suspension of cells and ^125I in 1 mL PBS at 25°C, 30 μL of 0.3% H2O2 was added three times every 5 minutes. The cells were washed four times in PBS and solubilized in 1.0 mL buffer pH 8.0 containing 1% NP-40, 50 mmol/mL Tris, 150 mmol/mL NaCl, 0.02% NaN3, 5 mmol/mL EDTA, 25 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 50 mmol/L iodoacetamide. This suspension was then centrifuged at 600 g x 5 minutes, passed through a 0.2 μm filter and preincubated with approximately 0.2 mL sepharose 4B beads coupled to protein A (Pharmacia, Piscataway, NJ) at 4°C. Approximately 0.5 mL of the preincubated sample was incubated for 1 hour at 4°C with 50 μL of goat-anti-mouse Ig agarose beads (Sigma, St Louis, MO) that were "armed" with the appropriate MoAb and washed in buffer pH 8.5 containing 0.5% NP-40, 0.045 mol/L NaCl, 50 mmol/L Tris, 5 mmol/L KI, and 0.02% NaN3. Antigens precipitated by these beads were denatured by boiling for 5 minutes in the presence of or absence of 2% 2-mercaptoethanol and were electrophoresed in 12.5% SDS-polyacrylamide gels.

**Assay of ecto-5'NT activity.** Ecto-5'NT activity on viable peripheral blood lymphocytes was quantitated essentially as described previously.24,25 Before assay, T lymphocytes were preincubated with medium alone or the MoAb 27.2. Briefly, ecto-5'NT was assayed in a 1 mL volume-containing 2 x 10^5 to 2 x 10^7 T cells, 8.6 mmol/L MgCl2, 50 mmol/L Tris HCl, pH 6.9, 20 μmol/L AMP and tracer amounts of ^3H-AMP (25.5 Ci/mol/L, NEN). Following a 30-minute incubation at 37°C, during which the cells remain intact, the reaction was stopped by the addition of 0.2 mL of 0.25 mol/L ZnSO4. Protein and unhydroyzed AMP were precipitated by the addition of 0.2 mL 0.25 M Ba(OH)2. Aliquots of supernatant-containing hydrolyzed AMP were counted in a Beckman β-scintillation counter. Ecto-5'NT activity, expressed as nmol/L/hr/10^6 cells, is based on linear regression analysis of five points with each point representing a sample that contains different cell numbers.

**RESULTS**

**Reactivity of MoAbs 27.1 and 27.2 with subpopulations of normal leukocytes.** As indicated by indirect immunofluorescence analysis in a FACS, (Fig 1), both 27.1 and 27.2 stained the HTLV-I +, CD25 + leukemic ATL cells from patient A.W. much more brightly than normal resting T cells, and reacted with unique subsets of the peripheral T cell pool. When peripheral blood T cells from 10 normal individuals were tested, 27.2 stained 17% to 31%, while 27.1 stained 12% to 23%.

Figure 1 also shows the FACS profiles of leukemic cells from two cases of CTCL stained with 27.1 and 27.2. MoAb 27.2 stained only one more brightly than normal T cells, whereas 27.1 stained both brightly. Although the FACS scatter plots of the cells are not shown, the sizes of the leukemic T cells from these three cases differed only slightly from normal T cells and therefore did not influence their relative fluorescence intensities.

To determine the distribution of the 27.1 and 27.2 epitopes on the two major subsets of normal T cells defined by the CD8 and CD4 surface markers, we isolated these subsets by a "panning" technique. As shown by two representative experiments depicted in Table 1 indirect immunofluorescence analysis of the isolated subsets indicated that the majority of 27.2 + T cells had the CD4-8 + phenotype, whereas the 27.1 epitope was primarily distributed on resting CD4 + T cells.

Experiment 2 in Table 1 also shows the effect of activating T cells in a mixed leukocyte culture on the expression of the 27.1 and 27.2 antigens. When stimulated for 5 days in a standard one-way MLC the number of 27.2 + cells declined,
Fig 1. Indirect immunofluorescence analysis in a FACS of normal T cells (A), A.W.'s leukemic ATL cells (B), and two other patients with leukemic CTCL (C and D, Sezary syndrome) stained with either a negative control (curve a), antibody 27.2 (curve b), or antibody 27.1 (curve c).

whereas the number of T cells expressing the 27.1 epitope increased. In other experiments (data not shown) using specific soluble antigen PHA or Con A to stimulate T cells, the number and fluorescence intensity of cells stained by 27.1 was found to correspond to staining by a MoAb that reacts with HLA-DR molecules, suggesting that 27.1 recognized a T cell activation antigen. In no case was the 27.2 antigen found to increase on normal T cells as the consequence of activation in vitro.

Table 2 shows the reactivity of 27.1 and 27.2 with different subpopulations of leukocytes. As with peripheral blood T cells these two antibodies were found to stain weakly small subpopulations of thymocytes and tonsillar T cells, although two of six thymic specimens did not have detectable numbers of 27.1+ or 27.2+ cells. 27.2 also stained a subset of tonsillar B cells whereas 27.1 did not. Monocytes, RBCs and polymorphonuclear leukocytes were unreactive with both antibodies. These results suggest that the antigen recognized by 27.2 is expressed by subsets of T and B lymphocytes.

MoAb 27.2 precipitates a 75 Kd cell surface antigen from ATL cells. To determine the size of the antigen defined by MoAb 27.2 the leukemic T cells from patient A.W. were labeled with 125I by the lactoperoxidase method and solubilized in buffer containing 1% NP-40. SDS-PAGE analysis of material precipitated by 27.2 revealed a 75-Kd band (Fig 2, lane b) when gels were run under reduced conditions. In other experiments this antigen was found to have a comparable molecular weight when precipitated by normal T cells and run under reduced and nonreduced conditions (data not shown). A discrete component precipitated by MoAb 27.1 was not seen in our gels (Fig 2, lane a).

Reactivity of MoAbs 27.1 and 27.2 with leukemias. Table 3 shows the reactivity of 27.1 and 27.2 with leukemic cells from cases of acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML) and chronic lymphocytic leukemia (CLL). 27.2 reacted with one case of non-T-ALL and with two of 16 cases of B-CLL. MoAb 27.1 reacted only with a case of T-ALL. These results are compatible with the observation that 27.2 reacted with a subset of normal B cells, whereas 27.1 did not (Table 2).

Leukemic cells from seven cases of ATL in Japan, four cases of CTCL, and one case of ATL (patient A.W.) in the US were studied. Table 4 shows the age, sex, white cell count, and percentage of cells stained with a panel of MoAbs and analyzed in a FACS. In all cases except no. 5 the cells were obtained from the peripheral blood. In case 5, which was a primary gastric lymphoma, the cells were derived from a cervical lymph node.

In 10 of 12 cases, there was good concordance between the number of cells stained with the pan-T cell antibody anti-Leu-1, and the subset-specific antibody, anti-Leu-3a. This was consistent with the absence of circulating Leu-2+ (CD8+) cells in these patients. An antibody to the IL-2 receptor (CD25) reacted with the majority of cells from five of eight cases of ATL and one of three cases of CTCL that were tested. Three cases of ATL (nos. 2, 3, and 5) were stained weakly by the CD25 antibody, whereas all cases of ATL and CTCL were stained brightly by MoAb 27.1 (Table 4).

MoAb 27.2 stained only a fraction of cells in two cases of chronic ATL (nos. 3 and 6) as did anti-Leu-3a. It should also
be mentioned that the subset of 27.2+ cells in these two cases fluoresced more brightly than normal 27.2+ T cells and also expressed the 27.1 epitope as determined by additive staining with 27.1 and 27.2 MoAb (Table 4). In fact, except for patient 8, all cases of 27.2+ leukemic T cells were stained more brightly than normal 27.2+ T cells. These findings suggest that the leukemic T cells in cases 3 and 6 were reactive with 27.2.

All four cases of CTCL were 27.2+ but only four of eight cases of ATL had this phenotype. Both cases of acute ATL and the lymphoma type ATL were 27.2+, but three of four cases of chronic ATL were 27.2+. The three cases of 27.2+ ATL did not have lymphadenopathy, hepatosplenomegaly, or hypercalcemia at the time of diagnosis.

The antigen recognized by MoAb 27.2 is the ecto-enzyme 5'NT. Recent reports of the distribution of the enzyme ecto 5'NT on subpopulations of T cells defined by CD4 and CD8 antibodies suggested that similar numbers of T cells in these subsets (20% and 50%, respectively) were positive for ecto-5'NT and 27.2. Moreover, the staining profiles of T cells reacted with 27.2 and analyzed by flow cytometry appeared very similar to the histogram plots reported for T cells stained with an anti-ecto-5'NT polyclonal serum, suggesting that both reagents defined an antigen of comparable density on the same number of peripheral T cells. Table 5 shows that preincubating plastic nonadherent peripheral blood mononuclear cell (PBMCs) at 37°C for 18 hours with MoAb 27.2 (5 µg/mL) before assay abolished the ecto-5'NT enzymatic activity. This result was reproduced in four separate experiments. When PBMC were preincubated with MoAb 27.2 for 1 hour at 4°C before assay of ecto-5'NT activity, inhibition (blocking) ranged between 37% and 49% in four separate experiments.

It is notable that partial blocking of enzymatic activity by anti-ecto-5'NT MoAbs has been reported by one group who found a marked variation in blocking capacity among five different MoAbs. The fact that MoAb 27.2 inhibited more effectively following an 18-hour incubation at 37°C than after a 1-hour incubation at 4°C is consistent with data indicating that this antibody modulates the 75 Kd antigen from the cell surface at 37°C (Table 5). Data depicted in Table 5 also indicate that incubating T cells with MoAb 27.2 for 18-hours at 37°C modulated the epitopes recognized by two MoAbs that were recently prepared to purified ecto-
Ecto-5'-NT activity or stained with anti-ecto-5'-NT MoAb and analyzed in a FACScan. 18 hours and assayed medium containing MoAb 27.2 (50 ng/mL) for did not increase their fluorescence. In fact, the epitope activated antigen failed to increase ATL and CTCL T cells than normal T cells; of this group expresses a variety of surface receptors that are not products of resting T cells. Our use of flow cytometry to screen 27.2-positive population high-density expression of the 27.2 antigen is increased on a that either normal T cells when normal T cells were activated. These results suggest that the 27.2 antigen on Sezary cells needs to be studied. eight of eight cases of Sezary syndrome were found to demonstrate that the majority of CLL cases lack the enzyme activity was "normal" in two. The identity of the 75 Kd 27.2 antigen as the enzyme ecto-5'-nucleotidase was revealed by demonstrating that MoAb 27.2 inhibited the enzymatic activity of ecto-5'-NT on normal lymphocytes. Binding by two MoAb (7G2 and I9E) that were recently prepared to purified placental ecto-5'-NT was also inhibited when PBMC were precultured with MoAb 27.2. (7G2 and I9E are two of three MoAbs that were used to assign ecto-5'-NT the designation CD73 in the Fourth International Conference on Human Leukocyte Differentiation Antigens.) While MoAb 27.2 may define a site on a cell surface molecule that is associated with but distinct from ecto-5'-NT, this possibility seems remote given the reported size (70 to 74 Kd) of this enzyme, and its density distribution on T cell subsets, including 20% of CD4 + and 50% of CD8 + T cells.

The enzymatic activity of ecto-5'-NT on leukemias has been studied by a number of groups but the reactivity of MoAbs to ecto-5'-NT on leukemias has not been reported. A rabbit anti-ecto-5'-NT serum was used in one study to demonstrate that the majority of CLL cases lack the enzyme protein, which is consistent with our finding that MoAb 27.2 stained only two of 16 cases of B-cell CLL. The activity of ecto-5'-NT on Sezary cells has been reported to be normal or depressed compared with normal T cells.

In one study, eight of eight cases of Sezary syndrome were found to have ecto-5'-NT activity that was comparable to normal T cells. In another study of six patients with Sezary syndrome, the ecto-5'-NT activity was "normal" in four and markedly depressed in two. Our results are consistent with the high frequency of ecto-5'-NT-positive cases of Sezary syndrome because four of four cases of Sezary syndrome were positively stained with MoAb 27.2. However, it was somewhat surprising to find that unlike some cases of cALL and an occasional case of B CLL, high levels of ecto-5'-NT activity have not been reported on Sezary cells. The density of the 27.2 antigen on Sezary cells needs to be correlated with ecto-5'-NT activity to determine whether or not the enzyme is inhibited when expressed in elevated densities on the surface of these leukemic T cells.

Increased ecto-5'-NT antigen expression might be causally related to T-cell leukemogenesis in one of two ways: (1)
ecto-5'NT may be functionally coupled to a cell growth signal with oncogenic properties, or (2) ecto-5'NT enzymatic activity or an unknown receptor function of this molecule may facilitate escape by a malignant T cell clone from the host's antitumor response. In regard to the first possibility, our data do not support the notion that elevated ecto-5'NT expression contributes to malignant transformation of T cells by upregulating the IL-2-IL-2R autocrine growth pathway. CTCL cells are frequently CD25- and we found that two of two cases of this phenotype had elevated amounts of ecto-5'NT on their surface (Table 4). Moreover, ecto-5'NT was elevated in four of eight cases of ATL, and in ATL IL-2R expression is activated by the HTLV-I transactivator p40X (tat) gene.19-20 Taken together, these results point away from the possibility that ecto-5'NT is functionally coupled to the IL-2-IL-2R autocrine growth pathway in CTCL and ATL.

It is notable that by dephosphorylating $5'$ monophosphate nucleotides to their respective nucleosides ecto-5'NT permits the uptake of purines and pyrimidines by the cell by a selective transport mechanism. Thompson has demonstrated that when endogenous purine synthesis is blocked with aminopterin in T cells stimulated with Con A, ecto-5'NT on these cells can meet their total purine requirements by catalyzing dephosphorylation of inosine monophosphate in medium lacking other purine nucleotides or nucleosides.18 Therefore, ecto-5'NT might facilitate the growth of malignant T cells by helping to meet their metabolic requirements under microenvironmental conditions of nucleoside deprivation when nucleotide synthesis de novo is limited. Alternatively, ecto-5'NT may have a primary function in normal and malignant T cells that is unrelated to the metabolic requirements of these cells.

The ecto-5'NT antigen was found in three of four cases of ATL in Japan that were clinically diagnosed as chronic type. These three patients did not have lymphadenopathy, hepatosplenomegaly, or hypercalcemia at the time of diagnosis. In two of these patients, with a mixture of normal and leukemic T cells in their peripheral blood, high densities of the 27.2 antigen helped to delineate the malignant subpopulation. This subset was also found to express elevated densities of the epitope recognized by MoAb 27.1. Studies are underway to determine whether or not ecto-5'NT antigen is a useful marker in detecting low numbers of circulating malignant T cells in ATL and CTCL or distinguishing early cases of CTCL from benign erythrodermas.

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Increased density of ecto 5’ nucleotidase antigen on leukemic T cells from patients with cutaneous T-cell lymphoma and adult T-cell leukemia/lymphoma

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