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Heterogeneity of Human Thymocytes and a Malignant T-Lymphoblast Cell Line, MOLT-3[1]

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ABSTRACT

The purpose of this paper was to study the heterogeneity of human thymocytes and leukemic cells of the T-cell line MOLT-3 by velocity sedimentation. Analysis of the subpopulations of thymocytes demonstrated that they represent a heterogeneous population of cells with respect to their size, proliferative activity, and presence and quantities of terminal deoxynucleotidyl transferase and human thymus leukemia-associated antigen, a thymic isozyme of adenosine deaminase (HThy-L/ADA). Only a minor subpopulation of thymocytes (large cells) was in active cycle. The highest level of HThy-L/ADA was associated with the main subpopulation of thymocytes sedimenting at 3 to 4 mm/hr while low amounts of the HThy-L/ADA antigen (enzyme) were found in the minor fractions of the small and large cells. The distribution of terminal deoxynucleotidyl transferase-positive cells indicated that most, but not all, thymocytes contain the enzyme.

Analysis of the T-cell line MOLT-3 showed that these cells could be separated into subpopulations with different biochemical and biological properties. More than one subpopulation of cells was capable of DNA synthesis. In contrast to the thymocytes, all fractions of MOLT-3 cells contained high amounts of HThy-L/ADA. The proportion of terminal deoxynucleotidyl transferase-positive cells as a function of sedimentation velocity was also quite constant although there was a slight but reproducible drop in the percentage of these cells in the slowly sedimenting fractions. The percentage of cells with receptors for sheep erythrocytes also remained high in fractions separated on the basis of size, although a consistently higher percentage was found in smaller cells. These studies indicated that thymus cells as well as the malignant T-cell line MOLT-3 can be separated on the basis of sedimentation velocity into subpopulations with different biological and biochemical properties. The data also indicated that the heterogeneity of MOLT-3 line cannot be explained solely on the basis of volume changes due to cell cycle, suggesting that they may represent heterogeneous populations of cells.

INTRODUCTION

The enzyme TDT[4] and HThy-L, recently identified (9) as a thymic isozyme of ADA (in view of this finding, HThy-L will be noted as HThy-L/ADA), both appear to be linked to differentiation of hematopoietic cells (7, 8, 14). However, the highest levels of TDT and HThy-L/ADA seem to be expressed at different stages of hematopoietic cell differentiation. Thus, the highest activities of TDT have been detected in normal thymocytes, a small subpopulation of bone marrow cells, in leukemic blasts of non-T-cell, non-B-cell, ALL, and a high proportion of T-cell ALL (4, 14, 24). The highest quantities of HThy-L/ADA have been demonstrated in normal thymocytes, leukemic cells of almost all T-cell ALL, and a small propotion of patients with non-T-cell, non-B-cell ALL (5, 6). It has been suggested that TDT may serve as a marker of immature lymphoid cells (14), whereas HThy-L/ADA is restricted to the intrathymic stage of T-cell differentiation (8). It seems that ADA plays an important role in the development of the lymphoid system, its absence being associated with a recessive form of immunodeficiency (12). Furthermore, ADA has also been related to the early stages of T-cell differentiation (1, 2, 10).

The prerequisite for clarification of the relationship between TDT and HThy-L/ADA with differentiation of murine and leukemic cells is a separation of different subpopulations of hematopoietic cells and their phenotypic characterization. In the present study, we have separated thymocytes and cells of the T-cell line MOLT-3 by sedimentation velocity at unit gravity and analyzed these cells using immunological and enzymatic markers as well as biological methods.

MATERIALS AND METHODS

Cells and Tissue Materials. Fresh thymus tissues from children between 2 and 8 years old were obtained from surgical and diagnostic material. Single-cell suspensions were prepared by mincing the tissue in serum-free tissue culture medium and filtering through glass wool. The MOLT-3 cell line which was established from a 19-year-old patient with ALL (7) was obtained from Dr. J. Kaplan, Detroit, Mich. The cell line is maintained in culture in medium supplemented with 10% fetal calf serum (27). The cell line had been repeatedly tested for the presence of *Mycoplasma* and had been found to be negative. Viable mononuclear cells from these cells were isolated by centrifugation of cell suspensions on Picoll-Inopaque gradients.

Sedimentation Velocity. Sedimentation velocity analysis was carried out according to the method of Miller and Phillips (23). Briefly, single-cell suspensions of thymocytes or MOLT cells were allowed to sediment through a shallow gradient of bovine serum albumin in a chamber with a diameter of 22 cm and a depth of 15 cm. This chamber permitted separation of between 10^8 to 3×10^8 cells without distortion of the sedimentation profile by "streaming." Sedimentation continued for 4 to 5 hr for thymocytes and for 2 to 3 hr for MOLT-3 cells. The

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[4] The abbreviations used are: TDT, terminal deoxynucleotidyl transferase; HThy-L, human thymus leukemia-associated antigen; ADA, adenosine deaminase; ALL, acute lymphoblastic leukemia; E-rosette, erythrocyte-forming rosette.

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sedimentation was carried out at 4°, and 50-ml fractions were collected. This procedure separated cells principally on the basis of size with small contributions attributable to density (21, 22). The viability of the thymocytes and MOLT-3 cells remained high (>90%) after separation with this method.

TDT. TDT activity and TDT-positive cells were assayed by 2 independent methods. TDT activities were assayed biochemically as described previously (25). Cells containing TDT were also detected by a single-cell indirect immunofluorescence assay using a specific $F(ab)'_2$ rabbit anti-TDT and a fluorescein isothyocyanate-conjugated $F(ab)'_2$ fragment of a goat anti-rabbit $F(ab)'_2$ as described before (24).

Radioimmunoassay for HThy-L/ADA. A competitive radioimmunoassay for HThy-L/ADA was performed as described previously (6). Briefly, different concentrations of cell extracts were incubated with a rabbit anti-HThy-L/ADA antiserum, followed by the addition of an iodinated preparation of HThy-L and normal rabbit serum and coprecipitation of "bound" labeled antigen with a sheep anti-rabbit γ-globulin. A standard curve was run for each experiment using the unlabeled purified preparation of HThy-L/ADA as the "unknown" specimen. The quantity of HThy-L/ADA in cell extracts was assessed from the standard curve at a value of 50% inhibition of the precipitation reaction.

[^{3}H]Thymidine Labeling. Single-cell suspensions of thymus cells or MOLT-3 cells were resuspended in medium (without nucleosides or deoxynucleosides), and [^{3}H]thymidine (50 Ci/mmol, Amersham/Searle Radiochemicals, Mississauga, Ontario, Canada) was added to a concentration of 5 μCi/ml. The cultures were labeled for 15 min at 37°, and the cells were washed 3 times with 0.14 M NaCl-0.02 M sodium phosphate (pH 7.2) before analysis by sedimentation velocity.

Colony Formation. The cloning efficiency of MOLT-3 cells was determined by plating in 0.8% methylcellulose. Cells from each fraction were washed twice with α-medium supplemented with 20% fetal calf serum.

E-Rosette Assay. The E-rosette assay using treatment with aminoethylisothiouronium bromide was carried out as described by Gelfand *et al.* (11).

RESULTS

Heterogeneity of Human Thymocytes. Thymocytes were separated into subpopulations according to size by sedimentation velocity. Chart 1 illustrates the typical results of one of 4 similar experiments. The profile of cell concentration and sedimentation velocity is shown in Chart 1*a*. As can be seen, the thymocytes sediment as a broad peak with sedimentation velocity of approximately 2 to 8 mm/hr with a peak at 3 to 4 mm/hr.

In order to assess DNA-synthetic capacities of the various subpopulations, their capacity to incorporate [^{3}H]thymidine was examined. As can be seen in Chart 1*a*, the cells capable of incorporating thymidine sediment as a discrete sharp peak with a minor population at approximately 7 mm/hr. These results indicate that thymocytes are heterogeneous with respect to their capacity for DNA synthesis, and only a minor population of large cells sedimenting at 7 mm/hr is capable of DNA synthesis.

Using a radioimmunoassay for HThy-L/ADA, the distribution of this antigen (enzyme) in the thymocytes was also examined.

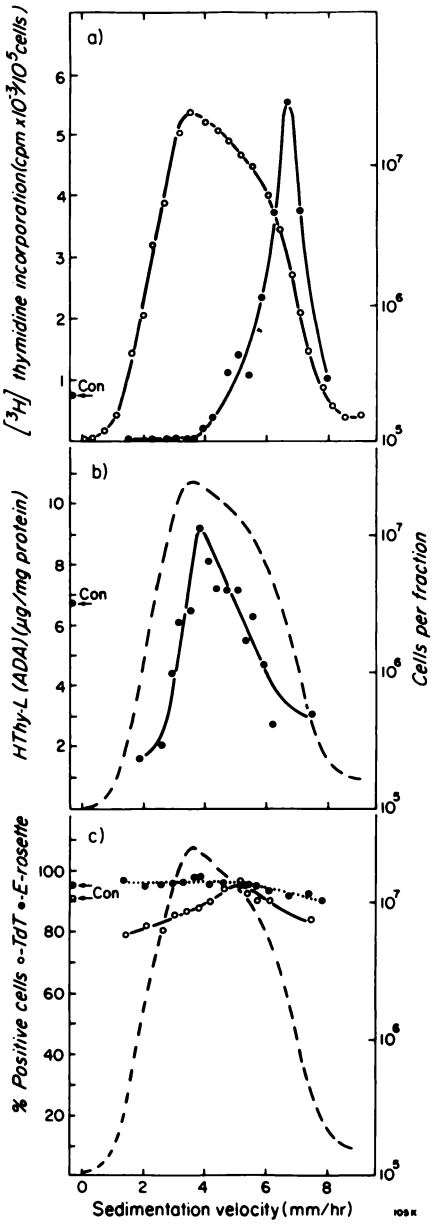

Chart 1. Analysis of the heterogeneity of thymocytes. Nucleated thymocytes (3×10^8) were separated at unit gravity on a 1 to 3% bovine serum albumin gradient. Forty fractions containing 50 ml each were collected, and the cells were analyzed with the following markers: *a*, cells/fraction (○); [^{3}H]thymidine uptake per cell (●); *b*, amount of HThy-L/ADA in μg/mg protein (●); and *c*, percentage of E-rosette-positive cells (●), percentage of cells containing TDT (○). *Con*, control values for the unfractionated thymocyte sample. – – – –, tracing of cell number in *a*.

Results in Chart 1*b* indicate that HThy-L/ADA was not distributed evenly among all thymocytes. The high levels of the antigen (enzyme) appear to be mainly associated with the major population of cells sedimenting at approximately 4 mm/hr. It is known that most thymocytes are capable of forming E-rosettes with sheep RBC (3, 16). Results in Chart 1*c* indicate that the majority of cells throughout the gradient were capable of forming such rosettes, although the percentage of these cells appeared to be slightly lower in the large cells sedimenting at 7 to 9 mm/hr while virtually all the smaller cells possessed this property.

Thymocytes from many species including humans are known

to contain a high level of TDT (4), and a high percentage of thymocytes contains this enzyme (4, 14, 24). In the search for heterogeneity in the distribution of TDT in these subpopulations, we examined these cells using a single-cell immunofluorescence assay. Results in Chart 1*c* confirm our earlier data that a high percentage of thymocytes contains TDT (24). This high proportion (80 to 100%) of the cells that were stained by the immunofluoresence assay can also be found throughout the gradient.

Heterogeneity of the Malignant T-Cell Line MOLT-3. In order to ensure that a representative culture was being examined, MOLT-3 cells were first cloned in methylcellulose, and single-cell clones were analyzed. The results and the analysis of one such clone are summarized below. The data in Chart 2 represent typical results of one of 9 similar experiments. Chart 2*a* illustrates the profile of cell concentration as a function of sedimentation velocity. As can be seen, MOLT-3 cells sediment as a broad peak of cells, ranging from 3 to 16 mm/hr. As can be seen in Chart 2*a*, the cells capable of thymidine incorporation were spread over a broad range of cells from 6 mm/hr to 14 mm/hr. These results suggest that there was more than one subpopulation of cells incorporating DNA.

Similar to thymocytes, malignant T-cell lines have also been shown to contain a high level of the antigen HThy-L/ADA (7). In order to examine if the amount of HThy-L/ADA is also heterogeneous in these MOLT-3 lines, the level of this antigen was also analyzed in these subpopulations of MOLT-3 cells separated by sedimentation velocity. Results in Chart 2*b* indicate that, contrary to the results found in thymocytes, the quantity of HThy-L/ADA was similar in all fractions. The slight variations in the level of HThy-L/ADA observed here were not evident in other experiments.

The ability of these cells to form E-rosettes was also examined, and results are summarized in Chart 2*c*. As can be seen, although the ability to form E-rosette remained relatively constant throughout the different fractions, a slight but consistent difference was observed. Cells with the lower sedimentation velocity (4 to 48 mm/hr) appear to have a high percentage (90%) of E-rosette cells, while the larger cells (>13 mm/hr) contain a lower proportion (70%) of E-rosette cells. The distribution of TDT was examined by 2 independent methods. Using a single-cell immunofluorescence assay, the percentage of TDT-positive cells in these fractions was determined. As can be seen in Chart 2D, all the fractions of MOLT-3 cells contain a high percentage of TDT-positive cells. The highest proportion (90 to 100%) of these cells appears to be associated with the larger cells (12 to 16 mm/hr), while a lower proportion (60 to 80%) of these cells that were positive with this assay was found with the smaller cells (3 to 8 mm/hr). Results using a biochemical assay supported these findings. The highest amount of enzymatic activity per cell was associated with the larger cells, while considerably lower (about 5-fold) activities were found with the smaller cells. This apparent discrepancy between the extent of reduction of TDT-positive cells and the enzyme levels in the smaller cells can be partially accounted for by the reduction of the size of these cells and thus lower levels of the enzyme activity per cell.

The cloning efficiencies of these cells in methylcellulose were also determined. Results summarized in Chart 2*e* indicate that, in contrast to the broad peak of MOLT-3 cells, only a narrow peak of cells sedimenting at between 6 and 10 mm/hr

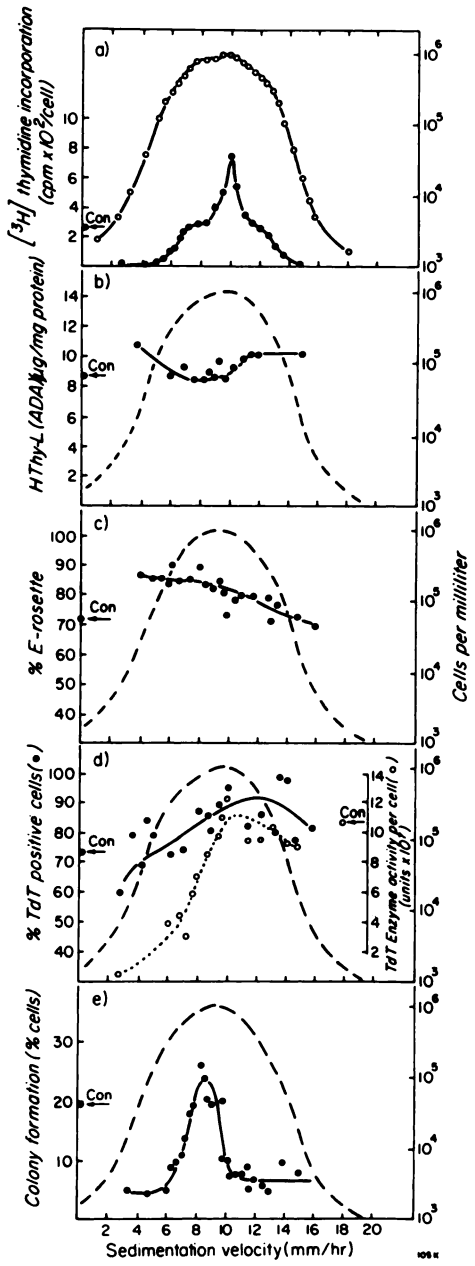

Chart 2. Analysis of the heterogeneity of the malignant T-cell line MOLT-3. MOLT-3 cells (1.5×10^8) were separated at unit gravity on a 1 to 3% bovine serum albumin gradient. Forty fractions containing 50 ml each were collected, and the cells were analyzed for the following properties: *a*, cells/ml (○); [^{3}H]thymidine incorporation per cell (●); *b*, amount of HThy-L/ADA in μg/mg protein (●); *c*, percentage of E-rosette cells (●); *d*, percentage of cells stained for TDT (●); amount of TDT enzyme activity per cell (○); *e*, colony formation in percentage of cells (○). *Con*, control values for the unfractionated MOLT-3 cell sample. – – – –, tracing of cell number in *a*.

is capable of forming colonies. These results are consistent with the results described above, that the MOLT-3 cells comprise a heterogeneous population of cells.

DISCUSSION

In the present paper, we have analyzed the cellular heterogeneity of human thymocytes and a freshly cloned culture of the leukemic T-cell line MOLT-3. Using unit gravity sedimentation velocity, we have separated thymocytes as well as the T-

cell line MOLT-3 into subpopulations on the basis of size, and the fractions recovered were studied using a variety of biological, immunological, and enzymatic assays.

Analysis of the subpopulations of human thymocytes confirmed previous studies that thymocytes are indeed heterogeneous (8, 26). Examination of their capacity to synthesize DNA indicates that, in agreement with previous findings (26), only a minor population of large cells in the thymus is in active cycle. Analysis of the cellular distribution of HThy-L/ADA also indicated that thymus cells are heterogeneous with respect to the amount of this antigen (enzyme). The high level of HThy-L/ADA was found to be associated with the main population of cells sedimenting at 3 to 7 mm/hr, while 3- to 5-fold lesser quantities of this antigen were found in the minor population of the small and the large cells. Thus, it has been shown that the subpopulations of thymocytes capable of synthesizing DNA contained relatively low amounts of HThy-L/ADA.

The distribution of TDT-positive cells indicated that most, but not all, of the thymocytes contain this enzyme. This finding is consistent with the observation by Goldschneider *et al.* (13) that the enzyme is not found in all thymocytes in the rat but is associated mainly with the cells from the thymic cortex, while only low amounts of the enzyme are found in the cells from the medullary region. These results also indicate that, although HThy-L/ADA and TDT are present in the majority of thymocytes, their distributions in these subpopulations are not entirely overlapping.

Analysis of the leukemic T-cell line MOLT-3 by sedimentation velocity also indicated that these cells can be separated in populations with different biochemical and biological properties. The results, however, also indicated that the heterogeneity of these cells cannot be explained solely on the basis of cell size changes due to cell cycle. The wide range in the sedimentation velocities (3 to 16 mm/hr) of these freshly cloned cells exceeds the expected change in sedimentation velocity of single populations of cells before and after mitosis, where only a change by a ratio of about 1.6 is expected (21). The analysis of the DNA-synthetic abilities of these cells indicated that more than one population of cells was capable of DNA synthesis. This finding is also inconsistent with a single-cell population where only one discrete peak of DNA-synthesizing cells would be expected (18, 20, 26). Consistent with these analyses is the observation that only a minor population of these cells (6 to 10 mm/hr) was capable of colony formation. Taken together, these data suggest that the MOLT-3 cultures may contain a heterogeneous population of cells with different proliferative capacities probably as a result of spontaneous differentiation in culture. Thus, in addition to the cell size changes due to cell cycle, some of the changes in cell volume and heterogeneity of DNA-synthetic abilities may be due to "cellular maturation," which is known to be accompanied by changes in the physical characteristics of volume and shape (22) and density (17) as well as in proliferation potentials (19) in different systems.

The proportion of TDT-positive cells as a function of sedimentation velocity was also quite constant, although there was a slight but reproducible drop in the percentage of these cells in the slowly sedimenting fractions. These observations are in agreement with those reported for TDT activities in murine leukemia cell lines separated by unit gravity sedimentation (15).

Finally, the results described herein not only substantiate the heterogeneity of thymocytes and the leukemic T-cell line MOLT-3, but they also showed that these cells can be fractionated into discrete subpopulations. The ability to separate such cells should permit further analysis of the functional properties of these subpopulations and their interactions. Of particular interest is the antigen HThy-L/ADA which has recently been identified as a thymic isozyme of ADA (9). The finding that the main subpopulation (but not the smaller or larger cells in the thymus) contains high-level HThy-L/ADA may help to further define these subpopulations.

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