Nucleoside Transport and Proliferative Rate in Human Thymocytes and Lymphocytes

By Carole L. Smith, Linda M. Pilarski, Mark L. Egerton, and James S. Wiley

The thymus is a site of active T-lymphoid cell proliferation and DNA synthesis. In this study, the capacity of human thymocytes for nucleoside transport was assessed both by cytosine arabinoside influx and by equilibrium binding of nitrobenzylmercaptopurine riboside (NBMPR), a specific ligand for the equilibrative nucleoside transporter of leukocytes. The proportion of freshly isolated thymocytes synthesizing DNA was 8.6% ± 2.1% (n = 12) by 3H-thymidine labeling index and 7.8% ± 2.9% (n = 4) S-phase cells by flow cytometric analysis of DNA content. In comparison, both methods gave proliferation S-phase values <1% for peripheral blood lymphocytes (PBLs). Thymocytes expressed a high density of specific NBMPR binding sites (26,060 ± 6,776 sites per cell, n = 12) as compared with PBLs (1,123 ± 553 sites per cell, n = 8). The initial influx of cytosine arabinoside into thymocytes was 14-fold greater than into PBLs, and in both cell types the influx of nucleoside was totally inhibited by 0.5 μmol/L NBMPR, which is known to inhibit the major equilibrative nucleoside transporter in white blood cells. Depletion of mature CD3+ cells from the thymocyte preparation by anti-CD3 antibody left a residual population with both increased labeling index and up to twofold greater density of NBMPR binding sites. When PBLs were cultured for 48 hours with the T-cell mitogen phytohemagglutinin, a 40-fold increase in labeling index was observed, together with a 30-fold increase in the density of specific NBMPR binding sites. Thus, fresh thymocytes from human thymus are actively proliferating and express high densities of a functional nucleoside transporter. The more immature cells in the thymocyte population which are proliferating more actively have a greater density of nucleoside transporters than the whole population. In contrast, mitotically inactive PBLs have few nucleoside transporters, but after mitogenic stimulation PBLs express large numbers of this transmembrane molecule.

MATERIALS AND METHODS

Materials. Imidazole-buffered saline (145 mmol/L NaCl, 5 mmol/L KCl, 5.0 mmol/L imidazole.Cl, 1.0 mmol/L MgCl2, 5.0 mmol/L glucose, pH 7.4) was ultraltered before use by passage through 0.22-μm filter units (Millipore, Bedford, MA). Deoxyribonuclease I (1 U/mL, Sigma Chemical, St Louis, MO) was added during cell washes to reduce clumping of cells. Ficoll-Paque (1.077 g/mL) was from Pharmacia Fine Chemicals, Uppsala, Sweden. Dilazep (N,N'-bis(3-(3,4,5 trimethoxybenzoyloxy)propyl)-homopiperazine) was from Hoffmann-La Roche, Basel, Switzerland. NBMPR was from Sigma and 3H-NBMPR was from Moravek Biochemicals, Brea, CA. After storage, the latter was purified by Dr A. R. P. Paterson by high-performance liquid chromatography with a C18u Bondapak column (Waters Scientific, Mississauga, Ontario) eluted with methanol-water solutions. The concentration was checked spectrophotometrically using a molar extinction coefficient of 27.3 ± 104 at a wavelength of 284 nm. Unlabeled NBMPR solutions (in imidazole-buffered saline) were prepared by vigorous stirring for 4 hours to yield a saturated solution of about 30 μmol/L. 3H-water (1 μCi/mL), 14C-polyethylene glycol 4,000 (20 μCi/g), and methyl-3H-thymidine (40 to 60 Ci/mmol) were from Radiochemical Center, Amersham, England. Cell culture reagents were purchased from Gibco (Burlington, Ontario). Fresh thymuses were removed routinely from children undergoing cardiac surgery and obtained from the Royal Children’s Hospital, Parkville, Victoria. The children’s ages are shown in Table I. Peripheral blood from normal donors was obtained through the Melbourne Red Cross Blood Bank.

Isolation of thymocytes. Cell suspensions of thymocytes were obtained by gentle mincing of thymic tissue with scissors, followed by passage through a fine sieve; thymocytes were separated from dead cells and erythrocytes by Ficoll-Paque (Pharmacia). Cells at the interface were harvested and washed three times in imidazole-buffered saline containing deoxyribonuclease. Immunologic characterization of this population was described previously. Peripheral blood lymphocytes were separated from venous blood by density-gradient centrifugation over Ficoll-Paque, followed by washing in imidazole-buffered saline (composition in mmol/L: NaCl 145, KCl 5, imidazole Cl, pH 7.4 ± 20, and glucose 10.

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From the Department of Haematology, Austin Hospital, Heidelberg, Victoria, Australia; Walter & Eliza Hall Institute, Melbourne, Australia; and Department of Immunology, University of Alberta, Edmonton, Canada.

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Address reprint requests to James Wiley, MD, Haematology Department, Austin Hospital, Heidelberg, Victoria, Australia.

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Labeling index. The labeling index (percentage of S-phase cells) was measured by autoradiography. Cell suspensions of 2 × 10⁷ cells/mL were incubated at 37°C for 60 minutes with 5 μCi/mL ³H-thymidine (40 to 60 Ci/mmol) at a final concentration of 0.06 to 0.12 μmol/L. Cells were washed, and autoradiography was performed on cytospin preparations. Cells with at least five grains per nucleus were scored as positive.

Propidium iodide measurement of DNA. Propidium iodide measurement of DNA was performed according to the method of Taylor. Eighty units of bovine RNase A, 100 μL 5% Triton X-100 (1C1), and 300 μL 100 μg/mL propidium iodide were added to 5 × 10⁶ cells in 500 μL serum-free medium; 10,000 cells were analyzed on an EPICS IV flow cytometer, and the resultant DNA content histogram was analyzed with the EPICS Para 1 (version 3.3) program to determine the population of cells in G1/G0, S, G2, and M phases of the cell cycle.

Measurement of ³H-NBMPR binding. The number of ³H-NBMPR binding sites per cell was determined as described previously. Cell suspensions were incubated with ³H-NBMPR (0.1 to 6.0 nmol/L) for 15 minutes at 37°C. Incubation was terminated by centrifugation of cells through silicone oil (SG 1.031), and radioactivity in the solubilized pellet was determined by scintillation counting. Aliquots of the supernate were also counted to estimate free ³H-NBMPR concentration. Nonspecific binding of ³H-NBMPR was measured in parallel incubations of cells that had been preincubated (5 minutes, 37°C) with 5 μmol/L NBMPR. Specific binding was analyzed by Scatchard plots, from which the maximum number of binding sites per cell was derived.

Intracellular water space. For determination of mean cell volume, cell suspensions were incubated (5 minutes, 20°C) with ³H-water (5 Ci/mL) and aliquots were layered over silicone oil mixture (5) and centrifuged at 8,000 g for 4 minutes. The trapped extracellular pellet volume was determined with ³H-polyethylene glycol in parallel tubes. The intracellular water space was calculated by subtracting extracellular space from the total water space. An equivalent cell volume was calculated by dividing cell mass by cell density, assuming a cell density of lymphocytes of 1.087 g/mL and cell water contributing 76.5% to cell mass.

AraC influx into thymocytes. Two hundred microliters of 2 μmol/L ³H-cytosine arabinoside (araC) (30 Ci/mmol) in RPMI-1640 medium was preincubated over 150 μL silicone oil in 1.5-mL microfuge tubes at 37°C. Cells were resuspended at 4 to 5 × 10⁷ cells/mL in RPMI 1640 alone or with NBMPR at a concentration of 1 μmol/L. AraC influx was initiated by addition of 200 μL cells at 37°C to the 200 μL ³H-arac. After incubations of 1 to 5 seconds, uptake was terminated by addition of 800 μL 750 μmol/L cold dilaze. The tubes were immediately spun at 8,000 g for 1 minute. We estimated extracellular radioactivity associated with the cell pellet separately by mixing cells and dilaze before adding ³H-arac and then spinning through oil. Medium was removed above the oil, and pellets were solubilized overnight in 0.5 mol/L NaOH for scintillation counting. Uptake values were expressed as pmol/10⁶ cells.

CD3-depletion of thymocytes. To enrich for immature thymocytes, cells were treated with the monoclonal antibody OKT3 followed by cell lysis with absorbed rabbit complement. Dead cells were removed by separation over a Ficoll-Paque density gradient followed by two washes with RPMI 1640 and 10% fetal calf serum. The remaining cell population was 90% to 95% pure as defined by the number of cells unable to bind fluorescent-conjugated anti-CD3. Contaminant cells had a low density of CD3. The cell population included two peaks of small and large thymocytes as determined by forward-angle light scatter with a FACS II (Becton Dickenson, Mountain View, CA).

Phytohemagglutinin stimulation of peripheral blood lymphocytes. Ficoll-Paque purified PBLs were cultured at 10⁷ cells/mL in RPMI 1640 with 10% fetal calf serum, 1% human AB serum, 0.31% bicarbonate, 25 μmol/L Hepes and 1% phytohemagglutinin (PHA) for 24 to 48 hours at 37°C in a 10% carbon dioxide-air mixture. Cytospin preparations showed 76% lymphocytes, 18% monocytes, and 6% polymorphs at zero time; monocytes and polymorphs decreased to 6% and 0% at 24 hours and to 4% and 0%, respectively, at 48 hours.

Statistics. Mean ± 1 SD are shown; significance of differences between means was analyzed by a Student’s t test. Regression lines were fitted by the least-squares method.

RESULTS

Proliferation rate of fresh thymocytes from human thymus. Proliferation of thymocytes was assessed by two independent techniques: ³H-thymidine labeling index and flow cytometric analysis of percentage cells in S phase. The mean labeling index for thymocytes isolated from 12 fresh thymic lobes from children was 8.6% ± 2.1% (Table 1). In contrast, PBLs from normal subjects had a labeling index <1%. Propidium iodide analysis of DNA content showed that the percentage of cells in S, G2, and M phase was 7.8% ± 2.9% (Table 1) for thymocytes as compared with <1% for PBLs.

Specific ³H-NBMPR binding sites in thymocytes. The values for specific binding of ³H-NBMPR to both thymocytes and lymphocytes are shown in Table 1. Analysis yielded linear Scatchard plots, suggesting that the inhibitor always interacted with a single class of binding sites (Fig 1). The mean value for maximal ³H-NBMPR binding to fresh thymocytes was 26,068 ± 8,776 sites per cell (n = 12), which is 20-fold higher than that of PBLs from normal subjects (P < .001). The affinity of ³H-NBMPR binding (kd) was calculated from the slope of the Scatchard plots and had a mean value of 0.51 nmol/L, which is not significantly different from that established from a range of fresh normal lymphocytes and leukemic cells.
Fig 1. Typical Scatchard analysis of specific $[^3H]$-NBMPR binding to fresh thymocytes obtained from a single human thymus. Regression lines were fitted by the method of least squares. Intercept on x-axis ($B_{max}$) estimates maximum number of binding sites ($r = 0.80$, $K_d = 0.62$ nmol/L, $B_{max} = 35,500$ sites/cell).

$[^3H]$-NBMPR binding sites in resting and mitogen-stimulated PBLs. The results show that the low proliferative rate of PBLs is associated with a low density of specific $[^3H]$-NBMPR binding sites. Peripheral blood lymphocytes were stimulated with the T-cell mitogen PHA, and $[^3H]$-NBMPR binding was measured at 0, 24, and 48 hours of incubation. The number of $[^3H]$-NBMPR binding sites increased 30-fold during this period, concurrent with an increase of labeling index from 0.8% to 3.1% (Table 2). Flow cytometry analysis of DNA content also showed that the percentage of cells in $S + G_2 + M$ phases increased from <1% to 45% during the 48-hour incubation (Fig 2). Phytomenadione stimulation also produced an increase in cell size which was calculated from the intracellular $[^3H]$-water space. Mean cell volume increased from 250 to 430 fl during the 48-hour incubation (Table 2).

Nucleoside transport rates by thymocytes. To determine whether the high density of $[^3H]$-NBMPR binding sites on thymocytes was associated with a high rate of nucleoside transport, we measured the influx of $[^3H]$-araC. Thymocytes were incubated at 37°C and $[^3H]$-araC (1 μmol/L final concentration) added to initiate transport. The uptake of araC was linear with time over 5 seconds (Fig 3) and the mean influx was 0.14 ± 0.05 pmol/10^7 cells/s (n = 3). The transport inhibitor NBMPR at 0.5 μmol/L completely inhibited araC influx (Fig 3). Influx of araC into PBLs was substantially lower (0.01 pmol/10^7 cells/s) (n = 2). The turnover rate of the nucleoside carrier was calculated assuming a stoichiometry of 1:1 of specific NBMPR binding to nucleoside transport carriers. This turnover rate (for araC at 1 μmol/L) was 0.42 ± 0.11 molecules per site per second at 37°C (n = 3). The corresponding turnover rate for PBLs was 0.59 molecules per site per second.

Table 2. Increased Expression of Specific $[^3H]$-NBMPR Binding Sites After Mitogen Stimulation of PBLs

<table>
<thead>
<tr>
<th>Hours After PHA Stimulation</th>
<th>Mean Cell Size (fl)</th>
<th>Specific $[^3H]$-NBMPR Binding (Sites/Cell)</th>
<th>$[^3H]$-Thymidine Labeling Index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>250</td>
<td>2,203</td>
<td>&lt;1</td>
</tr>
<tr>
<td>24</td>
<td>305</td>
<td>13,402</td>
<td>10</td>
</tr>
<tr>
<td>48</td>
<td>430</td>
<td>68,024</td>
<td>45</td>
</tr>
</tbody>
</table>

Cytospin preparations showed 18% monocytes at 0 hours, 6% monocytes at 24 hours and 4% monocytes at 48 hours.

Fig 2. DNA histograms of lymphocytes after PHA stimulation for 0, 24, and 48 hours. Each histogram represents the red (DNA) fluorescence of propidium iodide-stained cells. Proportion of cells in each cell cycle phase is indicated in the lower histogram. PHA stimulation caused an increase in percentage of $S$, $G_2$, and $M$ cells to 45% by 48 hours.

Fig 3. $[^3H]$-araC uptake by fresh human thymocytes at 37°C. Uptake was inhibited by 0.5 μmol/L transport inhibitor NBMPR. Regression lines were fitted by the method of least squares.
yielded a population with an increased $^3$H-NBMPR binding density associated with an increase in proliferative rate (Table 3).

Thymocyte cell size. Thymocyte size was evaluated by measurement of the intracellular water space. The mean value was 0.94 ± 0.10 μl/10^7 cells (n = 10), which is equivalent to 114 fl mean cell volume. This compares with the values of PBLs of 1.44 ± 0.24 μl/10^7 cells, which gives a calculated mean cell volume of 174 fl.

**DISCUSSION**

Equilibrium binding of the nucleoside analogue NBMPR has been widely used to estimate the numbers of nucleoside transporters on the cell membrane, as this ligand binds with high affinity and stoichiometry of 1:1 to the major nucleoside transporter of leukocytes. This transporter is a transmembrane protein with a molecular size of 55,000 daltons, which facilitates the transmembrane fluxes of various purine and pyrimidine nucleosides and their analogues.5-7 Table 1 shows that human thymocytes freshly isolated from thymic tissue express 20-fold more specific $^3$H-NBMPR binding sites than do PBLs. Measurements of nucleoside transport rates in thymocytes suggest that the increased $^3$H-NBMPR binding sites in these cells were fully functional in nucleoside transport. Influx of the nucleoside analogue araC, into the cells occurred at a rate 14 times that of PBLs at 37°C. AraC influx in both cell types was totally inhibited by 0.5 μmol/L NBMPR, indicating that nucleoside transport was almost entirely facilitated by the NBMPR-sensitive equilibrative nucleoside transporter. Calculations yielded a turnover rate of this carrier (for araC at 1 μmol/L) of 0.59 molecules per site per second for normal PBLs and 0.42 molecules per site per second for thymocytes. These values compare with a turnover rate of 0.67 molecules per site per second in the rapidly proliferating RC2a leukemic cell line, which has 120,000 NBMPR binding sites per cell.18 Thus, this major nucleoside transporter functions as effectively in proliferating thymocytes as in the more quiescent PBLs, although the density of transporters in the two cell types differ widely.

The extensive proliferation characteristic of human thymocytes is shown by their high $^3$H-thymidine labeling index (5.0% to 12.2%) and high proportion of cycling cells, as determined by flow cytometry. The data in Table 1 show an association between rapid proliferation and high $^3$H-NBMPR site expression in thymocytes, which is in striking contrast with the low proliferation and few $^3$H-NBMPR binding sites of PBLs. This association suggests that the density of $^3$H-NBMPR binding sites on the cell surface may depend on the fraction of cycling cells in the cell population. The density of $^3$H-NBMPR binding sites may also be influenced by cell surface area, but this parameter cannot be measured directly. An indirect estimate of surface area is given by mean cell size, which was less for thymocytes (114 fl) than for PBLs (174 fl). Thus, cell surface area does not appear to be a major factor regulating expression of $^3$H-NBMPR binding sites, although any calculation of surface area from mean cell volume overlooks the known heterogeneity in size of proliferating populations.

Proliferating leukemic blast cells have been reported to have a higher density of $^3$H-NBMPR binding sites than their mature nonproliferating end-cells of differentiation,5,6 and a close correlation has been shown between proliferative rate and the number of nucleoside transporters in lymphomas and the myeloid leukemias.10 Moreover, if cultured HL-60 cells are induced to differentiate, a marked loss of $^3$H-NBMPR binding sites occurs within 2 to 3 days coincident with a decrease in proliferative rate.19 The present study provided further evidence for a linkage between proliferation rate and density of nucleoside transporters by experiments in which PBLs were stimulated by mitogen to divide. Fresh pooled PBLs with low nucleoside transporter density (2,200 sites per cell), were stimulated with the T-cell mitogen PHA. After 48 hours, the density of nucleoside transporters increased by more than 30-fold to 68,000 sites per cell, while the labeling index and analysis of DNA content showed the expected increase (Table 2). Mean cell size also increased by 1.7-fold during PHA stimulation, but the increase in NBMPR binding site density was far greater than would be predicted from the increased surface area of the cells. Further evidence for a linkage between proliferative rate and density of nucleoside transporters was obtained by fractionation of the thymocyte population. Mature thymocytes (CD3+) were depleted with monoclonal anti-CD3, and $^3$H-NBMPR binding was measured before and after this procedure. Immature CD3- thymocytes showed both increased $^3$H-NBMPR binding (up to twofold) and a greater thymidine labeling index (up to threefold) than that of the unfractonated population.

We previously showed a high density of nucleoside transporters in the malignant cells of T-acute lymphoblastic leukemia, an aggressive malignancy with a phenotype similar to immature thymocytes (CD3-, CD2+, CD4-, CD8+) in most cases. Our findings show that thymocytes have a high density of nucleoside transporters and, by selection of a population of less mature thymocytes (CD3+), even higher densities are found, with levels attained comparable to those reported in T-acute lymphoblastic leukemia.5,18 Stimulation of PBLs by the T-cell mitogen PHA also increases the density of nucleoside transporters to a level similar to that reported in T-acute lymphoblastic leukemia. These findings suggest that both normal and neoplastic thymocytes are capable of expressing a high density of nucleoside transporters, although this potential is only apparent at high proliferative rates.

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CL Smith, LM Pilarski, ML Egerton and JS Wiley