Polyamine Content of AKR Leukemic Cells in Relation to the Cell Cycle

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SUMMARY

In AKR mice that develop spontaneous lymphoid leukemia, the thymus is the organ of primary involvement. Thymic cells from normal mice, as well as from mice with advanced leukemia, were separated according to size by sedimentation in a 1 x g sucrose gradient, the larger cells sedimenting further than the smaller cells. The fractions obtained from this gradient, which correspond to successive phases of the cell cycle, were collected and subjected to polyamine analysis. In the G0 phase of the cell cycle the spermine content was higher than in the G1 phase, whereas the putrescine and spermidine content were similar in these two phases. The cellular content of putrescine, spermidine, and spermine increased progressively as the cells traversed the cell cycle from G1 to M. In fact, the amount of all these amines was increased in the G1 + S fraction. In the fraction corresponding to the G2 + M phases, the polyamine content was twofold that in the G1 phase. This increase in the polyamine content was seen first for putrescine, then for spermidine, and finally for spermine. This sequential pattern reflects the order of synthesis of the polyamines, i.e., putrescine being the precursor of spermidine and spermine the precursor of spermine.

INTRODUCTION

The information that is presently available about the metabolism of the polyamines, putrescine, spermidine, and spermine, in relation to the mammalian cell cycle, has been largely the result of studies on cells that have been stimulated to divide from a quiescent state (3, 5, 6, 16, 18). Shortly after stimulation there is a burst of ornithine decarboxylase activity (5, 18) which results in an accumulation of the product, putrescine (6, 9, 19). These events, which take place prior to the synthesis of DNA, are followed by spermidine synthesis and accumulation (3, 5, 6, 9, 16, 19). Putrescine stimulates the 1st step in spermidine synthesis, the decarboxylation of S-adenosylmethionine (14), and is the acceptor of the propylamine moiety which is transferred from "decarboxylated S-adenosylmethionine." The increase in the cellular content of spermidine seems to parallel that of RNA (3, 6, 16), whereas spermine, which is formed by the transfer of a propylamine moiety to the spermidine molecule, shows a pattern of accumulation more similar to that of DNA (21).

So far, only a few studies have been performed to establish changes in polyamine metabolism that occur in continuously dividing cells as they traverse the cell cycle. In all these cases, synchronous systems were used, i.e., heat-synchronized Tetrahymena populations (8), colcemid-synchronized Don C cells (4), developing sea urchin embryos (10), and plasmodia of Physarum polycephalum (12).

In the present study, we have used a sucrose gradient sedimentation technique (15), which separates cells by size, for separation of AKR leukemic cells in various phases of the cell cycle (13, 17). We exploit the fact that the order of cells in the gradient represents the order in the cell cycle, i.e., samples separated by spatial intervals down the gradient are equivalent to samples separated by temporal intervals in a synchronous culture. This method is advantageous since cells at different stages of a spontaneous cell cycle can be compared with the original unseparated cells and analyzed without the need to assess the possible effects of a synchronizing agent. In order to assay quantitatively the polyamine content of samples containing no more than 2 to 20 x 10^6 cells, we used the very sensitive dansylation method developed by Seiler and Wiechmann (22) which relies on the conversion of amines to intensely fluorescent DANS-\W^amides, separation of these derivatives by TLC, and subsequent in situ measurement of their fluorescence.

MATERIALS AND METHODS

Experimental Animals. Female AKR mice, 6 to 8 weeks old without evidence of leukemia ("normal") and 6 to 12 months old with advanced spontaneous leukemia ("leuke-

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1 An abstract of this work was presented at the 8th Congress of the Nordic Society for Cell Biology, June 27, 1973, in Oslo, Norway (7).
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1 The abbreviations used are: DANS-, 1-dimethylamino-naphthalene-5-sulfonyl-; TLC, thin-layer chromatography; TdR-\W^H, thymidine-\W^H.

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mic were obtained through the Mammalian Genetics and Animal Production Section, National Cancer Institute, Bethesda, Md. Younger mice were selected for normal controls since mice of 6 to 12 months of age may have occult preleukemic changes. The criteria for diagnosis of AKR leukemia have been described elsewhere (13). Leukemic mice were selected at approximately the same stage of their disease as determined by clinical examination. The weights of the leukemic thymuses were >350 mg and the viability of the leukemic cell as determined by trypan blue exclusion was >80%. The weights of the normal thymuses ranged from 50 to 120 mg, and the viability of the thymic cells was almost 100%.

**Cell Separation.** Cell suspensions of 3 leukemic thymuses and 2 normal thymuses were prepared in Hanks' balanced salt solution supplemented with 0.05% sucrose. From each suspension, approximately 2.6 x 10^8 cells were separated according to size by sedimentation at unit gravity in a sucrose gradient at room temperature, as previously described (13, 17). To avoid cell crowding, each suspension was sedimented in 2 equal aliquots (approximately 1.3 x 10^8 cells) applied simultaneously to 2 identical sedimentation chambers. Cells were separated over approximately 4 hr and then collected in 30 sequential 50-ml fractions after an initial discard of 150 ml. The fractions collected represented >90% of the cells applied. The procedures for cell suspension preparation and cell separation have been described in detail previously; smaller cells appear in the earlier, low-number fractions, and larger cells appear in the later, high-number fractions (13, 15, 17).

In the leukemic cell separations, the fractions from each sedimentation chamber were pooled into 5 groups of increasing cell size (Chart 1). Group I, the pool of the earliest fractions, encompassed the smallest cells. Groups II to V consisted of successively larger cells. For each thymus suspension, the equivalent groups from each of the 2 separations were then pooled for polyamine analysis.

In normal Thymus 1, the fractions were pooled into 4 groups of increasing cell size (Chart 2, top). As above, the 2 separations were then appropriately combined. In normal Thymus 2, however, one-half of the cells to be separated was preincubated with TdR-3H, 1 μCi/ml, (specific activity, 6.7 Ci/m mole) at 37°C for 30 min, then washed twice in Hanks' balanced salt solution, and resuspended for separation. Incorporation of TdR-3H into the resultant fractions was then assessed by liquid scintillation counting after digestion of the cells (17). The remaining one-half of the cells (not incubated with TdR-3H) was simultaneously separated by sedimentation. The resultant fractions were pooled into 5 groups for polyamine analysis, the same groups as in normal Thymus 1 plus an additional intermediate group designated 1a (Chart 2, bottom).

The final pooled groups were counted by hemocytometer in order to determine cell concentration. Viable cell concentration in each group was determined by trypan blue exclusion. Known volumes of each group were then centrifuged at 300 x g; the supernatant was discarded and the pellet was frozen at -20°C for assay of polyamine content.

**Preparation of the Cell Extracts.** The designated cell fractions (2 to 20 x 10^6 cells per fraction) were thawed and homogenized with an Electro-Mechanics Corp. ultrasonic cell disrupter equipped with a 4.5-inch probe (Electro-Mechanics Instrument Corp., Perkasie, Pa.) in 400 μl of 0.2 M HClO₄ at 0–2°C. The homogenates were centrifuged at 1000 x g for 15 min.

**Dansylation of the Cell Extracts.** Cell extracts and polyamine standards (mixtures of putrescine, spermidine, and spermine at concentrations from 2.5 to 25 μM in 0.2 M HClO₄) were allowed to react with an excess of DANS-CI according to the method of Seiler and Wiechmann (23); 200-μl volumes of the cell extracts and polyamine standards were transferred to 1.5-ml Mini-Aktors (Applied Science Laboratories, Inc., State College, Pa.) and dansylated by the addition of 400 μl of DANS-CI solution (acetone, 30 mg/ml) and 100 μl of a saturated Na₂CO₃ solution. DANS-CI reacts rapidly with primary and secondary amino groups under suitable conditions (20, 23), but to ensure quantitative dansylation of the polyamines, the reaction mixtures were kept for 16 hr in the dark at room temperature and in the presence of an excess of reagent. Since DANS-CI is easily hydrolyzed by silica gel to DANS-OH (23), which causes blue-green fluorescent streaks on the chromatogram, 100 μl of a proline solution (150 mg/ml) were added to, and thoroughly mixed with, the reaction mixture to remove the excess DANS-CI by conversion to DANS-proline. After 30 min the DANS-amides were extracted into 500 μl of benzene by vigorous shaking, and the layers were separated by centrifugation.

**TLC.** Aliquots (5 to 20 μl) of the benzene extracts were applied to activated (1 to 2 hr at 110°C) TLC plates (20 x 20-cm glass plates, precoated with 250-μm silica gel G layers; E. Merck, Darmstadt, Germany) with a Hamilton syringe. The spots were applied 1.5 cm from the edge of the plate. Eight samples and 3 concentrations of polyamine standards (10 to 100 pmoles in 10 μl) were applied to each TLC plate. The separation of bis-DANS-putrescine from tri-DANS-spermidine and tetra-DANS-spermine as well as from other fluorescing substances in the benzene extracts was achieved by a 1-dimensional ascending development of the TLC plates with ethyl acetate/cyclohexane (2/3, v/v) (2). Development was stopped when the solvent front was 5 mm from the top edge of the plate (>90 min). Immediately after development the plate was sprayed with 10 ml of triethanolamine/propan-2-ol (1/4, v/v) to enhance the fluorescence intensity of the DANS-derivatives and to stabilize their fluorescence. To further increase the fluorescence, the TLC plates were dried in vacuo for 16 hr in a desiccator containing anhydrous CaSO₄ (room temperature, in the dark) and then equilibrated for 30 to 60 min at atmospheric pressure. The increase in fluorescence intensity is accomplished by the removal of water and possibly some volatile quenching substances (23). By equilibration, quenching of the fluorescence by water absorption during scanning was avoided.

**Direct Fluorometry on TLC Plates.** In situ quantitative analyses were obtained with an Amino-Bowman spectrophotofluorometer (American Instrument Co., Inc., Silver Spring, Md.) equipped with an Amino TLC scanner and
The excitation wavelength was 365 nm and the emission wavelength was 500 nm. In Chart 3, the linear relationship between heights of the fluorescence peaks and the amounts of the polyamines is illustrated. The cellular putrescine content was estimated more accurately by spotting larger volumes of the benzene extracts and then assayed separately. The use of either peak heights or peak areas (peak height times peak width at half-height) yielded similar results and both methods were used in the polyamine determinations. DANS-polyamine standards were included on each TLC plate since the fluorescence intensity varied slightly even when plates were treated identically.

**Chemicals.** TdR-methyl-\(^{3}H\) (specific activity, 6.7 Ci/m mole) was obtained from New England Nuclear, Boston, Mass. DANS-Cl was obtained from Calbiochem, Los Angeles, Calif., and Pierce Chemical Co., Rockford, III., from the latter as a 10% solution in acetone. L-Proline and the hydrochloride salts of putrescine, spermidine, and spermine were purchased from Calbiochem. The polyamines were recrystallized 3 times before use; i.e., dissolved in 50% ethanol, precipitated by the addition of cold acetone, and washed with cold acetone on a filtering funnel. Baker Instra-Analyzed GC-Spectrophotometric Quality Solvents (Baker Chemical Co., Phillipsburg, N. J.) were used.

**RESULTS**

**Separation of Leukemic Cells.** Three separation experiments with AKR leukemic cells were performed. Cell size distribution and composition of the pooled Fractions I to V are depicted in Table 1 representative leukemic cell separation (Chart 1, top). Cell size and, therefore, sedimentation velocity correlated with increasing fraction number, as previously reported (13, 17). In all cell separations a significant portion of debris appeared prior to Fraction I. This debris (0 to 5% viability) was also collected and subjected to polyamine analysis. About 85% of the thymus cells in the original sample was demonstrated to be viable by the trypsin blue exclusion method, whereas the viability of the cells in each fraction was **\( \approx 75\% \).**

Previous studies of the cytokinetics of AKR leukemia (13, 17) indicate that the cell size distribution generated by sucrose gradient cell separation correlates with cell cycle phases. Therefore the leukemic cells were pooled into groups (Fractions I to V) representative of \( G_0, G_1, G_1 + S, S, \) and \( G_2 + M \) populations, respectively. A portion of the evidence from an earlier study (13, 17) illustrating the correlation of TdR-\(^{3}H\) uptake and DNA content to determine the viability of the cells in each fraction is seen for comparison in Chart 1 (bottom).

**Separation of Normal Cells.** Two separation experiments with normal AKR thymus cells were performed. The cell size distributions and compositions of the pooled Fractions I to IV are shown in Chart 2. Almost 100% of the thymus cells in the original sample was viable compared to **\( \approx 90\% \)** viability of the cells in each fraction. The cytokinetics of the normal AKR thymus is not as well documented as that of the leukemic thymus. Therefore the fractions were divided arbitrarily for polyamine assay. TdR-\(^{3}H\) uptake into normal thymic cells was analogous to the leukemic AKR data and therefore suggests that Fractions I la consist of \( G_0 \) and \( G_1 \) cells, Fraction II consists of \( G_1 + S \) cells, and beyond Fraction II consists of \( S \) and/or \( G_2 \) and/or \( M \) cells.

**Polyamine Content of AKR Leukemic Cells.** Despite the high cell count of the fraction containing debris (up to \( 2 \times 10^8 \) cells with 0 to 5% viability) and despite its high DNA content, it was not possible to identify any polyamines in this fraction. The subsequent fractions (I to V), however, contained considerable amounts of the polyamines (Charts 3 and 4). Since nonviable cells did not seem to contain any appreciable amounts of the polyamines, the cellular polyamine content in each fraction was calculated on the basis of viable cells. The content of putrescine and spermidine were similar in the \( G_0 \) (Fraction I) and \( G_1 \) phases (Fraction II) of the cell cycle, whereas the spermine level was higher in \( G_0 \) than in \( G_1 \). In Fraction III, which contained cells both in \( G_1 \) and \( S \), the amounts of putrescine, spermidine, and spermine per \( 10^6 \) viable cells were markedly increased. Also, from Fractions IV and V.
**DISCUSSION**

**Techniques.** The method of sucrose density gradient fractionation of both normal thymic cells and AKR leukemic cells into discrete size groups representative of phases of the cell cycle presents a powerful tool to study biochemical

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**Chart 2.** Top and bottom, cell size distribution and composition of the pooled Fractions I to IV in the $1 \times g$ sucrose density gradient separations of normal thymic cells from 2 AKR mice. Bottom, TdR-$^3$H incorporation in normal thymic cells is presented as related to the separation pattern.

**Chart 3.** Record of the fluorescence intensity pattern of a typical thin-layer chromatogram of a dansylated extract, Fraction I of AKR leukemic cells that had been separated by density in a $1 \times g$ sucrose gradient. Superimposed are standard curves of fluorescence intensities (peak heights) versus amount (10 to 75 pmoles each) of bis-DANS-putrescine (Pu), tri-DANS-spermidine (Sd), and tetra-DANS-spermine (Sp).

**Chart 4.** Amounts of putrescine (A), spermidine (B), and spermine (C) in AKR leukemic cells separated in a $1 \times g$ sucrose density gradient. Three cell populations were analyzed after their separation into 5 fractions (I to V) composed of cells representative of the $G_0$ (I), $G_1$ (II), $G_1 + S$ (III), S (IV), and $G_2 + M$ (V) phases of the cell cycle. On abscissa, control values; cellular polyamine content before separation of the cells.
events of the cell cycle without the complications inherent in the utilization of synchronizing techniques.

To examine whether the endogenous levels of the polyamines were affected by the separation procedure itself, we compared the polyamine concentration of cells suspended for the same length of time in the same medium used in the sedimentation chamber with cells of the original suspension frozen immediately. No significant differences were found and thus the possibility of leakage of polyamines during the cell separation procedure can be excluded. It also appeared that the 4-hr separation time at room temperature did not markedly affect the viability of the cells, since the viability count of the cells, as determined by the trypan blue exclusion method, decreased by less than 10% during the 4-hr separation procedure.

**AKR Leukemia.** Leukemic cell populations, derived from the thymus of AKR mice with advanced spontaneous leukemia, contain a significant population of quiescent cells. In previous studies (13, 17) it has been shown that the smallest cells, which constitute 5 to 10% of the population, neither incorporate TdR-3H with repetitive labeling nor arise immediately from dividing cells; they are felt to be G_0_ cells. When transplanted into nontumorous AKR mice, however, these small cells, like the larger proliferating cells, can induce leukemia. When compared to G_1_ cells, the G_0_ cells had a higher spermine content, whereas the putrescine and spermidine contents were approximately the same in these 2 phases of the cell cycle (Chart 4). In subsequent phases the content of the polyamines increased progressively as the cells traversed the cell cycle (Chart 4).

The putrescine/spermidine and spermidine/spermine ratios were previously shown to decrease with the decreasing growth rate of an experimental tumor (1) and to increase following the stimulation of quiescent cells to divide by an appropriate stimulus (6, 16, 19). Accordingly, in AKR leukemic cells these ratios are higher in cells being in the S, G_2_, and M phases of the cell cycle than in G and G_1_.
The greatest increase in the putrescine/spermidine ratio occurred from $G_1$ to $G_1 + S$, whereas the greatest increase in the spermidine/spermine ratio occurred from $G_1 + S$ to $S$. These changes actually reflect the order of synthesis of the polyamines, putrescine being a precursor of spermidine and spermine being a precursor of spermine. We conclude from these data that substantial putrescine synthesis occurs during late $G_1$ and/or early $S$ phase of the cell cycle.

Normal Thymic Cells. The cell composition in normal AKR thymuses is largely different from that in leukemic thymuses. In studies by Metcalf and Wiadrowski (11), it was shown that the smallest lymphocytes, which do not incorporate TdR-$^3$H and are considered to be nonproliferating cells, constitute almost 90% of the entire thymic cell population. These cells contained the same amount of putrescine, but only one-half as much spermidine and spermine as noncycling leukemic cells. Fractions I to II, which did not incorporate TdR-$^3$H with flash labeling (Chart 2, bottom) and are therefore probably cells of low proliferative activity, had a content of the polyamines that was far less than the cells of the subsequent fractions (Chart 6). The medium and large lymphocytes (Fractions III to IV), which were the only cells in the population that incorporated dT-$^3$H (Chart 2, bottom), had putrescine/spermidine and spermidine/spermine ratios which were much higher than the nonproliferating lymphocytes (Chart 7). Their spermidine and spermine content approximated that of small leukemic cells but their putrescine content approximated that of medium and large leukemic cells.

Conclusions. (a) Polyamine synthesis is initiated early in the cell cycle, i.e., in the $G_1$ phase, in both normal and neoplastic cells. (b) Elevated ratios of putrescine/spermidine and spermidine/spermine are established in late $G_1$ to early $S$ in both normal and neoplastic cells. In AKR leukemic cells, the higher spermidine/spermine ratio exhibited in the $G_1$ compared to the $G_0$ phase is not due to $G_1$ synthesis of spermidine but to the higher content of spermine in the $G_0$ cells. A decreased spermine concentration upon entry of $G_0$ cells into the cell cycle has been observed in liver cells after partial hepatectomy (6, 16, 19). (c) The cellular content of putrescine, spermidine, and spermine increased progressively as the cells traversed the cell cycle from $G_1$ to $M$, and at the end of the cell cycle, their content was 2-fold that in the $G_1$ phase. (d) The discrete ratios of polyamines that are expressed during the cell cycle would suggest precise timing and regulatory mechanisms for polyamine biosynthesis in growth processes.

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