Analyses of Cyclic Nucleotide Phosphodiesterases in Lymphocytes from Normal and Aged Leukemic Mice

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ABSTRACT

The activity of cyclic 3':5'-nucleotide phosphodiesterase was measured in thymocytes and splenic lymphocytes of AKR mice of various ages. Concurrently, the cell distribution profiles of these cell populations were studied by laser cytometric analysis. The results indicate that lymphocyte and thymocyte populations from young, control animals are relatively homogeneous with respect to cell size, whereas cell populations from old, leukemic mice showed increases in the percentage of larger, blast-like cells. These latter cell populations also showed increases in cyclic nucleotide phosphodiesterase activity when compared with that of young animals. Old, non-tumor-bearing mice had lower levels of enzyme activity than did their leukemic counterparts and showed only modest increases in the percentage of blast-like cells. Correlation analyses revealed that increases in enzyme activity correlated with increases in the percentage of large cells but not with increases in thymus weight. These data suggest that increases in phosphodiesterase activity are associated with the appearance of leukemic cells and not with a putative preleukemic state.

Preparative polyacrylamide gel electrophoresis revealed multiple forms of both cyclic adenosine and cyclic guanosine 3':5'-phosphodiesterases. The pattern of the phosphodiesterases showed distinct differences between normal and leukemic thymocytes. The major differences were a reduction in the more slowly migrating forms and a large increase in the activity of one form of the enzyme in the leukemic cells. This suggests that selective inhibition of this form of phosphodiesterase might be effective therapy for certain types of leukemias.

INTRODUCTION

It has been shown previously that the intracellular concentration of cyclic AMP is related to the proliferative potential of both normal and neoplastic cells (3, 16). Cells transformed by oncogenic viruses have lower levels of cyclic AMP than do the corresponding types of normal, nontransformed cells, and there is evidence that these decreased levels are related, at least in part, to the malignant properties of these cells (2, 21). The addition of dibutyryl cyclic adenosine 3':5' monophosphate, cyclic AMP itself, or agents that increase its intracellular concentration has been shown to decrease the proliferation of transformed cells (6, 12, 25) and to reverse the morphological changes often associated with the transformed state (13, 14, 24).

The intracellular concentration of cyclic AMP is regulated by adenylate cyclase, the enzyme that converts 5'-ATP to cyclic AMP, and by cyclic nucleotide phosphodiesterase, the enzyme that degrades cyclic AMP to 5'-AMP. It has been reported that some transformed cells are deficient in adenylate cyclase (1, 2, 4, 22, 33) and that certain neoplastic cells have a higher phosphodiesterase activity than do their normal counterparts (10, 11, 19, 20). Furthermore, it has been reported recently that significant changes in cyclic AMP metabolism are detectable even before neoplastic cells are found in the thymus of AKR mice undergoing spontaneous leukemogenesis (15).

If, in fact, there is an abnormally high activity of phosphodiesterase in leukemic lymphocytes, then by selectively inhibiting this activity with drugs it may be possible to raise the intracellular concentration of cyclic AMP in this tissue. Previous studies showed that there are multiple forms of phosphodiesterase in several tissues (9, 26-29) including normal and leukemic lymphocytes (W. N. Hait and B. Weiss, unpublished observations) and that the various forms in these tissues can be selectively inhibited with pharmacological agents (9, 30, 31).

This report describes studies of the leukemic process in the AKR mouse. The goals of these experiments were 3-fold: (a) to develop a methodology for early detection of leukemic cells in the tissues of animals undergoing leukemogenesis; (b) to analyze the cyclic nucleotide phosphodiesterase activity in these tissues to ascertain whether changes in the activity of this enzyme are associated with a predilection toward the leukemic state or whether these changes are merely part of the general biochemical abnormality associated with leukemia itself; and (c) to determine and compare the pattern of the multiple forms of cyclic AMP and cyclic GMP phosphodiesterase in normal and leukemic cells with the hope of detecting whether any of the changes in phosphodiesterase activity are due to alterations in 1 particular form of the enzyme.

MATERIALS AND METHODS

Animals. AKR/J mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. Two groups of animals were maintained for the duration of the experiment. Group
1 consisted of young female mice that were 8 weeks old at the start of the experiment. Group 2 consisted of female retired breeders. These animals were 8 to 9 months old at the beginning of the study.

**Preparation of Cell Suspensions.** At weekly intervals, 5 mice from each group were sacrificed. The mice were lightly anesthetized with ether and were exsanguinated from the axillary blood vessels. The spleen and thymus were then removed and kept in Petri plates over ice. The organs were lightly blotted and weighed, and cell suspensions were prepared by gently pressing the tissues through fine stainless steel wire mesh with the use of PBS. After large clumps of tissue were allowed to settle, the cell suspension was drawn off, centrifuged in the cold, and resuspended. At this point spleen cell suspensions were resuspended briefly in distilled water and were quickly brought to isotonicity with 154 mM sodium chloride. Thymus cells were resuspended in PBS. Both thymus and spleen cell preparations were washed 2 additional times in PBS, the cells were counted, and aliquots were distributed for laser cytometry and enzyme analyses.

**Laser Cytometric Analysis.** Thymic and spleen cell suspensions were diluted to a final concentration of 3 to 5 x 10^6 cells/ml in PBS, and 0.4 ml of this suspension was added to 10 ml of 0.83% NH_4Cl and incubated for 10 min to lyse any remaining erythrocytes. Without further treatment, this suspension was analyzed on a Model 6301 cytograph equipped with a Model 2100 distribution analyzer (Biophysics Instruments, Mahopac, N. Y.). This instrument permitted the analysis of single cells in the suspension with the use of a helium-neon laser beam with a wavelength of 6328 Å. For a given cell type, the absorption of light is proportional to the size and morphology of the cell. It is possible, therefore, to distinguish normal lymphocytes from larger blast-type cells (34). For each analysis, 10,000 cells were examined, and a histogram plotting light absorbance (size) versus the number of cells was obtained. Cells were distributed over 100 channels (0 to 99); smaller cells with diminished absorbance were distributed in the lower channels while larger cells are distributed among the higher channels. In practice few or no cells were distributed in the channels beyond Channel 50. It is also possible to obtain a subtotal count of all cells distributed between any 2 preselected channels and a printout of the total number of cells in each of the 100 channels.

**Cyclic Nucleotide Phosphodiesterase Analysis.** Individual cell suspensions were aliquoted at 1 to 3 x 10^7 cells/tube, centrifuged, and resuspended in 0.5 ml ice-cold 10 mM glycylglycine buffer, pH 8.0, containing 3 mM MgCl_2. This suspension was then sonically disrupted for 15 sec with a Branson Model W140 Sonifier (Branson Instruments, Plainview, N. Y.) equipped with a microtip at a setting of 2. The sonic product was then quick frozen in alcohol-dry ice and stored at -70° until enzyme analysis.

Cyclic AMP phosphodiesterase activity was determined by the firefly luciferin-luciferase technique as described previously (11, 32). Briefly, the tissue sample is incubated at 37° for varying periods of time with 400 µM cyclic AMP in the presence of 25 milliunits myokinase and 50 milliunits pyruvate kinase in 50 mM glycylglycine buffer, pH 8.0, containing 0.26 mM phosphoenolpyruvate. The reaction is terminated by placing the samples in a boiling water bath for 5 min. An additional quantity of myokinase (75 milliunits) and pyruvate kinase (150 milliunits) is then added, and the samples are reincubated for 1 hr at 37°. The myokinase and pyruvate kinase convert the 5'-AMP, formed from the hydrolysis of cyclic AMP, to ATP. ATP is then quantitated by the addition of firefly luciferin-luciferase, the generated light being detected with a luminescence biometer (E. I. Dupont de Nemours and Co., Wilmington, Del.).

Cyclic GMP phosphodiesterase activity was determined as described earlier (8). In this procedure the tissue is incubated with 200 µM cyclic GMP in 50 mM glycylglycine buffer, pH 8.0. The reaction is terminated by placing the samples in a boiling water bath for 5 min. The 5'-GMP formed from cyclic GMP is then reacted with a known quantity of ATP in the presence of 10 milliunits guanylate kinase. This converts the 5'-GMP and ATP to GDP and ADP. The decline in ATP, therefore, is a measure of the quantity of 5'-GMP formed in the phosphodiesterase reaction.

**Electrophoretic Separation of the Multiple Forms of Cyclic Nucleotide Phosphodiesterase.** Cells were prepared and sonically disrupted as described above and were stored at -70° until assayed (within 1 week). Sonic products of the purified lymphocytes were centrifuged at 100,000 x g for 60 min. Approximately 70% of the phosphodiesterase activity in thymocytes of 11-week-old mice and 60% of that of 40-week-old mice were recovered in the soluble supernatant fluid. This soluble fraction was subjected to polyacrylamide gel electrophoresis on a preparative column as described previously (29). One hundred thirty fractions (2 ml each) were collected, and cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activities were determined in each fraction as described above with 400 µM cyclic AMP or 200 µM cyclic GMP as substrates.

**RESULTS**

Two groups of animals were studied in the experiments described in this report. Group 1 consisted of young AKR mice between 8 and 20 weeks of age, and group 2 consisted of old AKR mice between 34 and 47 weeks of age.

**Cytometric Analysis.** Chart 1 illustrates the laser cytometric analysis of the distribution of thymocytes and splenocytes from AKR mice. Chart 1A depicts the data from 2 individual populations derived from young AKR mice (Group 1). These animals were 11 and 12 weeks old, and neither showed any gross evidence of tumor. The histogram indicates a rather narrow distribution of cell sizes (between 6 and 8 µm) with the mode falling between Channels 13 and 16. Approximately 88% of the cells were included in the channels between 0 and 20, while 94% were included between Channels 0 and 25. Analysis of thymocyte populations from 30 young AKR mice varying in age between 8 and 20 weeks indicated that, on the average, only 5.8% of the cells were distributed beyond Channel 25 (see Table 1).

Chart 1B shows the distribution of 2 populations of splenic lymphocytes from 1-week-old AKR mice from Group 1. When compared with the thymic lymphocytes, the histograms of splenic lymphocytes were skewed slightly more to the right. In 1 case 94% of the cells and in the second case about 89% of the cells were distributed be-
Chart 1. Laser cytometric analysis of the distribution of thymocytes and splenocytes from AKR mice. For each population 10,000 cells were monitored. Abscissa, cells distributed into channels according to light absorbance (size); ordinate, number of cells per channel. A. , thymocytes from an 11-week-old AKR mouse; , Mouse 45; , Mouse 46; , Mouse 47. B. Plots of 2 individual populations of splenic lymphocytes from 13-week-old AKR mice. C. Thymocytes from 38-week-old mice. , Mouse 45; , Mouse 46; , Mouse 47. D. Splenocytes from 38-week-old mice. , Mouse 45; , Mouse 46; , Mouse 47.

Chart 1C illustrates the distribution of thymocytes, and Chart 1D shows the distribution of splenic cells derived from animals taken from Group 2 which were then 38 weeks old. The first of these mice (Mouse 45) had both a thymoma and an enlarged spleen, the second (Mouse 46) had only a slightly enlarged thymus but a greatly enlarged spleen, while the organs from the third mouse (Mouse 47) were of normal size and were indistinguishable from those taken from young mice of Group 1. From Chart 1C, it is evident that the distribution of thymocytes from Mouse 47 is similar to that of a normal, young AKR mouse. Channels 0 to 25 included 92% of the cell population. In Mouse 45, the thymus weighed 795 mg and the spleen weighed 540 mg. Nearly 19% of the thymocytes were distributed beyond Channel 25, and the histogram shows comparatively fewer cells than normal distributed in the range occupied by small lymphocytes. Mouse 46 was unusual in that, whereas the spleen weighed nearly 800 mg, the thymus weighed only 141 mg and appeared essentially normal. However, the histogram revealed that 34% of the thymus cells from this animal were distributed beyond Channel 25. Morphological examination of smears from this cell population confirmed that these cells were indeed blast types resembling tumor cells.

Chart 1D shows the patterns of spleen cell populations from these same 3 mice. Although the shift to the right in the cells derived from the tumor-bearing mice is less dramatic than that seen with the thymocytes, nevertheless, 19% of the splenocytes from Mouse 45 and 14% of those from Mouse 46 were distributed beyond Channel 25. By contrast, only 9.7% of the splenocytes from Mouse 47 (which had a normal-sized spleen) were found beyond Channel 25. Thus, the data show that laser cytometric analysis is capable of detecting changes in both thymus and spleen cell populations from tumor-bearing mice and, more importantly, is capable of detecting these changes even when the organs themselves appear essentially normal.

The mice in Group 2 were examined weekly when they were between 34 and 47 weeks old. Concurrently, young mice from Group 1 were also examined. From these studies it became evident that many of the mice from Group 2 were similar to Mouse 47; i.e., they appeared essentially normal at the time they were sacrificed. Consequently, on the basis of data derived from organ weights and laser cytographic analysis, Group 2 was divided into subgroups; Group 2a included those animals with no evidence of tumor development and Group 2b included those mice with some evidence of tumor. The data derived from these studies are summarized in Table 1. It can be seen that the thymus weights are not significantly different between Group 1 (the young mice) and Group 2a (the old mice that had no evidence of tumor). On the other hand, the mean thymus weight of mice from Group 2b (365 mg) was significantly different from that of the other groups (p < 0.01). The spleen weights showed a similar pattern.

Cytographic analysis of the cells derived from these mice showed that both the lymphocytes and the splenocytes of mice in Group 2a had a higher number of cells distributed beyond Channel 25 than did those of Group 1. This shift in the cell size may be related to a preleukemic state since tumor-bearing mice from Group 2b showed large increases in the number of cells distributed beyond Channel 25 for both thymus and spleen cell populations (p < 0.01) when compared with cells from Group 1.

Phosphodiesterase Activity. The cyclic AMP phosphodiesterase activities from these mice are also presented in Table 1. As may be seen, phosphodiesterase activity in thymus cells from mice from Group 2a (aged mice with no evidence of tumor) was not significantly different from that found in young AKR mice (Group 1). However, the thymocytes of mice that showed evidence of tumor (Group 2b) exhibited nearly a 4-fold increase in phosphodiesterase activity as compared with that of the young control mice (Group 1).

The phosphodiesterase activity in splenocytes from aged, non-tumor-bearing mice was lower than that of the young control group. However, the enzyme activity in cells from aged, tumor-bearing mice (Group 2b) was 1.7-fold greater than that of young control animals (p < 0.05) and 2.5-fold
greater \( p < 0.05 \) than that found in aged non-tumor-bearing animals (Group 2a). Thus, increases in phosphodiesterase activity correlated fairly well with tumorigenesis and cell size distribution and do not appear to be due to age-related changes.

To determine whether there are any statistically significant correlations between organ weight, cell distribution, and phosphodiesterase activity, we conducted additional statistical analyses of the data of Groups 1 and 2. In these analyses no distinction was made with regard to whether or not the mice in Group 2 showed evidence of tumor development. No statistically significant correlations were found from comparisons of any of these parameters from Group 1. However, when these analyses were performed on the data from Group 2 mice, several correlations were evident.

First, although there was no significant correlation between thymus weight and thymic cell distributions or between thymus weight and thymic phosphodiesterase activity, there was a statistically significant correlation \( p < 0.001 \) between thymus cell distribution and thymus cell phosphodiesterase activity. Furthermore, there was a significant correlation \( p < 0.001 \) between the spleen cell distribution and the thymic cell distribution and between the spleen weight and the thymic cell distribution.

**Multiple Forms of Cyclic AMP and Cyclic GMP Phosphodiesterase Activity.** Chart 2 shows the electrophoretic pattern of the multiple forms of cyclic AMP and cyclic GMP phosphodiesterase found in the 100,000 × g soluble supernatant fraction of thymocytes from 11-week-old mice. As can be seen there were 5 distinct peaks of cyclic AMP phosphodiesterase activity: 2 relatively small peaks of activity emerging first from the polyacrylamide gel column followed by the major peak at Fraction 75 and then by 2 other peaks of activity (Chart 2A). By contrast, only 4 peaks of cyclic GMP phosphodiesterase activity were detectable (Chart 2B). The latter 3 peaks coincided with the latter 3 peaks of cyclic AMP phosphodiesterase although the relative proportions were somewhat different. However, the first peak of cyclic GMP phosphodiesterase did not correspond completely with either of the first 2 peaks of cyclic AMP phosphodiesterase.

Chart 3 shows the pattern of the cyclic nucleotide phosphodiesterase in the soluble fraction of lymphocytes isolated from thymomas of 40-week-old AKR mice. In these experiments, although material from approximately the same number of cells was added to the column, as in the previous experiments the total activity eluted from the polyacrylamide gel column in the thymoma samples was substantially greater than that eluted from the normal lymphocytes, a reflection of the higher phosphodiesterase activity in thymomas. As can be seen, the pattern of phosphodiesterase activity from the thymomas has some similarities, but some marked differences from that of the normal lymphocytes. As in the cyclic AMP phosphodiesterase pattern of lymphocytes from young AKR mice, there were 2 small peaks of activity followed by the major peak at Fraction 75 (Chart 3A). Also as in the lymphocytes of young AKR mice, there was no detectable peak of cyclic GMP phosphodiesterase in the early samples (i.e., Fraction 35) (Chart 3B). However, a major difference between lymphocytes from young and old mice was the lack of significant phosphodiesterase activity following the major peak at Fraction 75 in the thymomas.

Each of these forms of phosphodiesterase was analyzed for its ability to be activated by the calcium-dependent, heat-stable modulator protein (5), which selectively activates 1 particular form of phosphodiesterase (18, 30). None of the fractions was activated by this protein, suggesting that, like the lymphocytes from DBA mice of L1210 leukemic lymphocytes (11), these cells do not contain the activable form of phosphodiesterase.

**DISCUSSION**

Malignant tissue differs from normal tissue in several biochemical, cytological, and metabolic properties, and the essence of cancer depends upon the uncontrolled replication and invasiveness of the tumor cells themselves. One of the main questions concerning neoplasia is the biochemical or metabolic reason for this uncontrolled proliferation. Recent reports have suggested that cyclic nucleotides and the enzymes that control their intracellular levels may be involved in malignant disease. Numerous studies, including several on various leukemic cell lines, support this concept.

AKR mice, beyond the age of 6 months, spontaneously develop murine leukemia, virus-induced tumors, evident
Chart 2. Multiple forms of cyclic AMP and cyclic GMP phosphodiesterase activities in thymocytes of young AKR mice. Thymocytes from 11-week-old mice were purified, sonically disrupted, and centrifuged for 1 hr at 100,000 x g. The soluble supernatant fluid of these sonic products was subjected to polyacrylamide gel electrophoresis, and each fraction was assayed for cyclic nucleotide phosphodiesterase activity as described in “Materials and Methods” with the use of 400 μM cyclic AMP (A) or 200 μM cyclic GMP (B). The phosphodiesterase activities of the material added to the polyacrylamide gel column were 2.8 nmol cyclic AMP hydrolyzed per mg protein per min and 1.2 nmol cyclic GMP hydrolyzed per mg protein per min. Approximately 32% of the cyclic AMP phosphodiesterase and 34% of the cyclic GMP phosphodiesterase activity were recovered from the electrophoresis column. This is representative of 2 similar experiments.

Chart 3. Multiple forms of cyclic AMP and cyclic GMP phosphodiesterase activities in thymomas of old AKR mice. Thymocytes from thymomas of 40-week-old mice were purified, sonically disrupted, and centrifuged for 1 hr at 100,000 x g. The soluble supernatant fluid of these sonic products was subjected to polyacrylamide gel electrophoresis, and each fraction was assayed for cyclic nucleotide phosphodiesterase activity as described in “Materials and Methods” with the use of 400 μM cyclic AMP (A) or 200 μM cyclic GMP (B) as substrate. The phosphodiesterase activity of the material added to the polyacrylamide gel column was 11.3 nmol cyclic AMP hydrolyzed per mg protein per min and 4.0 nmol cyclic GMP hydrolyzed per mg protein per min. Approximately 37% of the cyclic AMP phosphodiesterase and 26% of the cyclic GMP phosphodiesterase activity were recovered from the electrophoresis column. This is representative of 2 similar experiments.

most often as thymomas. In this model, the development of leukemia is preceded by a preleukemic state in which there are demonstrable changes in cyclic AMP levels, epinephrine-sensitive adenylate cyclase activity, and cyclic AMP phosphodiesterase activity (15, 19). It might be suggested from these data that changes in cyclic nucleotide metabolism in a presumed preleukemic animal signal the onset of leukemogenesis and may even be responsible for the sequence of events leading up to leukemogenesis. It was of critical interest, therefore, to ascertain whether the changes in cyclic nucleotide metabolism were associated with a preleukemic state or merely with the leukemic process itself. Moreover, since cyclic nucleotide phosphodiesterase exists in different molecular forms (9, 29) the activities of which can be selectively modified with drugs (9, 30, 31), it is important to compare the pattern of phosphodiesterase activity in the nonleukemic and leukemic cells.

With laser cytometry it is possible to measure changes in the population of blast cells. This procedure has been used to quantitate the in vitro immune response (34). Since many types of tumors contain a predominance of larger, blast-like cells, we undertook to apply this methodology to the analysis of the AKR model. Since this tumor occurs spontaneously in vivo, it is difficult to predict accurately the advent of leukemogenesis. Therefore, in these studies we examined each animal and used the presence of thymomas as...
the major sign of neoplasia. By laser cytomeric scanning of a large population of cells (10,000 cells/organ), we hoped to find early evidence of leukemogenesis and to determine whether changes in the metabolism of cyclic nucleotides are associated with tumor development itself or with a putative preleukemic state.

The evidence presented in this report demonstrates that thymic tissues from mice in the preleukemic state (Group 2a) show a small although statistically insignificant increase in cyclic AMP phosphodiesterase activity. Significant increases in the activity of this enzyme, however, were clearly demonstrable in those animals that showed definite signs of neoplasia (Group 2b) as indicated by changes in both thymus and spleen weights and by increases in the percentage of blast-like cells detectable by laser cytometry. Our findings of a higher phosphodiesterase activity in leukemic cells of the AKR mouse are in agreement with the results of Menahan and Kemp (19) and Kemp et al. (15) in the AKR mouse and with those of Hait and Weiss (10, 11) who showed severalfold elevations of cyclic nucleotide phosphodiesterase activity in L1210 and L5178Y leukemic cell lines.

Laser cytomeric analysis of the thymic and splenic lymphocytes from preleukemic mice revealed increases in the percentage of blast-like cells and spleen weight. Tumor-bearing mice (Group 2b) showed definite evidence of thymoma and exhibited large increases in spleen weight. Laser analysis of the cells from these populations also indicated dramatic increases in the percentage of blast-like cells. Morphological examination of cells from this population confirmed the fact that these cells did indeed resemble tumor cells. Correlation analyses established a significant relationship only between thymic cell cyclic AMP phosphodiesterase activity and the percentage of blast-like cells present in the thymus. In some mice the thymus weight was essentially normal, but the percentage of blast cells in the thymus was markedly elevated. Thymus phosphodiesterase activity from these animals was also high.

The studies on the multiple forms of phosphodiesterase found in the soluble 100,000 g supernatant fraction of cell sonic products showed that leukemic cells exhibited a pattern of isozymes different from that found in the nonleukemic cells. The major difference was a marked reduction in leukemic cells of phosphodiesterase isozymes that migrated relatively slowly on the polyacrylamide gel. In leukemic cells, 1 form of the enzyme (which peaked at about Fraction 75; see Chart 3) accounted for approximately 90% of the total phosphodiesterase activity. By contrast, in nonleukemic cells this same form of the enzyme (Chart 2) accounted for less than 40% of the total phosphodiesterase activity. This suggests that if one could inhibit this form of phosphodiesterase selectively one might be able to alter specifically the concentration of cyclic nucleotides in the leukemic cells. This suggestion must be tempered, however, by the observation that our studies deal with only the soluble phosphodiesterase. The particulate phosphodiesterase, which in our experiments accounts for 30 to 40% of the total activity, may have entirely different properties from that of the soluble phosphodiesterase. Indeed Menahan and Kemp (19), who also reported that much of the phosphodiesterase activity of thymic lymphocytes was particulate, found that this fraction contained a greater percentage of the high-affinity phosphodiesterase than did the soluble fraction.

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