Lymphocyte Aromatic Hydrocarbon Responsiveness in Acute Leukemia of Childhood

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Aryl hydrocarbon hydroxylase (AHH) activity and inducibility were examined in mitogen-stimulated cultured lymphocytes from children with acute leukemia in remission, with nonleukemic malignancies, and with no family or personal history of malignant disease. Neither morphological differences nor differences in mitogen responsiveness were observed among the three sources of cells studied. Levels of constitutive and dibenzanthracene-induced AHH activity were found to be similar among the three groups by analysis of variance. However, when results were analyzed in terms of inducibility ratios, it was found that cells from leukemic children were significantly less inducible (p < 0.005) than cells from unaffected children or children with nonleukemic malignancies. The reason for this difference became apparent when statistical criteria were employed for the phenotypic separation of individuals who were highly aromatic hydrocarbon responsive and minimally responsive. A significantly larger proportion (p < 0.001) of leukemic children than unaffected children or children with nonleukemic malignancy were found to be minimally aromatic hydrocarbon responsive. Moreover, in patients with acute lymphoblastic leukemia relapsing while on therapy, longer durations of the first remission were correlated (r = 0.63, p < 0.05) with the highly inducible AHH phenotype.

Acute leukemia ranks as the malignancy of highest incidence in the pediatric population of the United States. The possibility that genetic factors might play a role in the leukemogenesis was first recognized by Videbaek; however, subsequent studies specifically related to childhood acute leukemia have not supported his conclusions. Nevertheless, it has been convincingly demonstrated that certain chromosomal disorders (e.g., Bloom's syndrome, Fanconi's anemia, Down's syndrome) are associated with an increased risk of acute leukemia. In addition, in studies involving 22 pairs of monozygotic twins, the cotwin was affected within weeks or months of the initial diagnosis, suggesting a genetic predisposition to the disease. Finally, Miller concluded that siblings of a leukemic child have a fourfold greater risk of developing the disease than the general population of children between birth and 15 yr of age.

To date, all of the studies that propose a role for genetics in leukemogenesis have been epidemiologic in nature. Epidemiologic studies have also suggested that environmental chemicals may be responsible for a considerable portion of all human cancer. The mechanisms whereby these environmental agents result in neoplastic transformation remain highly speculative; however, the data suggest that, at least in part, an interaction between the putative carcinogen and a group of intracellular enzymes responsible for its metabolic activation is required.

Of the enzymes whose activities have been associated with the metabolic activation of chemical carcinogens, aryl hydrocarbon hydroxylase (AHH) has been most thoroughly studied. AHH is a cytochrome P-450 oxygenase found in essentially all tissues of the body. P450 monoxygenases are known to play a role in the metabolism of a large number of endogenous and exogenous compounds including drugs, steroids, fatty acids, pesticides, hormones, cosmetics, and environmental pollutants. Metabolic reactions catalyzed by AHH may result in substrate activation to highly reactive intermediates. AHH activity has been demonstrated in mitogen-stimulated cultured human lymphocytes. Recent work by Boobis et al. has shown that these lymphocytes can catalyze the activation of benzpyrene to metabolites that covalently bind to DNA. Lymphocyte AHH activity is inducible by exposure of the cultured cells to certain polycyclic aromatic hydrocarbon (PAH) carcinogens, and this inducibility has been shown in twin studies to have a strong genetic component.

In the present study the lymphocyte aromatic hydrocarbon responsiveness (i.e., inducibility) of children with acute leukemia is compared to that of unaffected children. It was found that the incidence of minimal aromatic hydrocarbon responsiveness was significantly increased in leukemic children when...
compared with unaffected children. Further, the AHH inducibility phenotype significantly correlated with response to chemotherapy.

**MATERIALS AND METHODS**

**Patient Population**

Patients were enrolled only after informed consent was obtained from parents and/or the patients themselves. This study was approved by the Institutional Review Board for Human Subject Experimentation of University Hospitals of Cleveland.

A summary of patient characteristics is shown in Table 1. Three groups of children were studied. Unaffected children were surgical patients at Rainbow Babies and Children’s Hospital. None of these children had a history of malignancy.

Children with acute leukemia and with nonleukemic malignancies were participants in chemotherapeutic protocols sponsored by the Children’s Cancer Study Group. Unless otherwise specified, all leukemic patients were in complete remission as defined by the apparent absence of leukemic blasts from peripheral blood, bone marrow (M-1 marrow), and cerebrospinal fluid in the presence of a normal physical examination at the time of the study. The duration of remission ranged from 2 mo to more than 10 yr. All acute lymphoblastic leukemias were of the non-T-cell, non-B-cell type.

Children with nonleukemic malignancies were included in an attempt to control for possible effects of cytotoxic chemotherapy on enzyme activity and inducibility. The nonleukemic malignancies included were: rhabdomyosarcoma, 4; Hodgkin’s disease, 7; neuroblastoma, 3; medulloblastoma, 2; osteogenic sarcoma, 2; Wilms’ tumor, 2; and pinealoblastoma and Burkitt’s lymphoma, 1 each. It is recognized that such a group of patients constitutes an imperfect control, because both the disease processes and the drug regimens differ from those of the study population. Nevertheless, in terms of drug effects, the antitumor actions of these agents are due to a relatively limited number of mechanisms (e.g., alkylation, intercalation, antimetabolite, mitotic inhibition, etc.), and the pharmacologic half-lives of most are quite short. Thus, any effect of these therapeutic agents on the in vitro AHH inducibility in patients’ lymphocytes would most likely be related to somatic cell mutations induced as a consequence of their antitumor mechanisms and would be expected to be manifest in both cancer patient populations. Any more precise control would be ethically unacceptable.

In addition to the three groups of patients studied, blood samples were obtained at frequent intervals from a group of laboratory personnel. These subjects were studied to control for any changes that might occur in culture conditions throughout the study period. None were apparent.

**Lymphocyte Isolation and Culture**

All cultures were prepared under aseptic conditions. Mononuclear cells were isolated from whole blood by sedimentation on Ficoll-Hypaque (density = 1.080) as described by others16,17 (Fig. 1). Dibenz[a, h]anthracene (DBA) (Aldrich Chemical Co., Milwaukee, Wisc.) was chosen as the PAH-inducing agent for lymphocyte AHH after preliminary studies revealed it to be the most efficacious aromatic hydrocarbon among seven that were tested. Similar observations have been reported recently by others.18 DBA was added to a final concentration of 1 μM, which produced maximal induction in responsive individuals.

**Cell Characterization**

A white cell and differential count were obtained on all samples prior to lymphocyte isolation. In general, 30% of the peripheral blood lymphocytes were recovered and postculture viability was routinely 85%–90% as determined by trypan blue dye exclusion.

Cytocentrifuge slides were prepared pre- and postculture for each patient. They were all stained with May-Grünewald-Giemsa19 and the percent lymphocytes or lymphoblasts, respectively, was determined after counting 100 cells. Where indicated, 1 μCi [3H]-thymidine, 23 mCi/m mole, (Amersham, Arlington Heights, Ill.) was added to one control and one DBA-treated culture each containing 2 x 10⁶ cells concomitant with the addition of inducer. These cultures were used to measure the extent of mitogen-induced blastogenesis.10

Recovery of cells from culture was determined by counting and by DNA determination according to the method of Hill and Whatley;20 each of 10⁶ cells was found to contain 10 ± 0.8 μg of DNA.
Determination of cell surface markers was performed according to the procedure of Stadecker et al.27

Enzyme Assay

AHH activity in freshly isolated and cultured human lymphocytes was measured in terms of the formation of alkali-extractable phenolic products from 3,4-benzpyrene (Aldrich Chemical Co., Milwaukee, Wis.) by the fluorometric assay of Nebert and Gelboin28 as modified by Gurtoo et al.22 Each reaction mixture contained in 1 ml: 2-10 × 10^6 cells, 50 mM Tris-HCl, pH 8.6, 10 mM Gelboin22 as modified by Gurtoo et al.22 Each reaction mixture was incubated at 37°C for 3-5 min. All assays were performed under subdued lighting and terminated by the addition of 4 ml of acetone:heptane (1:3).

Preliminary studies showed that the product(s) formed by cultured human lymphocytes had fluorescence excitation and emission spectra analogous to those obtained with the products of rodent liver microsomal metabolism of 3,4-benzpyrene. It was also determined that product formation was linear with respect to cell number (from either leukemic or non-leukemic children) up to at least 10 × 10^6 cells/ml and with respect to time up to at least 45 min. Routinely, reaction mixtures contained 4 × 10^6 cells/ml and the cells were incubated for 30 min prior to the addition of acetone:heptane.

Analysis of Data

Overall comparisons of AHH activity in control and induced cells as well as the AHH inducibility ratio for the three study groups were performed by the analysis of variance method.23 Scheffe's multiple comparison technique was applied for the testing contrasts.24 The cutoff value for the AHH phenotype was obtained by utilizing a maximum likelihood estimation procedure.21

RESULTS

Characterization of Isolated Cells Before and After Culture

Cytocentrifuge preparations of cells sedimenting at the plasma-polymer interface after Ficoll-Hypaque sedimentation were stained as described. When examined by light microscopy, preparations from children with acute leukemia in remission and from children with nonleukemic malignancies were indistinguishable from those from unaffected children. In each case, approximately 95% of the cells were mononuclear, and of these more than 85% were clearly recognizable as small lymphocytes.

After culture for 72 hr in the presence of mitogen or mitogen plus DBA, slides were again prepared by cytocentrifugation. All cells present after culture were blast-like with one or more prominent nucleoli, euchromatin, and a highly basophilic cytoplasm containing several clear vacuoles.

Cell viability was estimated before and after culture by trypan blue dye exclusion. Irrespective of source or culture conditions (i.e., presence or absence of DBA), apparent cell viability was always greater than 90%.

The lack of discernible morphological differences among the cells of the three groups of children studied was supported further by determination of T and B surface markers in ten children from each group. In freshly isolated cells, there were no differences in the relative numbers of T and B cells among the three groups; 68% ± 12% formed E-rosettes and 26% ± 8% formed EAC-rosettes. The values obtained were in good agreement with those reported by others in normal controls.26 Moreover, after culture in the presence of mitogen and/or DBA the percentage of T and B cells did not change.

Response to Mitogen

Work in other laboratories, confirmed in the present study (see below), has shown that mitogen stimulation is required for the expression of AHH activity in human lymphocytes.27 It was therefore necessary to determine that cells from leukemic children in remission and cells from children with nonleukemic malignancies responded to mitogen stimulation in a manner analogous to cells from unaffected children. This was evaluated by measuring 3H-thymidine incorporation during the last 24 hr in culture. Preliminary experiments showed that 3H-thymidine uptake into cells from both unaffected and leukemic children cultured in the presence or absence of DBA was linear for at least 72 hr. No significant difference in 3H-thymidine uptake among the three groups of children studied, either in the presence or absence of DBA, occurred. Similar results were obtained with a 4-hr pulse of label just prior to harvesting the cells.

| Table 2. AHH Activity in Mitogen-Stimulated Cultured Lymphocytes From Pediatric Patients |
|---------------------------------|-------------------|-------------------|
| Group                          | Treatment         | Control (pmole/30 min/10^6 cells) | DBA (pmole/30 min/10^6 cells) |
| Unaffected                     |                   | 1.53 ± 0.22*       | 5.23 ± 0.80                  |
| Nonleukemic malignancy         |                   | 1.61 ± 0.38       | 5.04 ± 1.17                  |
| Acute myelogenous leukemia     |                   | 1.82 ± 0.21       | 3.88 ± 1.41                  |
| Acute lymphoblastic leukemia   |                   | 2.09 ± 0.21       | 3.71 ± 0.37                  |
| F                              |                   | 1.19 (p > 0.05)   | 1.13 (p > 0.05)              |

*Values represent the mean ± SE.
†F. value from analysis of variance.
**AHH Activity in Cultured Cells**

In confirmation of previous reports, AHH activity was undetectable in cells from any of the three groups of children prior to culture even at cell densities as high as $10 \times 10^6$ cells/ml.

Table 2 summarizes the AHH activity in mitogen-stimulated cultured lymphocytes from the three groups of children studied. Unaffected children were studied only once. Children with leukemia in remission and children with nonleukemic malignancies were studied from 2 to 5 times, with all but 12 being studied on at least three separate occasions. No significant differences in control activity were noted. Following 24 hr of exposure to DBA, the AHH activity in cells from unaffected children and from children with nonleukemic malignancies was increased to the same extent. In contrast, cells from patients with acute leukemia showed less of an increase in activity after DBA treatment, although the difference was not statistically significant.

**AHH Inducibility Ratio and AHH Phenotype**

As depicted in Fig. 1, our experimental design requires that each patient serves as his/her own control. This permits the derivation of a quotient, the inducibility ratio, which is defined as the ratio of AHH activity in mitogen-stimulated DBA-treated cells to the AHH activity in cells cultured in mitogen alone. The use of the inducibility ratio served to minimize the day-to-day intrapatient variation in the absolute levels of constitutive and induced AHH activity so that the coefficient of variation for individual patients on repetitive sampling is approximately 0.20.

Table 3 shows the average inducibility ratios for the various groups of children studied. The group means were significantly different ($p < 0.05$) by analysis of variance. Because of the skewed nature of the distribution of inducibility ratios in our study population (see Fig. 2), a log-transformation of the inducibility ratios was performed in an attempt to normalize the data. It was found that the log inducibility ratio can better discriminate the four groups than the nontransformed inducibility ratio (Table 3).

When pairwise comparisons of the mean inducibility ratios were made by Scheffé’s method: (1) there was no difference between the unaffected group and the group with nonleukemic malignancies; (2) there was no difference between children with acute lymphoblastic and acute myelogenous leukemia; (3) the unaffected group has a significantly higher mean inducibility ratio than the pooled acute leukemia group ($p < 0.05$).

Figure 2 shows a comparison of the distribution of inducibility ratios of unaffected children (top) and children with acute leukemia in remission (bottom). Using these distributions, AHH inducibility phenotypes were determined on a statistical basis by a computerized iterative maximum likelihood procedure. Individuals with an inducibility ratio $> 2.5$ are phenotypically inducible, while those with an inducibility ratio $\leq 2.5$ are phenotypically noninducible.

### Table 3. Comparative Lymphocyte AHH Inducibility in Pediatric Patients

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Inducibility Ratio (IR)</th>
<th>log IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unaffected</td>
<td>37</td>
<td>4.50 ± 0.82*</td>
<td>0.55 ± 0.05</td>
</tr>
<tr>
<td>Nonleukemic malignancy</td>
<td>22</td>
<td>4.39 ± 0.73</td>
<td>0.53 ± 0.08</td>
</tr>
<tr>
<td>Acute myelogenous leukemia</td>
<td>6</td>
<td>2.46 ± 0.97</td>
<td>0.21 ± 0.18</td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>48</td>
<td>2.78 ± 0.40</td>
<td>0.30 ± 0.05</td>
</tr>
<tr>
<td>$F^\dagger$</td>
<td></td>
<td>3.12 ($p &gt; 0.05$)</td>
<td>5.22 ($p &gt; 0.005$)</td>
</tr>
</tbody>
</table>

*Values represent the mean ± SE.

†$F$ value from analysis of variance.
dure.* A common cut-off value at the inducibility ratio of 2.5 was found. Thus, individuals with an average inducibility ratio >2.5 were considered aromatic hydrocarbon responsive (highly inducible phenotype), while those with an average inducibility ratio ≤2.5 were considered aromatic hydrocarbon nonresponsive (minimally inducible phenotype).

Table 4 summarizes the phenotypic expression of AHH inducibility in our study population. The proportion of children phenotypically highly inducible was significantly lower in the leukemic population than in either the unaffected group or the group with nonleukemic malignancies (p < 0.001). However, within a phenotypic class there were no significant differences in inducibility ratios. Thus, the observed difference in mean inducibility ratios among our patient groups (Table 3) are due strictly to differences in the proportion of each phenotype within each group and not to variations in enzyme activity within a phenotypic class. It is noteworthy that, despite some variation in the individual inducibility ratios with repeated sampling, there were only 12 instances of 287 samplings (4.2%) where there was discord with respect to AHH phenotype in individual determinations on the same patient. In all such cases, the final phenotype is based on the average inducibility ratio.

Prognostic Significance of AHH Phenotype

While children with acute myelogenous leukemia continue to have a rather bleak prognosis,28 certain factors of prognostic importance have been identified among those children with acute lymphoblastic leukemia.1 These include age, white blood cell count at the time of diagnosis, sex, and race. No significant correlation was found between AHH phenotype and age, sex, or white blood cell count at diagnosis. (By Mann-Whitney U test29). Since only three nonwhite children are included in the present study, no conclusions concerning race could be drawn.

Recently, the French-American-British (FAB) Cooperative Group published a system of classification for acute leukemias based on morphological appearances of the bone marrow at diagnosis.29 In lymphoblastic leukemia, these morphological classes have been ascribed some prognostic significance. For the present study, the diagnostic bone marrow slide was reviewed for each patient and classified as L1, L2, or L3 according to the FAB criteria. No association was found between AHH phenotype and FAB classification.

Children with acute lymphoblastic leukemia were also studied with respect to the incidence of relapse while on therapy. Of the total of 48 children with acute lymphoblastic leukemia, 10 were found to have relapsed while on chemotherapy. If relapse were unrelated to AHH phenotype, then the proportion of inducible and noninducible patients relapsing should be the same as the proportion of each of these phenotypes in the study population (Table 4). When the observed number of relapses in each of the two phenotypic groups was compared with the number expected, it was found that there were fewer inducible and more noninducible children relapsing than expected, however, the difference was not statistically significant.

Figure 3 depicts the relationship between the AHH inducibility ratio and the duration of first remission among the ten children with acute lymphoblastic leukemia relapsing while on chemotherapy. A statistically significant positive correlation was noted between the duration of first remission and the inducibility ratio (r = 0.73, p < 0.05). This correlation remains significant even if the one extreme (phenotypically highly inducible) case is removed (r = 0.63, p < 0.05).

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* A model of two overlapping Gaussian distributions was applied to the distribution of inducibility ratios. In this model, the overlapping Gaussian distributions may be expressed mathematically by the equation for the probability function, f(x) as:

\[
f(x) = \frac{\alpha}{\sqrt{2\pi\sigma_1^2}} \exp\left(-\frac{(x - \mu_1)^2}{2\sigma_1^2}\right) + \frac{1 - \alpha}{\sqrt{2\pi\sigma_2^2}} \exp\left(-\frac{(x - \mu_2)^2}{2\sigma_2^2}\right)
\]

Where \(\mu_1\) and \(\mu_2\) are the means of the two distributions, \(\sigma_1\) and \(\sigma_2\) are their respective standard deviations, and \(\alpha\) the proportion of individuals belonging to the first curve. These parameters were estimated by a computerized iterative maximum likelihood procedure. From the estimated parameters the cut-off value separating inducibility phenotypes was determined by minimizing the sum of the two probabilities of misclassification. In each case, at least one cut-off value at <2.5 was found.
DISCUSSION

The results of the present study have suggested that children who are phenotypically minimally aromatic hydrocarbon responsive are more susceptible to the development of acute leukemia. In the past, attempts have been made to relate the inducibility of AHH activity in mitogen-stimulated cultured human lymphocytes to the incidence of bronchogenic carcinoma. In 1973, Kellermann et al. studied AHH inducibility in cultured lymphocytes in a normal white population in Houston and in a group of 50 patients with bronchogenic carcinoma. It was concluded that susceptibility to bronchogenic carcinoma was associated with a highly inducible form of the enzyme. While this association has been questioned, more recent studies have provided support for the relationship between bronchogenic carcinoma and aromatic hydrocarbon responsiveness.

In general attempts to relate the expression of certain enzyme activities to a genetic predisposition to a certain disease are shrouded in uncertainty. Simultaneous characterization of enzymes in tumors and surrounding normal tissue has revealed marked differences presumably related to an effect of the disease state on the phenotypic expression of the enzymes' activity. However, several lines of evidence suggest that this is not the case for AHH and acute leukemia of childhood. First, it is evident from studies in animals that the AHH inducibility phenotype predisposes to certain forms of cancer. Consistent with this concept our recent studies have indicated that there is no change in AHH inducibility phenotype during the course of chemotherapy for acute leukemia. Thus, 10 children whose AHH inducibility phenotypes were determined on leukemic blasts at diagnosis all were found to have the same phenotype when their apparently normal lymphocytes were tested while in remission (Blumer et al., to be submitted). Moreover, in 15 children with acute leukemia, AHH phenotypes should be accurately predicted from studies of parents, grandparents, and siblings, assuming a mendelian mode of inheritance (Blumer et al., manuscript in preparation).

Aromatic hydrocarbon responsiveness in mice is defined with respect to the response of hepatic microsomal AHH to PAH-inducing agents. Recent studies have suggested a similar relationship between aromatic hydrocarbon nonresponsiveness and leukemia in mice. Duran-Reynals et al. showed that after painting with 3-methylcholanthrene, the incidence of skin tumors and leukemia were mutually exclusive events in certain inbred strains of mice. With one exception, aromatic hydrocarbon nonresponsive strains developed thymic leukemia.

Nebert and Jensen, studying the effects of ingesting small doses of 3,4-benzpyrene in C57BL/6N and DBA/2N mice, noted that the nonresponsive DBA/2N strain developed leukemia with greater frequency and after a shorter latency than the responsive C57BL/6N mice. They found that this response could be almost completely prevented by addition of α-naphthoflavone, an inhibitor of cytochrome P-450 (AHH) catalyzed carcinogen activation, to the carcinogen diet. These results suggested that metabolism of benzpyrene by an α-naphthoflavone-sensitive enzyme in bone marrow was responsible for leukemogenesis in these mice.

One explanation for the association of aromatic hydrocarbon nonresponsiveness with leukemia in mice has been advanced by Nebert. He suggests that with inducible mice, the rapid metabolism of carcinogens at their portals of entry (i.e., the skin, gastrointestinal tract, lung) and the further rapid elimination by the liver of that fraction of carcinogen absorbed precludes the arrival of sufficient potential carcinogen to the bone marrow to cause neoplastic transformation. This "first-pass elimination" concept has been recently supported by pharmacokinetic studies of labeled benzpyrene in mice. This has led Nebert and his associates to conclude that proximate tissues (i.e., those in contact with the administered drug) exhibit more toxicity/malignancy in responsive mice, whereas distal tissues play more toxicity/malignancy in nonresponsive mice.

While this "first-pass elimination" concept probably contributes to the observed difference in leukemic
susceptibility, it is clearly not the whole answer. Data on the profile of benzpyrene metabolites formed by the bone marrow and/or other lymphoreticular organs of aromatic hydrocarbon responsive animals are lacking. Further information derived from family studies (Blumer et al., manuscript in preparation) suggests that the expression of enzyme activities in addition to AHH may be important for the development of acute leukemia in childhood.

The apparent association between aromatic hydrocarbon responsiveness and acute leukemia in childhood suggests a role for environmental chemicals in leukemogenesis. Even though epidemiologic studies indicate that environmental chemicals may be responsible for a considerable portion of all human neoplasms,8 the role of chemical carcinogens in the pathogenesis of pediatric neoplasms has been largely overlooked. Nevertheless, it is clear that: (1) chemical carcinogens can cross the placenta and affect the fetus;43 (2) fetal and neonatal tissues have the capacity to metabolically activate chemical carcinogens;43,44 (3) fetal and neonatal animals appear to be more sensitive to the tumorigenic effects of some chemical carcinogens than adult animals;45,46 (4) AHH activity is transplacentally inducible by chemical carcinogens relatively early in gestation;47 and (5) the transplacental inducibility of AHH activity by PAHs is genetically determined.48 Thus, the potential interactions between the fetus and its chemical environment must not be overlooked as a determinant of malignant disease in childhood.

The relationship between AHH phenotype and the response to chemotherapy was unexpected. In cancer research the relationship between AHH activity and carcinogenesis is so often emphasized that it is easy to lose sight of the fact that the metabolism of many antitumor agents is catalyzed, at least in part, by cytochrome P-450 containing monoxygenases. In fact, just as many carcinogens require metabolic activation to their ultimate carcinogenic form, some chemotherapeutic agents must likewise be activated. The relationship described in Fig. 3 requires a great deal of further substantiation but may prove important in the evaluation of our present chemotherapeutic regimens and in the design of new approaches.

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