Cytogenetic Studies of Long-Term Survivors of Ch Lymphoblastic Leukemia

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ABSTRACT

Ten long-term survivors of childhood acute lymphoblastic leukemia were studied to determine if cytogenetic abnormalities were present in lymphocytes following discontinuation of therapy. The study included patients diagnosed between 1969 and 1974 who had received radiation therapy and a minimum of 3 years systemic chemotherapy. At study, the patients had been off all therapy from 1.3 to 6.5 years (median, 4 years). Peripheral blood lymphocytes were examined for spontaneous chromosome breakage and sister chromatid exchanges. In addition, G-banded metaphase and prometaphase chromosomes were analyzed. Chromosome breakage was found to be within normal limits for all patients. Likewise, there was no significant difference between patients and controls with respect to sister chromatid exchange frequency. However, seven of the ten patients were found to have one or more cells with nonclonal karyotypic abnormalities. Our results indicate that, although long-term survivors of childhood acute lymphoblastic leukemia treated with intensive radiation and combination chemotherapy do not demonstrate chromosome instability or DNA damage as measured by breakage and sister chromatid exchange, a majority of these patients have a subpopulation of lymphocytes with nonclonal chromosome abnormalities years after stopping therapy.

INTRODUCTION

The survival of children with ALL has increased markedly during the last 2 decades (23). Presently, more than 50% of children diagnosed with ALL are surviving free of disease 8 years from diagnosis following treatment for 3 to 5 years (17). There are data to suggest that effective treatment of childhood ALL can be associated with impaired growth, neuropsychological dysfunction, and endocrine dysfunction (5).

Cytotoxic chemotherapeutic agents, commonly used in combination with radiation, are standard therapy for ALL. Both of these treatment modalities are known to be mutagenic (3, 4, 16) and carcinogenic (1, 8). In an effort to determine whether or not sustained DNA damage occurs in these patients, we have examined peripheral blood lymphocytes using 3 cytogenetic methods from 10 patients who have been successfully treated for ALL and have discontinued antileukemia therapy.

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2 To whom requests for reprints should be addressed, at Box 484, Mayo Memorial Building, Department of Pediatrics, University of Minnesota, Minneapolis, Minn. 55455.
3 The abbreviations used are: ALL, acute lymphoblastic leukemia; SCE, sister chromatid exchange.

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PATIENTS AND METHODS

Patients

All patients were diagnosed with ALL and treated at the University of Minnesota Hospitals. Our objective was to study a relatively homogeneous group of patients who had received chemotheraphy and radiation therapy for ALL. Patients selected were free of disease, had been treated with standard induction and maintenance regimens, had by present norms received extensive radiation, had received at least 3 years of maintenance chemotherapy, and had been off all chemotherapy for at least 1 year. Ten consecutive patients seen in Oncology Clinic for long-term follow up of ALL who met these 5 criteria were included.

The 4 males and 6 females were from 2 to 15 years (median, 4 years) of age at the time of diagnosis of their ALL and ranged from 9 to 23 years (median, 12 years) at the time of this study. Nine of the patients received induction therapy consisting of vincristine, prednisone, and L-asparaginase. The remaining patient (Patient 7) was treated with an induction regimen of vincristine, prednisone, methotrexate, and 6-mercaptopurine. Central nervous system prophylaxis consisted of 2400 rads (Patients 1 to 5 and 8 to 10) or 1200 rads (Patients 6 and 7) craniospinal radiation. Prophylactic cranial radiation consisting of 1200 rads to the abdomen including the kidneys, spleen, liver, and gonads was also received by 7 of the patients (Patients 2 to 4, 6, and 8 to 10). Remissions were maintained using 6-mercaptopurine, methotrexate, vincristine, and prednisone in all but one of the patients. Patient 7 received 6-mercaptopurine, methotrexate, vincristine, cyclophosphamide, 1-β-d-arabinofuranosylcytosine and nitrogen mustard. Patient 4 had a persistent relapse 5 years from diagnosis and subsequently was treated with an additional 2400 rads to the testes and 1200 rads cranial radiation. The median duration of therapy was 3.4 years (range, 3.2 to 6.3 years), and the median time from discontinuation of chemotherapy was approximately 4 years (range, 1.3 to 6.5 years).

Controls

In all cases, a known normal healthy control was studied concurrently for spontaneous chromosomal breakage and SCE. In 6 of 10 cases, the patient and control were sex matched. In general, the controls were older, ranging in age from 3 to 35 years (median, 27 years). The control for Patients 2 and 3 was the normal son of a Robertsonian translocation carrier t(13q14q), and Control 6 was a normal donor for bone marrow transplantation to his brother who had idiopathic aplastic anemia. The remaining 7 controls were technologists in the Cytogenetics Laboratory; 2 of them smoked one pack of cigarettes per day (Controls 4 and 5), and one had been taking Aldomet for hypertension for 9 years (Control 8). Previous studies have shown that age and sex do not appear to affect baseline SCE levels but that cigarette smoking may increase the frequency of SCE (6, 10, 14).

G-banded karyotype analysis was done on the concurrent controls for Patients 2, 3, and 6 but was not done on the 7 technologists. To assess the incidence of abnormalities induced in vitro by methotrexate synchronization, an additional 16 subjects were used as controls. These 16 subjects included one female and 4 male children and 5 male and 6 female adults consecutively studied with methotrexate synchron-
Cytogenetic Studies

The cytogenetic studies were done using 10 ml heparinized peripheral blood from each patient and control.

Spontaneous Chromosomal Breakage. Phytohemagglutinin-stimulated lymphocytes were cultured for 72 hr at 37°C in Roswell Park Memorial Institute Medium 1603 supplemented with 10% fetal calf serum. Cells were harvested 30 min after Colcemid was added using routine methods. Slides were stained with Wright’s stain, and 50 cells were scored for chromatid and chromosome breaks. Major chromosome abnormalities were also noted; however, less obvious band abnormalities could have been easily missed.

SCE. The method used for SCE was similar to that described by Perry and Wolff (20). Phytohemagglutinin-stimulated lymphocytes were cultured in Dulbecco’s minimal essential medium for 24 hr before adding 10⁻⁴ M bromodeoxyuridine. Cultures were harvested at 69 and 72 hr after 1 hr exposure to Colcemid. The slides were allowed to age at least 4 days before staining with Hoechst 33258, exposure to UV, and counterstaining with Giemsa. The number of SCE was recorded in 20 cells for each patient and control at both harvest times. Individual patients were compared to their controls, and group data were analyzed using Student’s t test.

G-banded Chromosome Analysis. To obtain a variety of chromosome lengths for analysis (prometaphase → midmetaphase), a modification of the methotrexate synchronization technique of Yunis et al. (25) was used. Approximately 30 G-banded cells were analyzed in each case, and multiple photokaryotypes of normal and abnormal metaphase aneuploidy were constructed. Due to technical difficulties, banded studies were done on nonsynchronized cultures for patients 2 and 3. Grossly abnormal cells ascertained during the breakage studies were also photographed and analyzed after destaining and restaining for G-bands. The latter are not included in the calculation of the frequency of karyotypically abnormal cells since minor abnormalities could have been missed in the remaining nonbanded cells.

RESULTS

Spontaneous Chromosomal Breakage. None of the 10 patients demonstrated an increase in spontaneous chromosomal breakage (Table 1). Chromatid breaks were found in 3 patients (Patients 2, 3, and 8) who had 1, 2, and 1 chromatid breaks per 50 cells counted, respectively. Controls 4 and 6 had 4 and 1 chromatid breaks, respectively. Patients 1, 6, and 9 each had one chromosome break per 50 cells counted. None of the controls had chromosome breaks detected. Control 10 had too few mitoses to do a complete analysis.

SCE. At 69 hr, 3 of the patients (Patients 2, 3, and 9) had a significantly higher frequency of SCEs than the controls, 2 patients (Patients 6 and 7) demonstrated a significantly lower number, and the remaining 5 patients showed no significant difference. Only one significant result was obtained at 72 hr; Patient 4 was found to have a higher number of SCEs. In no case was there a significant difference between patient and control at both 69 and 72 hr (Table 1).

Overall, the distribution of SCE between patients and controls was not significantly different (p > 0.80). The average

Table 1

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<th>Experimental pair</th>
<th>Age at study (yr)</th>
<th>Sex</th>
<th>Yr on therapy</th>
<th>Yr off therapy</th>
<th>Chromatid (no. of breaks/50 cells)</th>
<th>Chromosome</th>
<th>Spontaneous breakage (no. of breaks/50 cells)</th>
<th>SCE Mean ± S.D.</th>
<th>Range</th>
<th>SCE Mean ± S.D.</th>
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<td>4-14</td>
<td>7.15 ± 2.64</td>
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a p < 0.01.

b p < 0.05.
c Cigarette smokers (1 pack/day).
d Medication (Aldomet).

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controls. The median number of SCE per cell was 7 for both patients and controls. The frequency of SCE per cell ranged from 1 to 30 for the patients and from 1 to 26 for the controls (Chart 1).

**Karyotypes.** Results of the G-banded karyotype analysis are given in Table 2. Seven of the 10 patients were found to have one or more karyotypically abnormal cells. Eleven of the abnormal cells were pseudodiploid, i.e., diploid with structurally abnormal chromosomes. The other 3 cells were hyperdiploid with multiple structural abnormalities present. None of the abnormalities were clonal. Examples of the chromosome abnormalities are shown in Figs. 1 and 2.

The frequency of abnormal cells in methotrexate-synchronized cultures was 3.85% (9 of 234 cells studied), and in nonsynchronized cultures was 1.9% (1 to 52 cells) giving an overall frequency of 3.5% (10 of 286) abnormal cells. In addition, abnormal cells were observed in nonsynchronized cultures used for breakage studies in Patients 1, 3, and 6. The number of abnormal cells seen in the patients was markedly increased as compared to the 18 controls, all of whom were studied using methotrexate synchronization. A total of 388 G-banded cells were studied from the controls, and only 2 abnormal cells were found (0.5%). The abnormalities in the 2 control cells were numerical rather than structural. The karyotypes of the abnormal cells (one from each of 2 controls) were 47,XY,+21 and 45,XY,+2,+4,+13, respectively.

Comparing the results shown in Tables 1 and 2, there was no correlation between the amount of therapy received or time off therapy and cytogenetic abnormalities, nor was there a correlation among the results found on the breakage, SCE, and karyotype studies.

**DISCUSSION**

Based on the annual incidence rate for ALL in the United States and the current long-term survival rate, it can be estimated that by the year 2000 approximately one of every 7500 persons in the United States under 35 years of age will have been diagnosed with ALL. As persons diagnosed and treated for ALL become more prevalent in the population, it will be important to determine the potential long-term iatrogenic effects of their treatment. Clearly, the benefits associated with chemotherapy and radiation for the treatment of ALL far outweigh the known risks.

Although the exact nature of the DNA lesion(s) that lead to the formation of SCE is currently unknown, SCE are believed to be a sensitive indicator of mutagenesis (12, 19, 24). The number of SCE is increased at diagnosis of ALL and during treatment with chemotherapeutic agents and radiation but returns to normal shortly after discontinuing treatment (11, 18, 21). In our study, no increase in SCE was observed in patients off therapy from 1 to 6 years. Our data confirm those of others who showed no increase in the frequency of SCE following treatment for lymphoma or leukemia in children (2, 7, 9, 22). Our results also showed no increase in chromosome fragility as measured by spontaneous chromosomal breakage.

In contrast, 7 of the 10 patients in our series (70%) had circulating lymphocytes with striking cytogenetic abnormalities. Schuler et al. (22) reported only 33% (4 of 12) of their patients had abnormal karyotypes following therapy for ALL consisting of vincristine, methotrexate, cyclophosphamide, and daunomycin plus cranial radiation of 2400 rads. Miller et al. (13) analyzed banded karyotypes from 7 patients diagnosed with ALL who had been off chemotherapy from 2 to 11 years. They found that 57% of patients (4 of 7) demonstrated the presence of abnormal cells. The therapy that their patients received varied widely, ranging from single-agent chemotherapy to multiagent regimens. Moreover, only one of the 7 patients had received radiation therapy at a dose of 400 rads. The overall frequency of abnormal cells in our series was low (3.5%). The karyotypic abnormalities were different in each cell and thus were nonclonal. An abnormal clone is defined here as 2 cells with an identical structural anomaly, 2 cells with identical extra chromosomes, or 3 cells with identical missing...
chromosomes. It is important to note that abnormal cells were seen in both synchronized and nonsynchronized cultures. In addition, karyotypic abnormalities were seen in only 0.5% of cells from 18 controls. These findings rule out the possibility that these abnormalities were cultural artifacts resulting from in vitro methotrexate treatment.

We were unable to show a correlation between type or duration of therapy and the presence of karyotypically abnormal cells. The addition of 1200 rads abdominal radiation did not appear to result in an increase in the frequency of chromosomal abnormalities as compared to patients who did not receive the additional radiation.

The significance of this small population of abnormal lymphocytes in survivors of ALL is currently unclear. It is possible but unlikely that these abnormalities were present at the time of diagnosis, perhaps being associated with the etiological leukemic event. More likely, the finding of these abnormalities suggests that a subpopulation of progenitor cells sustained genetic damage as a result of the multiagent effect of treatment. Whether or not these abnormal cells could become clonal at some time in the future is not known; however, data currently available on patients with second malignant neoplasms would suggest that cases of hematological cancers in survivors of ALL are rare (15).

Based on this pilot study of 10 long-term survivors of childhood ALL, it appears that future cytogenetic analyses of these and other similar patients are indicated to confirm and further clarify the significance of our findings.

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REFERENCES

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