Induction of Chromosome Breaks and Sister Chromatid Exchanges in Patients with Hodgkin's Disease by Two Combination Chemotherapy Regimens of Different Leukemogenic Potential

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ABSTRACT

The success of various combination chemotherapies in the treatment of cancer is compromised by their potential to cause secondary leukemia. Previous studies have suggested that the alkylating agents used in some regimens are the major etiological factor in these leukemias. In this study, we compared the abilities of two standard regimens used in the treatment of Hodgkin's disease to cause chromosome breaks and sister chromatid exchanges; the two most common types of chromosomal damage induced by alkylating agents. These regimens are MOPP [mechlorethamine-vincristine (Oncovin)-procarbazine-prednisone] and CVPP-ABDIC [cyclophosphamide-vinblastine-procarbazine-prednisone-doxorubicin (Adriamycin)-bleomycin-dacarbazine-l-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea]. Our study demonstrated that (a) levels of spontaneous chromosome breaks and sister chromatid exchanges were low in untreated Hodgkin's disease patients; (b) significantly higher levels of these damages were induced in patients receiving eight cycles of CVPP-ABDIC, as compared with their pretreatment levels; (c) significantly elevated levels of sister chromatid exchanges, but not chromosome breaks, were induced in patients receiving two cycles of MOPP; and (d) no differences in the effect of these two regimens on cell cycle kinetics were observed. Although MOPP therapy has been reported to have higher rates of secondary leukemia than CVPP-ABDIC, our studies show that eight cycles of CVPP-ABDIC are more potent than two cycles of MOPP in inducing chromosome damage in patients during treatments.

INTRODUCTION

Since the advent of combination chemotherapy, an increasing number of patients are being cured of cancer. However, follow-up studies of cancer survivors have shown that such treatment is not without long-term hazards, one of the most significant of which is the development of secondary cancers. The classic example of a successful therapy that causes secondary cancers is MOPP, used in the treatment of Hodgkin's disease (1-4). More recently, increased incidence of therapy-related leukemia has also been reported in patients following chemotherapeutic treatments for other cancers (5-7). Current evidence supports the view that alkylating agents used in various regimens are responsible for causing this leukemia (3, 4, 8). However, the specific types of DNA damage induced by these agents that lead to carcinogenesis are unknown.

In this study, we examined rates of chromosome breakage and SCE in peripheral blood lymphocytes of adult Hodgkin's disease patients receiving either MOPP or CVPP-ABDIC chemotherapy. Chromosome breakage and SCE are the most common types of chromosomal damage induced by carcinogenic agents (9). MOPP and CVPP-ABDIC are both among the most effective therapies in Hodgkin's disease yet differ in their capacity to cause secondary leukemia, which by 7 years posttherapy is seen in approximately 2% of patients who received only two cycles of MOPP, compared with 0.5% in patients who received eight cycles of CVPP-ABDIC (10). Comparison of the types of chromosome damage induced by these regimens may clarify which is (or are) crucial to the development of secondary leukemia. Because most of the drugs in the above two combinations are widely used to treat patients with other forms of cancer, information gained from studies of these two treatments may elucidate the actions of other chemotherapeutic regimens as well.

MATERIALS AND METHODS

Patients. Sixteen patients, ranging in age from 19 to 45 years, were studied. All had newly diagnosed Hodgkin's disease, and none had received any prior therapy. Eight were treated by the MOPP protocol and eight by CVPP-ABDIC.

Chemotherapy. Patients with stages I or II Hodgkin's disease who had minimal mediastinal or abdominal disease and those in stage III without mediastinal involvement were given two cycles of MOPP, each lasting 28 days (11, 12). In each cycle, mechlorethamine at 6 mg/m² and vincristine at 1.4 mg/m² were given i.v. on days 1 and 8, and procarbazine at 100 mg/m² and prednisone at 40 mg/m² were given p.o. on days 1 to 10. Patients with stage III disease with mediastinal involvement and those with stage IV disease were given 8 cycles of CVPP-ABDIC, with CVPP and ABDIC given in alternate cycles, each lasting 28 days (10). In each CVPP cycle, cyclophosphamide at 300 mg/m² and vinblastine at 6 mg/m² were given i.v. on days 1, 8, and 15; procarbazine at 100 mg/m² and prednisone at 40 mg/m² were given p.o. on days 1–10. In each ABDIC cycle, doxorubicin at 50 mg/m² (i.v.) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea at 40 mg/m² (p.o.) were given on day 1; bleomycin at 5 mg/m² was given i.v. on days 1 and 5; dacarbazine at 200 mg/m² (i.v.) and prednisone at 100 mg/m² (p.o.) were given on days 1–10.

Methods. Samples of peripheral blood were obtained from each patient before and at various time periods during chemotherapy, depending on availability (see Figs. 1 and 2 for exact time points). Lymphocytes from each sample were cultured under two different conditions. In one culture, lymphocytes were incubated in RPMI 1640 medium (Gibco) supplemented with 20% fetal bovine serum (Gibco) and 0.18 mg/ml phytohemagglutinin (Burroughs Wellcome) for 72 h at 37°C. In another culture, cells were incubated as above except that 15 μg/ml BrdUrd (Sigma) was added to the cultures for the last 40 h of incubation. BrdUrd-containing cultures were completely protected from light to minimize the photolysis of BrdUrd-containing DNA. Colcemid at a concentration of at 0.4 μg/ml (Gibco) was added to all the cultures for the last 1 h of incubation, and air-dried slides were made by standard procedures previously described (13). Briefly, cells were treated with a hypotonic solution (0.075 M KCl) for 15 min at room temperature, fixed in methanol-acetic acid (3:1) for 30 min with two or three changes, and dropped on wet slides.
For analyses of chromosome breakage, slides were stained with Giemsa, and at least 50 metaphases were analyzed from each culture to quantitate the frequencies of various types of chromosomal aberrations, i.e., chromatid-type breaks and gaps, chromosome-type breaks and gaps, fragments, dicentrics, and rings. For enumeration of the total number of chromosome breaks, each dicentric and ring is counted as two breaks, and each break, gap, and fragment as one break. The frequency of chromosome breakage was determined by dividing the total number of breaks observed by the total number of metaphases examined and expressed as breaks per cell. Because large numbers of aberrations in a cell could not always be accurately enumerated, any cell with 20 or more aberrations was assigned a value of 20 breaks.

For analyses of SCE, slides were stained with Hoescht 33258 (50 μg/ml) for 15 min and exposed to UV radiation (peaked at 254 nm) for 2 h from a distance of 5–7 cm. Slides were subsequently incubated in 2x 0.15 M NaCl and 0.015 M trisodium citrate before staining with Giemsa for 5 min. Whenever possible, 50 metaphases were evaluated in each sample to determine the total number of SCEs. The frequency of SCE was expressed as SCE/cell. To determine if there is any difference in the cell division rate between samples collected from patients treated by MOPP and CVPP-ABDIC, the percentages of cells that had completed the first, second, and third division during the 40-h BrdUrd-labeling period were determined in each culture by examination of 100 metaphases. These percentages were then compared among samples collected from the above two groups of patients.

Statistical Analyses. The nonparametric Mann-Whitney test was used to examine the differences in the frequencies of chromosome breaks and SCEs between pretreatment and during-treatment samples in each chemotherapy group of patients, as well as to examine the differences in the frequencies of breaks and SCEs induced by MOPP versus CVPP-ABDIC therapy. Furthermore, this test is used to compare the percentages of cells in the first, second, and third divisions among samples collected from the above two groups of patients.

RESULTS

Frequencies of Chromosome Breaks in HD Patients before Treatment. The frequencies of chromosome breaks in individual patients prior to therapy are shown for the MOPP-treated group in Fig. 1A and for the CVPP-ABDIC group in Fig. 1B. Detailed summaries of the specific types of chromosome aberrations observed in preparations from each patient are available from the authors upon request. The average number of breaks/cell in the MOPP group is 0.05 and that in the CVPP-ABDIC group is 0.06. No significant difference was seen between these two groups of patients before treatment. The spontaneous frequencies of chromosome breaks observed in HD patients are similar to those reported in healthy individuals (14, 15).

Frequencies of Chromosome Breaks in HD Patients during Treatment. The frequencies of various types of chromosome aberrations in individual patients by chemotherapy group at multiple time periods during treatment are graphically displayed in Fig. 1. The difference between the frequencies seen in the pretreatment and during-treatment samples was significant only in the CVPP-ABDIC group (P < 0.01 versus P < 0.5 in the MOPP group). Furthermore, CVPP-ABDIC was found to induce a significantly higher level of chromosomal breakage than MOPP (P < 0.01).

Interindividual variations in break frequency during treatment were observed in both groups of patients, but were more obvious in the CVPP-ABDIC group (Fig. 1B). An example of this variation was seen by comparing the break frequencies in patients 9, 11, 12, 13, and 16 at the end of the fourth cycle (4c) of therapy. As shown in Fig. 1B, these five patients exhibited different frequencies of breaks/cell ranging from 0.16 to 0.7 after identical doses and regimen of CVPP-ABDIC therapy.

The frequencies of chromosome breaks observed did not correlate with the number of cycles of chemotherapy received by the patients. For example, patient 9, who received CVPP-ABDIC therapy, had highly elevated levels of chromosome breaks at the end of cycles 1 and 5 (1c and 5c), representing 6- to 12-fold increases over the pretreatment level. However, the break frequency in the sample taken at day 15 of the third cycle (3d15) was not elevated. Such wide fluctuations in the break frequency during CVPP-ABDIC treatment were also seen in patients 10, 11, and 12 but not in patients 14, 15, and 16. In the MOPP-treated group, only one of eight patients showed significant variations in break frequency during therapy.

It is noteworthy that while cells with multiple aberrations were not observed in patients prior to treatment and in those treated with MOPP therapy, such cells were occasionally seen in samples taken during CVPP-ABDIC therapy. For example, in patient 9, 6% of the cells in the sample collected at the end of cycle 1 had 20 or more breaks, and 4–6% of the cells collected during cycles 2 and 5 had multiple aberrations such as dicen-
trics, rings, and chromosomal fragments. Similarly, in patient 10, the elevation at the end of cycle 3 was largely due to the presence of 16% of cells with more than 20 breaks/cell. Such findings suggest that a small percentage of T-lymphocytes in these patients are hypersensitive to the CVPP-ABDIC chemotherapy.

Frequencies of SCEs in Patients Prior to Chemotherapy. Fig. 2 shows the pretreatment SCE frequencies for individual patients by treatment group. The average frequency of SCE is 5.3 in both the MOPP and CVPP-ABDIC groups. These frequencies are similar to those reported for healthy individuals (16).

Frequencies of SCEs in Patients during Chemotherapy. Fig. 2 also shows SCE frequencies during CVPP-ABDIC and MOPP therapy. Both groups of patients had elevated levels of SCEs as compared with their pretreatment levels, at significance levels of \( P < 0.001 \) in the CVPP-ABDIC group and \( P < 0.01 \) in the MOPP group. However, not all patients showed an elevation. When the mean levels of SCEs induced by these two regimens were compared, CVPP-ABDIC was found to yield a significantly higher frequency than MOPP (\( P < 0.05 \)).

In most patients who showed elevated levels of SCEs during CVPP-ABDIC therapy, the SCE frequency generally peaked during the middle cycles of treatment, followed by a decline during the later cycles. This regimen was found to cause not only an overall increase in the number of SCEs in all cells examined, but also extraordinarily high numbers of SCEs (e.g., 50–70 SCEs/cell) in a small percentage of the cells. These results parallel those for chromosome breakage and suggest that there may be a subpopulation of T-lymphocytes that are hypersensitive to the therapy.

Effects of MOPP and CVPP-ABDIC Therapies on Cell Cycle Kinetics. The averaged percentages of first-, second-, and third-division metaphases in samples taken during MOPP therapy were 42, 38, and 20%, respectively. Those in patients treated with CVPP-ABDIC were 42, 34, and 24%, respectively. By the Mann-Whitney test, no significant difference was observed in the division rate of cultured cells from these two groups of patients.

**DISCUSSION**

Recent studies suggest a causal relationship between the use of alkylating agents in combination chemotherapy and increased risk of secondary leukemia in treated patients (4). Although alkylating agents are known to cause a variety of types of DNA damage, e.g., methyl- and alkyl-DNA adducts, single- and double-stranded DNA breaks, DNA-protein cross-links, gene mutations, chromosome breaks, and sister chromatid exchanges, the exact types of damage that lead to leukemia are unknown. Therefore, it is difficult to predict the leukemogenicity of various chemotherapeutic regimens and to identify individual patients who may be at risk of developing this complication.

Among the various types of DNA damage induced by alkylating agents, chromosome breaks and SCEs have been most frequently studied in patients receiving chemotherapy. Numerous studies have demonstrated elevated levels of these damages in patients receiving a variety of chemotherapeutic regimens containing alkylating agents (17–22). However, most previous studies were performed on patients who had a variety of cancers and who received different regimens of chemotherapy (23–26). Therefore, the increased levels of chromosome damages observed could not be attributed to any particular regimen. Furthermore, many of the patients studied had previously received radiotherapy or chemotherapy (24, 25, 27). Thus, the observed elevations in the levels of these damages are unlikely to reflect solely the effects of the chemotherapeutic drugs given. Despite much speculation that chromosome breaks and SCEs are important lesions responsible for the development of secondary leukemia (28), no previous study has been undertaken to determine the extent and duration of such damage in treated patients, and to determine whether the capacity of chemotherapeutic regimens to cause chromosomal damages correlates with their ability to cause secondary leukemia. Such studies require systematic evaluations of the frequencies of these damages in individual patients prior to any treatment and at multiple time periods during and following treatment with a particular regimen. Correlation can then be established if regimens that cause a higher frequency of chromosome breakage and SCE are more leukemogenic, or if individuals with high frequencies of these aberrations are associated with a higher risk of developing secondary leukemia.

In this study, we systematically evaluate the frequencies of...
chromosome breaks and SCEs in HD patients before and at multiple times during their treatment with MOPP or CVPP-ABDIC chemotherapy. Because patients under study receive no prior therapy and those in each treatment group are given identical doses and cycles of chemotherapy, we are able to compare the extent of chromosome damage induced by these two regimens during therapy and to determine individual variations in sensitivity. Because patients treated with MOPP have a much higher risk of developing secondary leukemia than those treated with CVPP-ABDIC, comparison of the frequencies of chromosome damages in these two groups of patients should be useful in determining if either of these two types of damage are indicative of the potential for the development of secondary leukemia. Our results show that patients receiving eight cycles of CVPP-ABDIC have significantly higher levels of chromosome breaks and SCEs as compared with their pretreatment levels. In contrast, patients receiving two cycles of MOPP generally do not have significantly elevated levels of chromosome breaks and only a few of them show elevated SCE frequencies. These data thus indicate that the CVPP-ABDIC regimen is more potent than the MOPP in causing chromosome damage in treated patients. We have shown further that the differential effects of these two regimens cannot be attributed to a difference of effect on cell cycle kinetics. For example, we showed that similar percentages of first-, second-, and third-division metaphases were analyzed for chromosome breaks in samples collected from the above two groups of patients. Thus, our comparison of the break frequencies in these two groups of patients is valid, despite the fact that the frequencies observed may be an underestimation of the damage present in cells in vivo.

Our data clearly show that there are interindividual differences in the sensitivities to drug-induced chromosome breaks and SCEs. These differences may be attributed to several factors, including drug absorption, metabolism, and efficiencies in the induction and repair of the above chromosome damages. Because all of the above factors influence the frequencies of these damages at any given time during chemotherapy, it is not surprising that wide fluctuations in the frequencies of these damages were observed in individual patients at different time periods of therapy. Although patients received increasing doses of chemotherapeutic drugs with increasing number of cycles of treatment, our data show that the highest frequencies of chromosome breaks and SCEs usually occur in the middle cycles of treatment. The reason for this observation is unknown.

There are several possible explanations for why an apparently less leukemogenic regimen causes more chromosome damage in treated patients. One possibility is that the leukemogenic potentials of these two regimens may not have been fairly compared. Whereas MOPP-treated patients have been followed for more than 20 years, CVPP-ABDIC is a more recently developed regimen and treated patients have been followed for only 7 years. It is possible that patients treated with CVPP-ABDIC may develop secondary leukemia more than 7 years after treatment. Although most secondary leukemias develop within 9 years after MOPP treatment (2), longer follow-up studies in patients treated with CVPP-ABDIC are needed to examine this possibility.

A second possible explanation of the findings that CVPP-ABDIC is more clastogenic but less leukemogenic than MOPP is that the induction of chromosome breaks and SCEs is less important than the persistence of such damage for initiation of carcinogenesis. The aberrations induced by MOPP might not be repaired as efficiently as those induced by CVPP-ABDIC, so that the cells with such damage could persist years after treatment and eventually progress to leukemic cells. Consistent with this hypothesis is the finding that chromosome breaks and SCEs induced by some antineoplastic agents are short lived, whereas these changes induced by other agents are persistent for months or years (17, 21, 22, 28–32). Of particular interest are the findings of Brown et al. (32) that mechlorethamine-vinblastine-procarbazine-prednisone induces high frequencies of SCE that persist for years. In contrast, Kadam et al. (33) reported that patients treated with cyclophosphamide-vincristine-procarbazine-prednisone show an elevated SCE frequency during treatment but not 3–4 months after treatment. Therefore, mechlorethamine appears to be more potent than cyclophosphamide in causing persistent SCEs. By long-term monitoring of the levels of chromosome breaks and SCEs in both of our groups of patients, we hope to determine if the persistence of cells with damage in some patients correlates with leukemia. Such monitoring may be especially important in view of the finding by Raposa and Varkonyi (28) that over 80% of all secondary leukemias are preceded by the administration of cytotoxic compounds inducing long-lasting SCE elevation.

A third hypothesis regarding our results is that chromosome breaks and SCEs are not the most crucial types of damage in the development of secondary leukemia. This possibility is supported by recent results of Schwartz et al. (34) that patients with cyclophosphamide vincristine-procarbazine-prednisone are not sensitive to the SCE induction by two alkylating agents. However, if the inclusion of alkylating agents in combination chemotherapy is indeed responsible for the appearance of secondary leukemia, other genetic alterations induced by these agents may be important in this process. Examples of such lesions are methyl- and alkyl-DNA adducts. For example, recently, Sager et al. (35) showed that HD patients have significantly lower levels of O6-methyltransferase during therapy, as compared with their pretreatment levels, suggesting that methylated DNA adducts that are induced by regimens containing alkylating agents may play a role in the etiology of secondary malignancies. However, their study also demonstrated significantly lower levels of O6-methyltransferase in patients treated with radiotherapy alone who do not have increased risks of developing secondary leukemia. Therefore, further studies are needed to elucidate the role of DNA adducts in leukemogenesis.

Since our ongoing studies are directed toward understanding the long-term effects of various chemotherapeutic regimens in HD patients, we hope to distinguish among the three possibilities described above by continual evaluation of chromosome aberrations and other genetic damage in these patients, and to correlate such damage with the occurrence of secondary cancers in individual patients.

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