Chromosome Damage and Polyploidization Induced in Human Peripheral Leukocytes in Vivo and in Vitro with Nitrogen Mustard, 6-Mercaptopurine, and A-649

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Summary

Chromosomal analysis of peripheral leukocytes in patients being treated with nitrogen mustard, 6-mercaptopurine, and A-649 demonstrated an increase in polyploidy, including cells showing endoreduplication. With nitrogen mustard, up to 15% of cells cultured were polyploids; with 6-mercaptopurine, 9% polyploidy was evident; and when A-649 was used, up to 14% polyploidy was seen. Chromosomal damage was evident with an increase in chromatid breaks and fragmentation. Dicentric chromosomes were the most common rearrangement found. These findings were not present in pretreatment cultures. When leukocytes from normal donors were incubated in vitro with these 3 agents, similar changes of polyploidization and structural abnormalities were produced. Care must be taken in the evaluation of chromosomal changes in patients undergoing active treatment.

Introduction

In a recent presentation from this laboratory it was demonstrated that radiation from radioactive 131I, 60Co, and X-rays has similar qualitative effects on human chromosomes in vivo and in vitro as measured in short-term leukocyte cultures (17). Chromosomal damage which characterized these effects was of 2 types. One type of damage was apparent in the genetic material, consisting of chromosomal breakage, inversions, deletions, dicentric forms, ring chromosomes, and other chromosomal rearrangements. The 2nd type showed damage in the mitotic apparatus of the cells, as evidenced by the induction of polyploidy and endoreduplication (P & E)1 of chromosomes. Previously, we described these “radiomimetic” effects in human leukocytes, both in vivo and in vitro, induced with N,N'-bis-(3-bromopropionyl) piperazine, an antitumor agent known as A-8103 (16). In leukocyte cultures, mitotic inhibition and chromosomal damage have been induced with mitomycin (18); and chromosome breakage and polyploidization have been demonstrated, utilizing streptonigrin or cyclophosphamide (15). Similar chromosomal changes have been induced by chloramphenicol in WBC cultures (C. E. Nasjleti and H. H. Spencer, unpublished data).

Although it is now apparent that certain chemotherapeutic agents induce cytogenetic changes in human leukocytes, studies showing these chromosome changes are not numerous. Therefore, probes in this direction may help to reveal the biologic effects of chemical agents upon human cells and, in particular, may show the frequency in which morphologic changes and chromosome polyploidization occur. This paper describes the structural chromosome aberrations and polyploidization that were induced by HN2, 6-MP, and A-649 on human WBC in vivo and in vitro.

Materials and Methods

Cytogenetic Studies In Vivo

Studies were obtained on 5 patients with metastatic carcinoma. Cases No. 1 and No. 2 had bronchogenic carcinoma and were treated with HN2. Case No. 3 had metastatic hypernephroma and was treated with 6-MP. Case No. 4 had neurofibroma-sarcoma and Case No. 5 had metastatic adenocarcinoma of the prostate gland; both of these were treated with A-649. For these studies, leukocytes from the peripheral blood were cultured by a modification of the method of Moorhead et al. (14). Samples of venous blood were obtained before, during, and after chemotherapy in these patients. As controls, leukocyte cultures from 10 normal men were also examined for chromosome damage and polyploidization.

Cytogenetic Studies In Vitro

Leukocytes from the peripheral blood of 3 healthy human donors were cultured for 100 hr instead of the 72 hr used in the in vivo work. Samples of 12-15 ml of venous blood were obtained from each donor and transferred to sterile vacuum tubes containing 0.2 ml of heparin solution (1000 units/ml). The vacuum tubes were placed at an angle of 60° and allowed to stand at room temperature (approximately 27°C) for 1 hr. From each tube duplicate aliquots of 2 ml of supernatant plasma containing viable leukocytes were placed in culture bottles with 8 ml of culture medium 199 (Difco), 700 units of penicillin G, and 0.7 mg of streptomycin.
of streptomycin. To each aliquot 0.5 ml of phytohemagglutinin M (Difco) was added. All aliquots were then incubated at 37°C in an atmosphere of 5% carbon dioxide and 95% air. After 48 hr of incubation the 6 aliquots were taken from the incubator. To each of 3 aliquots, 1 of the 3 chemotherapeutic agents was added in concentrations previously tested to induce maximal chromosome damage without being lethal to the cells. The concentrations used are shown in Tables 2 and 3. The remaining 3 aliquots were handled exactly like the treated cultures, but did not receive the chemical agents. Following both the actual and the sham chemical treatment, the 6 aliquots were reincubated for an additional 48 hr. Four hr before completion, 0.1 ml of Colecid (Ciba) in a concentration of 0.01 mg/ml was added to each culture. At the end of 100 hr, these cultures were ready for harvesting, fixation, and chromosome spreading. For this, cells were washed twice with Hank's solution, hypotonically treated with distilled water, then fixed in a mixture of methyl alcohol and glacial acetic acid (3:1). Drops of this hazy suspension of fixed cells were placed on tilted microscope slides and allowed to spread. Under these conditions the cells rupture, allowing the chromosomes to disperse so that they are individually recognizable. Slides were prepared from each aliquot, and when possible, a minimum of 100 metaphases were analyzed from each culture. The number of chromosome aberrations was recorded. Final analysis and karyotypes were prepared according to the Denver System (6).

Preparation of Chemical Solutions

Nitrogen mustard [mechlorethamine hydrochloride (Mustargen)] was dissolved in distilled water and used immediately after dissolving. 6-Mercaptopurine was dissolved in normal saline by heating in a bath at 85°C for 10 min. Stock solutions of 6-MP were stable for months in the refrigerator. A-649, an antibiotic of unknown empirical formula, was dissolved in ethyl acetate (1 mg/ml), and dilutions were made with sterile normal saline. The small amount of ethyl acetate present in the cultures was shown to be without effect on the chromosomes.

Results

Chromosome karyotypes from the 10 individuals of the control group were normal in number as well as in morphology. The only variations seen were those generally attributed to artifacts arising during preparations. Of 1000 cells counted and analyzed for chromosome damage, 93% had 46 chromosomes, 5% had missing or in excess 1 or 2 chromosomes, and 2% had chromosome breaks. Similarly, the chromosomal patterns in cultured WBC taken from

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* HN2, nitrogen mustard; 6-MP, 6-mercaptopurine; and A-649, which is NSC 38270, an investigational antitumor agent.
* B, before treatment; D, during; A, after. Number indicates chromosome studies performed.
* Indicates metaphases missing or having in excess 1 or 2 chromosomes.
* Indicates tetraploid metaphases with random chromosome distribution.
* Indicates tetraploid endoreduplicated metaphases with typical diplochromosomes.
* In each instance includes 1 octoploid metaphase.
ever, when using HN2, 6-MP, and A-649 in vivo and in vitro, a considerable frequency of structural chromosome aberrations as well as P & E of chromosomes resulted (Tables 1–3).

Types of chromosome morphologic changes are summarized in Tables 1 and 3, with an attempt at classification. The changes range from very small, hardly visible, acentric fragments to gross changes in which chromosome components are engaged in complex rearrangements (Fig. 3). Simple chromatid breaks were evident in these cultures, as were isochromatid breaks and associated displaced acentric fragments (Figs. 1, 2, 5). The most common rearrangement was that of dicentric chromosomes (Figs. 1, 5), and it should be pointed out that, in the analyzed karyotypes of cells containing dicentrics, it would appear that these chromosomes were formed by the fusion of 2 nonhomologous chromosomes. Tricentric chromosomes were also seen (Fig. 2). Ring chromosomes and typical quadriradial rearrangement of chromosomes were observed as well.

Although the following was not investigated or scored for the present work, it was noted that the 3 compounds under study are capable of inducing or enhancing secondary constrictions in the chromosomes. These constrictions appeared as short segments of under-stained chromatin of a hazy or puffy nature, and were seen most frequently in chromosomes Nos. 1, 2, 4, and probably 9. An example of these is shown in Fig. 4. It should be mentioned, however, that constrictions, breaks, dicentrics, and other types of rearrangements were present in diploid as well as in polyploid metaphases.

In addition to structural chromosomal aberrations, numerical variations of chromosomes were also evident. When patients were treated with HN2, up to 15% of cells counted showed polyploidization; with 6-MP, 9% polyploidization was evident; and when using A-649, up to 14% polyploidization was seen. These figures include the polyploid metaphases showing random distribution (Fig. 7) and in quadruplochromosomes (Fig. 8).

In addition to the structural chromosome disturbances and chromosome polyploidization, other anomalies were disclosed which seem worthwhile to report. In microscopic scanning of plates from the in vivo and in vitro treated cultures the presence of giant cells was evident, especially in HN2 preparations. Superfragmentation of genetic material could also be detected in these plates. It was clear that chromosomes had undergone excessive breakage, and fragments showing various degrees of abnormal condensation were scattered in the cytoplasm. It was noted that these octoploid cells were found with chromosomes in random distribution (Fig. 7) and in quadruplochromosomes (Fig. 8).

It is interesting that in the in vitro studies, although chromosome aberrations in these patients persisted for some time following therapy, a gradual decrease in the number of aberrations was noted. In no case were we able to detect chromosomal damage 3 months after treatment was stopped. Most cells containing chromosome aberrations seemed to lose their proliferative capacity. This was evident from the observation that, in cultures obtained 2–3 months following therapy in some of these patients, the large majority of their leukocyte metaphases were characterized by a diploid chromosome complement without apparent chromosomal changes.

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**Discussion**

The findings of structural chromosomal aberrations and polyploidization in our studies are not surprising. It is well known that HN2 is a radiomimetic substance, since investigations have disclosed that the cytologic effects, particularly the injuries to the chromosome material, are very similar to those induced by irradiation (10). Conen and Lansky reported the case of a patient who showed structural chromosome abnormalities in the cells cultured from peripheral blood after HN2 therapy for malignant
disease (4). Similarly, chromosome damage was reported following cyclophosphamide (1) or 6-azauridine (5) therapy. In our studies, although many different types of structural aberrations were induced with HN2, 6-MP, and A-649 chromosomal breakage and formation of dicentric chromosomes were among the most common. Chromosome breaks or fragments were easily detectable in both in vivo and in vitro plates. It was noted that broken chromosomes can behave in 2 different ways: (a) they may remain in a fragmented state, or (b) the fragments may reunite in a new chromosome combination. These chromosomal rearrangements did not follow any specific pattern. Dicentrics and a few tricentric chromosomes were seen in diploid as well as in polyploid mitoses in our treated preparations. It has been postulated that the formation of a permanent bridge by a dicentric chromosome results in cell death because such nuclei in subsequent division give rise to abnormal polytene figures and giant cells (19). These dicentric chromosomes were commonly observed in our fixed preparations when leukopenia occurred following therapy. Dicentrics were not observed in untreated human leukocytes in our study, although these chromosomes have been observed in euploid human cells (20) and rat cells (11).

Nowell, using mitomycin (18), and Jackson and Lindahl-Kiesling, with sulphydryl compounds (8), have shown separate effects on chromosomal material on one hand, and on the mitotic apparatus on the other hand, by treating leukocytes in cultures at various time intervals. It is postulated (17) that certain mutagenic agents might damage chromosomes and in addition might produce changes in the mitotic process of the cells. Thus, the induction of chromosome polyploidization may indicate mitotic damage.

As noted, we use the term polyploidization to designate polyploidy or endoreduplication (P & E) of chromosomes. Studies indicate that endoreduplicated cells represent a form of polyploidy. Jackson and Killander (7), in β-mercaptoethanol-treated 6-day cultures, observed that the amount of DNA synthesized was increased in proportion to the degree of polyploidy seen in cytologic examinations. DNA measurements obtained by microspectrophotometric studies were in agreement with cytogenetic determination of ploidy, in that tetraploid and octoploid mitoses, including endoreduplicated mitoses, contained amounts of DNA proportional to chromosome number. Endoreduplication was first described by Levan (12) on Allium cepa root cortex as a form of endomitosis and as a process in which internal doubling of chromosomes occurs singly and in multiples. In mouse ascites tumors, Levan and Hauschka (13) found cells in mitotic stages showing groups of 2, 4, or more chromosomes, indicating the doubling of chromosome number during previous interphases. Endoreduplication has been described in 2 cases of acute leukemia (3, 21). However, Sandberg et al. (22), studying chromosomal changes in 75 patients with acute leukemia have stated that the relationship of chromosomal changes to acute leukemias is not interpretable at present.

The tetraploid metaphase of Fig. 5 is highly interesting. It was found in a culture from the patient treated with 6-MP and has a unique structure. It contains 2 identical paired dicentric chromosomes and 2 sets of chromosome breaks. In addition, one can observe other paired chromosomes as seen in diplochromosomes of tetraploid endoreduplicated cells. These observations may indicate that (a) the structural chromosome aberrations occurred early in the damaging process of 6-MP, (b) that following this damage there were changes in the mitotic apparatus of the leukocytes with production of polyploid cells, and (c) that some if not all of the polyploidization induced in treated WBC occurred through endoreduplication. In this regard, Bell and Baker (2), in a study of the ratios of tetraploid cells to endoreduplicated cells sampled following irradiation, have suggested that tetraploidy was produced by the initial induction of endoreduplication. P & E of chromosomes have been demonstrated in cultured human leukocytes with a number of agents, including treatment with A-8103, a piperazine derivative (16); β-mercaptoethanol; β-mercaptopropionate; and β-mercaptopropylamine (8). Furthermore, exposing colchicine (23) to plant cells and kinetin (9) to animal cells has produced polyploidization of chromosomes.

It is apparent from the preceding references and the present investigation that one may well expect P & E of chromosomes as well as structural chromosome damage in human leukocytes when exposed to chemotherapeutic agents. However, all chemotherapeutic agents may not induce these chromosomal abnormalities, and if changes are produced, different molecular mechanisms may be at fault. It remains to be established if the administration of other chemotherapeutic agents will produce similar chromosomal aberrations.

Our cytogenetic studies of human leukocytes in vivo and in vitro indicate that HN2, 6-MP, and A-649 induce structural aberrations in addition to P & E of chromosomes. The 5 patients examined by us constitute too small a group for any general conclusions. Nevertheless, in view of our results, including the in vitro work, the interpretation of cytogenetic changes in patients receiving active therapy must be reserved.

Acknowledgments

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References

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**Figs. 1-8.** Metaphases of 72-hr cultures of peripheral leukocytes from patients treated with HN2, 6-MP, and A-649. Acetic orcein stain.

**Fig. 1.** B, Chromosomal breakage; D, dicentric chromosome. \(\times 1600\).
**Fig. 2.** Tetraploid-level mitosis. B, Chromosomal breakage; D, dicentric chromosomes; T, tricentric chromosome. \(\times 1400\).
**Fig. 3.** Note complex rearrangement which resulted from chromatid breakage and exchange. \(\times 1600\).
**Fig. 4.** Secondary constrictions are indicated by arrows. \(\times 1600\).
**Fig. 5.** Tetraploid-level mitosis with chromosomes randomly distributed. However, note paired chromosomes characteristic of endoreduplication (arrows). B, Chromosomal breakage; D, dicentric chromosomes. \(\times 1400\).
**Fig. 6.** Tetraploid endoreduplicated mitosis with typical diplochromosomes. \(\times 1400\).
**Fig. 7.** Octoploid mitosis with chromosomes randomly distributed. \(\times 1200\).
**Fig. 8.** Octoploid endoreduplicated mitosis with quadruplochromosomes. \(\times 1200\).

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