CHROMOSOME DAMAGE AFTER TREATMENT WITH CYSTEAMINE IN NONIRRADIATED AND IRRADIATED HUMAN LEUKOCYTES

Carlos E. Nasjleti and Herbert H. Spencer

Veterans Administration Hospital and The University of Michigan, Ann Arbor, Michigan

In association with the increasing use of high-energy radiation in medicine, industry and warfare, the question of radioprotection, particularly chemical, is acquiring great importance. An intense search has been made for chemical compounds which will protect the organism from exposure to the deleterious effects of ionizing radiation. Radioprotective activity has been investigated using: (1) biological amines such as histamine, adrenaline and serotonin; (2) disulfide compounds such as cystamine and cystine; and (3) sulfur-containing compounds such as cysteine and cysteamine. A review of this field has been published by Eldjarn and Pihl (*1*). In this study, one compound that appeared to be of value because of its striking radioprotective activity was cysteamine. Cysteamine (β-mercaptoethylamine, MEA) caused protection from the effects of radiation both *in vivo* and *in vitro*. Comparatively few studies have been reported on the ability of MEA to protect mammalian cells in tissue culture against radiation, and in the studies reported different mammalian cells were investigated and dissimilar criteria were used as indicators of radiation damage. Grant and Vos used survival curves of irradiated rat thymocyte suspensions as a criterion for protection (*2*), and Eker and Pihl studied the growth-inhibiting effects of radiation on strain L mouse cells (*3*). To show protection and using a method based on formation of clones, Kelley and Wheeler studied cells derived from a human epidermoid carcinoma (*4*), and Vos, Budke and Vergroesen studied an established heteroploid cell line derived from human kidney (*5*). Although MEA was found to protect these cells *in vitro*, it is not yet clear whether mammalian cells, in general, can be protected by MEA. The mechanism of action of MEA on the cellular level is still a matter of debate.

Recent cytogenetic studies from this laboratory showed that ionizing radiation induces two kinds of effects within the chromosomal complex in human leukocyte cultures (*6,7*). One apparently affects the spindle apparatus with formation of polyploidy and endoreduplicated cells; the other appears on the chromosomes, inducing breakage, dicentric chromosomes, ring chromosomes and chromosomal translocations. It was also shown that both disturbances can lead to degeneration and death of the affected white blood cells.

Therefore it seemed to be advantageous to investigate the radiation-protective ability of MEA using normal human somatic cells growing in tissue culture and subsequent cytogenetic analysis. In this system, chromosomal integrity of leukocytes is the actual parameter for protection. Cytological analysis of the chromosomal complement is well suited for the study of protective agents as has been shown by other investigators in plant (*9,10*), and mammalian cells (*11,12*).

To find out whether or not the reduction in the radiation sensitivity of human leukocytes by MEA is correlated with a reduction in the amount of chromosome injuries of individual cells, it was decided to make comparative analyses of the frequency of chromosome injuries in cultured leukocytes subjected to treatments by MEA and radiation. Corresponding controls were also analyzed.

MATERIALS AND METHODS

There is considerable evidence that mononuclear leukocytes, more specifically small lymphocytes, are the cells which divide in human blood cultures.

Cell cultures. Leukocytes from the peripheral blood of healthy human donors grown in culture were used throughout the course of the experiments.

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For reprints contact: Carlos E. Nasjleti, Veterans Administration Hospital, Ann Arbor, Mich. 48105.

These cultures, as well as the chromosome preparation from these cells, were carried out by the technique of Moorhead *et al* (*8*). Samples of 65–75 ml of venous blood were obtained from each donor and transferred to sterile vacuum tubes containing 0.2 ml of heparin (1000 units/ml). The vacuum tubes were placed at an angle of 60 deg and allowed to stand at room temperature 1–2 hr. From each tube, aliquots of 2 ml of supernatant plasma containing viable nucleated cells were placed in culture bottles (2-oz prescription bottles) with 8 ml of Culture Medium 199 (Difco), 700 units of penicillin G and 0.7 mg of streptomycin. To each aliquot 0.25 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. With the exception of the time required for treatment, both chemical and/or irradiation, these cultures remained uninterrupted under these conditions for a total of 100 hr, at which time the cultures were harvested for chromosome preparation. The cells were processed after 3-hr exposure to Colcemid (Ciba) in a final concentration of 10^{-6} *M*. Processing consisted of 10-min exposure to hypotonicity achieved by adding three times the volume of distilled water to the culture. Fixation was accomplished by exposure to absolute alcohol-to-glacial acetic acid, 3-to-1 for 30 min, and then the slides were prepared, air dried and stained by Giemsa stain.

Chemical treatment and irradiation. The effect of different concentrations of MEA with a constant dose of 200 R of x-rays was studied as follows. After 24 hr of incubation, the cultures were removed from the incubator, and each received MEA in dose ranging from 1×10^{-3} to 5×10^{-3} *M* final concentrations. Cultures were then returned to the incubator. After 24 hr the bottles were again removed from the incubator and irradiated with 200 R of x-irradiation. The cells were washed three times by replacing the culture fluid with fresh medium to which plasma from the same donor and antibiotics were added. The cultures were returned to the incubator for an additional 51 hr. Both MEA treatment and irradiation were carried out at room temperature (approximately 27°C), and 1 hr usually elapsed from the time the cultures were removed from the incubator until they were returned after the combined procedures. Each experiment contained all the necessary controls: (1) non-MEA, nonirradiated; (2) MEA, nonirradiated; and (3) non-MEA, irradiated.

MEA solution was prepared by diluting the chemical in Tissue Culture Medium 199 immediately before the addition to cultures. MEA was obtained from the Sigma Chemical Co., St. Louis, Mo.

The physical conditions of irradiation were as follows: batches of six culture bottles were irradiated at one time. The bottles were placed on a circular board so that the distance from the center of the beam was the same for all bottles. A conventional therapy machine operating at 250 KVP, 15 mA with 0.5 mm Cu and 1.0 Al filters was used. The doses were measured in air with a Philips universal dosimeter with the ionizing chamber placed in the center of the bottles, and the dose rate of 30.2 R/min for 6 min and 37 sec was used. We treated the cells by using two constant technical steps; cells were always exposed to MEA for 24 hr and were irradiated with 200 R of x-irradiation. There are two reasons for this: (1) There is always the danger that MEA in solution could be oxidized to cystamine, an inactive disulfide with a very poor record as a radioprotectant *in vitro*. Eker and Pihl working with MEA and L mouse cells have found that the sulfhydryl content in MEA solution decreased in the course of treatment (*3*). (2) The constant 200 R dose has been selected because in our laboratory we have recently characterized the chromosome damage induced by this level of irradiation in leukocyte cultures (*6,7*). We obtained reliable qualitative and quantitative data of cytogenetic changes in these cells. Other investigators have used this level of irradiation when studies were performed with x-rays (*13*) and gamma rays (*14*).

RESULTS

The present experiments were designed to investigate whether MEA affords some protection against the biological effects of irradiation in human leukocytes as judged by cytogenetic integrity. Following the treatment by MEA in nonirradiated cultures, a very high frequency of chromosomal aberrations resulted. MEA had a great deal of activity, both in the extent of chromosomal damage (which affected up to 75% of the 1,260 metaphase figures analyzed), and in the low concentrations necessary to show damage. Simple chromatid breaks and chromatid achromatic gaps of the interstitial type were seen in many cell metaphases while other cells showed constrictions of the erosion type in high number. Chromosomal breaks were seen less frequently (Fig. 1). The constrictions of the erosion type were present to a severe degree in many cells in which all the chromosomes were badly fragmented (Fig. 2). These constrictions had the general appearance of strongly stained segments alternating with segments with almost no stain. These segments were distributed in an irregular sequence along the chromosome length, thus giving a rugged appearance to the outline shown by these metaphases. In these MEA-

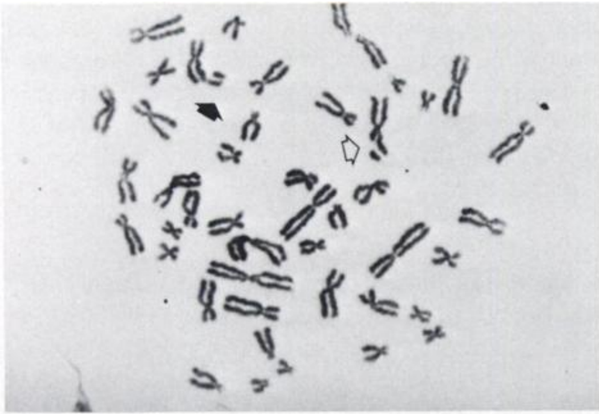

FIG. 1. Metaphase plate showing chromosome breakage (solid arrow) and chromatid break (open arrow). × 1,600.

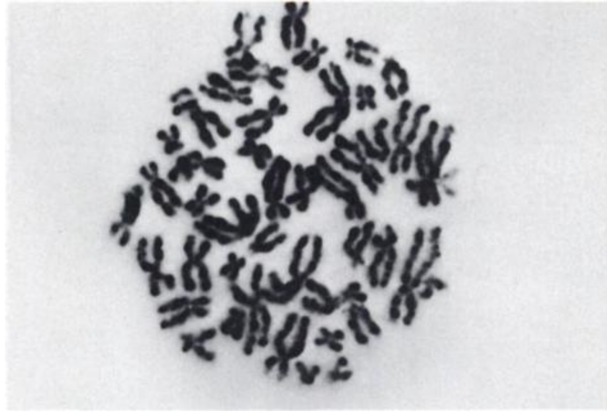

FIG. 2. Diploid metaphase with severe chromosome erosion. × 1,600.

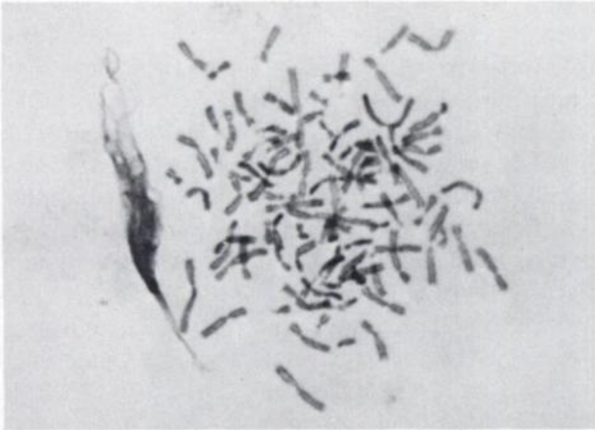

FIG. 3. Tetraploid metaphase with chromosomes randomly distributed. × 1,400.

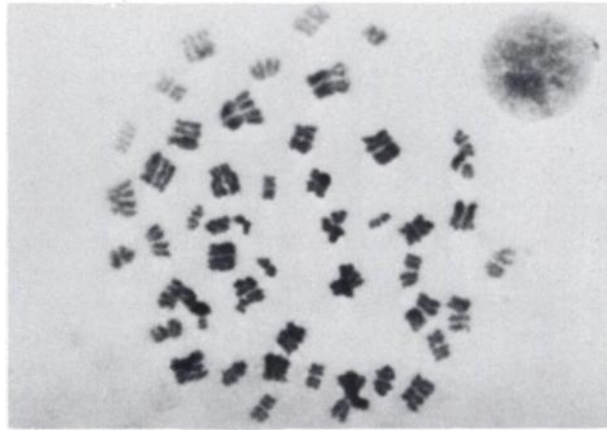

FIG. 4. Endoreduplicated tetraploid metaphase with 92 chromosomes arranged as 46 diplochromosomes. × 1,400.

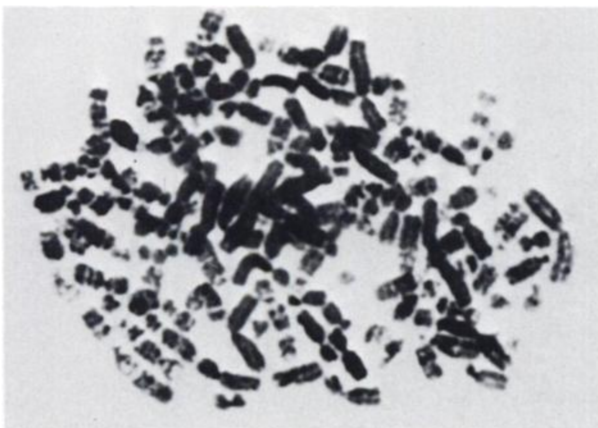

FIG. 5. Tetraploid metaphase showing extensive chromosome fragmentation. × 1,400.

treated cultures, dicentric and ring chromosomes as well as translocations and other types of structural chromosomal damage were not encountered. However, polyploidy and endoreduplication (P & E) of chromosomes were evident in these cultures. A significant proportion of tetraploid cells showed random location of chromosomes (Fig. 3) while others showed the paired diplochromosomes characteristic of endoreduplication (Fig. 4). The same type of chromosomal erosion described in diploid cells was also observed in tetraploid cells (Fig. 5). Besides the chromosomal aberrations in MEA-treated plates, microscopic examination showed an impressive number of giant cells. These cells had irregular shapes, sometimes with fragmented nuclei and very large diameters. The observed increase in chromosomal aberrations plus the induction of chromosomal polyploidy and giant cells in MEA-treated cultures rela-

TABLE 1. POLYPLOIDY IN HUMAN LEUKOCYTE CULTURES (100 HR)

Treatment		Cytological analysis	
X-rays (R)	MEA ($\times 10^{-3}$ M)	Metaphases counted	Polyploidy* (%)
0	0	300	1.0
0	1	300	2.0
0	2	300	3.0
0	3	200	3.0
0	4	200	4.5
0	5	260	3.4
200	0	300	6.3
200	1	300	6.0
200	2	180	9.4
200	3	120	7.5
200	4	100	11.0
200	5	50	8.0

* Includes both polyploidy with random distribution of chromosomes and diplochromosomes characteristic of endoreduplication.

tive to control cultures (non-MEA, nonirradiated), is almost certainly due to the effect of MEA treatment.

Since a very high frequency of metaphases from leukocytes treated by MEA showed chromatid gaps, constrictions breaks and chromatid and chromosome breaks, it was difficult to use chromosomal breakage as the criterion to determine the normality of each metaphase figure. We chose to use the number of polyploid metaphases to tabulate the results of the experiments (Table 1).

The chromosome aberrations in leukocyte cultures irradiated with 200 R of x-rays closely resemble those previously found by the authors (*6,7*) and other investigators (*13,14*). Observations made on the response of leukocyte chromosomes to ionizing radiation in this study can be summarized as follows: (1) breakage of chromatids takes place with a high frequency, and there is a random distribution of the breaks within the chromosomes; (2) breaks are usually followed by chromatid exchanges, formation of dicentric and ring chromosomes in addition to translocations and other chromosomal rearrangements; (3) an increased percentage of polyploid and endoreduplicated cells are present; (4) diploid, as well as tetraploid metaphases show structural chromosomal aberrations; and (5) cytological analysis of irradiated leukocytes show formation of giant cells.

After irradiation of MEA-treated cultures, an interesting pattern of response was found, mainly, a gross injury to the chromosomal complex which apparently caused cell death. Although diploid and tetraploid metaphases were observed in these cultures, there was also an increased percentage of pyknotic cells with superfragmentation of chromosomes (Fig. 6). These are assumed to be degenerating cells in which the mitotic mechanism has been greatly injured and in which fragmented chromosome segments have undergone pyknotic condensation in the cytoplasm (*6,7*). The cytological anomalies described after the combined treatment may have originated after the initial MEA damage.

DISCUSSION

Under the present experimental conditions MEA was unable to protect leukocytes against radiation, and it induced severe morphological chromosome damage in addition to chromosomal polyploidization. Chromosome aberrations were observed when MEA was administered alone, and the damage was increased when the chemical was given in conjunction with radiation. Although we have used a dissimilar experimental setting, our data confirm and extend Jackson and Hill's recent finding that the pretreatment of cultured leukocytes by MEA affords no protection against radiation damage, as ascertained by their cytogenetic study (*15*). In their investigation, the combined treatment with MEA in 5-day cultures and different doses of x-irradiation usually resulted in higher percentages of chromosomal polyploidy than the sum of percentages in cultures treated with either alone. It was suggested that the combined treatment is at least additive and perhaps synergistic. In their presentation, however, no mention was made of the striking chromosomal erosion seen in the present study.

MEA-treated cultures showed a high incidence of leukocyte metaphases with both interstitial chromatid erosions and constrictions breaks, simulating chromosomal fragmentations. They appear to be induced at random in the chromosomal complement,

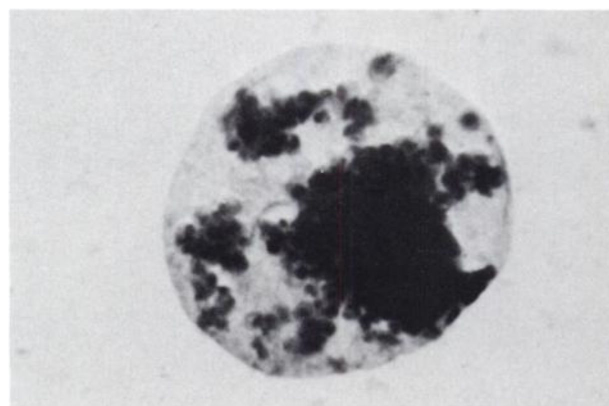

FIG. 6. Dividing human leukocyte, showing various degrees of superfragmentation of the chromosomal complement. $\times$ 2,000.

and the variability of appearance of the different chromosomes was striking. They probably represent exaggerations of constrictions breaks or possibly also negative heteropyknosis of the nucleic acid starvation type. They were observed in connection with the chromosome breakage induced by phenols in the experiments of Levan and Tjio 20 years ago and were called "erosions" at that time (*16*). Recently, Hampel and Levan described these chromosomal lesions in a human cell line Lu 106 induced by low temperature (*17*).

Polyploidy and endoreduplicated cells have been reported to occur regularly, although rarely, in human tissues *in vitro*. Several sulfhydryl compounds have been found to produce these changes in leukocytes stimulated by phytohemagglutinin in cultures. Jackson and Lindahl-Kiessling described an increased number of endoreduplicating and polyploid cells in human leukocytes after exposure to β-mercaptopyruvate or β-mercaptoethanol, together with chromosomal erosion (*18*). Nasjleti and Spencer observed this also happened when leukocytes were exposed to 6-mercaptopurine (*19*). Apparently, these sulfhydryl compounds disturbed the spindle apparatus of leukocytes, but the mechanisms involved are still obscure.

Our results would indicate that MEA does not have radioprotective ability and manifests chromosomal toxic effects as well. We have to consider the possibility that the observed MEA effects are at least partly exerted by an action on certain important cell constituents, above all on nucleic acid synthesis and turnover. Enzymes engaged in the oxidation-reduction system of the phytohemagglutinin-transformed lymphocytes are also likely to be affected, and possibly when MEA solution is applied, toxic oxidation products are formed. It seems reasonable to assume that the chromosomal damage found in this study was related to treatment with MEA.

The findings in the present study are in disagreement with the data of Vos, Budke and Vergroesen (*5*) and that of Kelley and Wheeler (*4*). Vos and coworkers used an established line of cells derived from human kidney to study the protective ability of MEA and suggested that it accomplished excellent radioprotection as measured by plating efficiency of the cells. Maximal protection was observed at 32 m*M* concentration. Similarly, Kelley and Wheeler, using cells derived from a human carcinoma, showed that MEA afforded protection by a method based on formation of clones and monolayers. Furthermore, Grant and Vos studied the effects of various chemical compounds on the survival of rat thymocytes irradiated *in vitro* and observed that the addition of MEA to the suspensions appreciably retarded the death-rate of the irradiated cells (*2*). They concluded that MEA protected by reacting with radiosensitive sites and not by causing anoxia in the cells.

Apparently MEA is an agent which is capable of producing chromosome damage *in vitro*, both at the first and second division of cultured leukocytes. However, it is difficult to extrapolate to a meaningful *in vivo* comparison. We have shown previously that individuals therapeutically irradiated (*6,7*), or treated with chemotherapeutic agents such as nitrogen mustard, 6-mercaptopurine (*19*), and N, N′-bis-(3-bromopropionyl) piperazine (*20*) showed increased frequencies of chromosome anomalies. It is noteworthy that these anomalies were similar although not identical to the chromosomal changes induced by MEA. Nevertheless, when irradiation was applied, or when those chemical agents were added to normal cultured leukocytes, the same chromosome aberrations resulted as were noted in the *in vivo* studies (*6,7,19,20*). These studies lend support to the possibility that MEA could induce those chromosomal aberrations *in vivo*.

The ability of MEA to damage chromosomes of human leukocytes may indicate its potential as a mutagenic agent. In this regard, it may be of some interest that in patients treated with ionizing radiation (*6,7*) and chemotherapeutic agents (*19,20*) a gradual decrease in the amount of aberrations was noted after treatment. It is possible that even if MEA induces chromosomal changes in humans, the cytogenetic damage may soon fade out after treatment. In addition to chromatid and chromosome breaks, MEA appears capable of producing at least three characteristic changes involving chromosomes. The first is chromatid achromatic gaps; the second is constrictions breaks simulating segmentations of chromosomes; and the third is chromosomal polyploidization. Probably most cells exhibiting any of the phenomena would eventually die and not go through more than two or three additional divisions. Many viruses and chemicals cause damage in a random fashion, and the apparent randomness of the constrictions and erosions caused by MEA suggests that it belongs to this class. These chromosomal manifestations in constrictions breaks simulating segmentations can also result from various physical and chemical procedures such as fixation (*21*), calcium deficiency (*22*), thymidine treatment (*23*) and low temperature (*17*). It seems unlikely that the chromosome damage resulting from these causes and also from MEA are associated with gene mutations. Nevertheless, the mutagenic potential of MEA as well as other radioprotectant agents could be tested in experimental animals.

SUMMARY

The radioprotective activity of cysteamine (MEA) at various concentrations was tested in an *in vitro* system based on a comparative analyses of the frequency of chromosomal injuries in cultured human leukocytes. These cells were treated alone or in combination with MEA and x-rays.

MEA did not protect these cells against radiation in this system. On the contrary, in leukocytes treated with MEA alone, a high frequency of chromosomal aberrations resulted. MEA-treated cultures showed cytogenetic damage which included chromatid breaks, achromatic gaps and constrictions of the erosion type. Increased number of polyploid cells were found in these cultures. After x-irradiation to previously MEA treated cultures, a striking number of death cells appeared in addition to the chromosomal abnormalities. There appeared to be a summation of deleterious effects with the combined treatment.

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