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Postreplication repair and the susceptibility of Chinese hamster cells to cytotoxic and mutagenic effects of alkylating agents

(V79 cells/methylnitronitrosoguanidine resistance/6-thioguanine/ouabain)

J. FRIEDMAN AND E. HUBERMAN

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

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ABSTRACT **A cell variant (VR-43) resistant to the cytotoxic effect of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and *N*-methyl-*N'*-nitro-*N*-nitrosourea was isolated from Chinese hamster V79 cells for use in studies of the relationship among cell survival, mutagenesis, and DNA repair by alkylating agents. Resistance to ouabain or 6-thioguanine was used as the genetic marker. After treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, the VR-43 cells exhibited mutation frequencies that were lower, on a dose basis, than those of the wild-type V79 cells. However, when analyzed at equicytotoxic doses, the VR-43 cells were more mutable than the V79 cells. No difference in cell survival or mutagenicity could be observed after treatment with other mutagens such as *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine, ethyl methanesulfonate, or x-rays. Postreplication repair was analyzed by determination of the molecular weight of the newly synthesized DNA by alkaline sucrose gradients. After treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, the VR-43 cells exhibited an enhanced postreplication repair relative to the V79 cells. No such enhancement was found after *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine or ethyl methanesulfonate treatment. Based on these results we propose that, after treatment of these and presumably other mammalian cells with some methylating mutagens, postreplication repair can cope with DNA lesions responsible for cytotoxicity and, to a lesser degree, with lesions responsible for mutagenicity.**

Studies on the relationship between carcinogenesis and mutagenesis (1–11) and on the involvement of altered DNA repair processes in some cancer-prone patients (12–18) support the concept that carcinogenesis may be initiated from somatic mutations in genes that control malignancy (6, 7, 19). It is therefore important to study the mechanisms involved in the induction of somatic mutations by environmental agents. A major factor in such a mechanism is DNA repair because insufficient or defective repair usually results in cell death or mutation. Excision and postreplication repair (20–25) are considered to be the major types of DNA repair. Excision repair involves the elimination of the DNA region bearing the altered nucleotides prior to gap-filling by the DNA polymerases; postreplication repair is the manner by which the DNA replication machinery copes with lesions in the parental DNA strands. Experiments with cells derived from xeroderma pigmentosum patients (13–17) have supported the idea that, as in bacteria, the mammalian excision repair is mainly an error-free process (17, 26, 27). However, the relationship among postreplication repair, cell survival, and mutagenesis is not clear (28).

The present studies were initiated to determine this relationship in cultured mammalian cells. We have compared DNA repair in the mutable Chinese hamster V79 cells with that in one of its cell variants which exhibited an enhanced cell survival after treatment with some methylating agents but not ethylating agents or x-rays and, at equitoxic doses, exhibited enhanced mutation frequencies.

Based on these experiments, we propose that, in these and presumably other mammalian cells, after treatment with some methylating mutagens, postreplication can cope with DNA lesions responsible for cytotoxicity and, to a lesser degree, with lesions responsible for mutagenicity.

MATERIALS AND METHODS

Chemicals. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), thymidine, deoxycytidine, 6-thioguanine, and ouabain were supplied by Sigma; *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) and *N*-methyl-*N'*-nitro-*N*-nitrosourea (MNU) were obtained from the Research Resources Section of the National Cancer Institute (Bethesda, MD); and ethyl methanesulfonate (EtMes) was from Eastman Kodak. [*methyl*-^{14}C]MNNG (specific activity, 20 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels), [^{3}H]thymidine (1.9 Ci/mmol), and [^{14}C]thymidine (559 mCi/mmol) were supplied by Amersham/Searle.

The cultured cells were treated with MNNG, ENNG, or MNU dissolved in acetone; EtMes was added directly. Ouabain and 6-thioguanine were dissolved in boiling water and dimethyl sulfoxide, respectively, prior to dilution into the culture media. The final concentration of acetone or dimethyl sulfoxide in culture medium was 0.5%.

Cell Culture. Chinese hamster V79 cells, derived from a subclone of V79-4 kindly supplied by E. H. Y. Chu (University of Michigan, Ann Arbor), and VR-43 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 0.25 μg Fungizone per ml (GIBCO) at 37 ± 0.5°C. Throughout the experiments, 60- or 100-mm plastic petri dishes (Falcon) were used. The cultures were incubated in a humidified incubator with 5% CO_2/95% air.

Isolation of Cells with an Increased Resistance to the Cytotoxic Effect of MNNG. To obtain cells with an increased resistance to the cytotoxic effect of MNNG, we treated 10^7 V79 cells cultured in 100-mm petri dishes with 2 μg of this mutagen per ml. After one subculture and about a week later, the cells were reseeded at 5×10^6 cells and on the following day were treated for 24 hr with MNNG at 2 μg/ml. This type of treatment with MNNG was repeated five times at 1-week intervals, after which a series of clones were isolated. Cells from one of these clones, designated as VR-43, exhibited an increased resistance to the cytotoxic effect of MNNG. These cells had a mean (±SD) generation time (16 ± 2 hr) and a chromosomal karyotype (around 22 chromosomes) similar to those of the parent cells.

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Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; ENNG, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine; MNU, *N*-methyl-*N'*-nitro-*N*-nitrosourea; EtMes, ethyl methanesulfonate.

Mutagenesis Assay. To test for ouabain and 6-thioguanine resistance, we treated 1-day-old exponentially growing 5×10^6 V79 or VR-43 cells cultured in 100-mm petri dishes with the mutagens. After 3 hr of treatment (in the case of x-rays, 3 hr after treatment), the cells were dissociated with a trypsin/EDTA solution and seeded at 200 cells per 60-mm dish in 5 ml of medium for determination of cloning efficiency and at 5×10^4 cells per dish in 4 ml of medium for determination of the number of ouabain-resistant mutants. Ouabain (to a final concentration of 1 mM) was added in 1 ml of medium 2 days after cell seeding. For selection of the 6-thioguanine-resistant mutants, 10^5 V79 or VR-43 cells that had been treated with the mutagens were seeded in 100-mm dishes. The cells were dissociated 6 days later and reseeded at 200 cells per 60-mm dish in 5 ml of medium for determination of cloning efficiency and at 2×10^4 cells per 60-mm dish in 4 ml of medium for determination of the number of 6-thioguanine-resistant mutants. Two days later 6-thioguanine (to a final concentration of 40 μM) was added in 1 ml of medium. Thus, an expression time of 8 days was used for selection of 6-thioguanine resistance, compared with a 2-day expression time for selection of ouabain resistance. These expression times were chosen because in both V79 and VR-43 cells, they yielded optimal mutation frequencies when tested with various doses of MNNG (4, 29, 30).

Cloning efficiency was determined by counting the number of Giemsa-stained colonies in six to eight dishes per point at 7–8 days after cell seeding, and the number of ouabain- or 6-thioguanine-resistant mutants was determined by counting Giemsa-stained colonies in 16–24 petri dishes per point at 12–14 days after cell seeding. Mutation frequencies of ouabain- and 6-thioguanine-resistant mutants were calculated per 10^6 or 10^5 survivors, respectively, based on the cloning efficiency and the number of cells seeded for mutant selection. The number of mutants after induction with the mutagens varied up to 30% between the different experiments.

DNA Repair Assay. DNA repair was analyzed by determination of DNA sedimentation in an alkaline sucrose gradient (21). The molecular weight of the newly synthesized DNA, after mutagen treatment, was determined in exponentially growing V79 and VR-43 cells seeded 40 hr earlier at 2×10^5 cells per 60-mm petri dish. Fresh culture medium was added 1 hr prior to treatment with the mutagen. After 20 min of treatment with the mutagen, the dishes were rinsed once with fresh medium, and the V79 or VR-43 cells were labeled with either 1 μCi of [^{14}C]thymidine per ml or 2 μCi of [^{3}H]thymidine per ml. In the case of EtMes, the time of treatment was extended to 60 min. No difference in the cells' response was observed with either label. After 25 min of pulse with labeled thymidine, the cells were chased with 0.5 mM unlabeled thymidine and 0.05 mM deoxycytidine. At different times of the chase, the cells were rinsed twice with cold phosphate-buffered saline, placed in 2.5 ml of ice-cold phosphate-buffered saline, and irradiated with x-rays at 1500 rad (15 grays) (31).

After irradiation the cells were incubated for 30 min at 4°C in 5 ml of trypsin solution. Then, 5 ml of culture medium (to neutralize the effect of the trypsin) was added. The cells were centrifuged and resuspended in 0.2 ml of EDTA/NaCl (14). Aliquots of the two cell types with approximately 2000 cpm of either isotope were combined in 50 μl (about 1.2×10^5 cells), lysed for 1 hr at room temperature in 0.2 ml of 1 M NaOH/0.1 M EDTA layered on top of 5 ml of 5–20% alkaline sucrose gradient in 0.1 M NaOH/0.9 M NaCl/0.003 M Na_2EDTA, and centrifuged at 25,000 rpm for 120 min in an SW 50.1 rotor. A 0.2-ml solution of 60% sucrose was on the bottom of each gradient. Ten-drop fractions were taken from the bottom of each tube and collected on paper strips, and the radioactivity and the weight-average molecular weight of single-strand DNA were determined as described (32, 33). Spillover corrections were made (^{14}C to ^{3}H channel, 10%; ^{3}H to ^{14}C channel, 2%).

RESULTS

Susceptibility of the V79 and VR-43 Cells to the Cytotoxic and Mutagenic Effect of Chemical and Physical Agents. To characterize biological properties of the VR-43 cells, we tested their susceptibility and that of the V79 cells to the cytotoxic effect of different mutagens. These included x-rays, the methylating agents MNNG and MNU, and the ethylating agents ENNG and EtMes. Both cell types exhibited a similar cell susceptibility to x-rays and the ethylating agents but not to the methylating agents (Fig. 1). In the case of MNNG, a reduction of the cloning efficiency of the V79 cells to 37% of the control value required a dose of about 0.3 μg/ml compared with about 1.0 μg/ml for the VR-43 cells. A similar situation was observed in the case of MNU.

Both cell types were also tested for their susceptibility to the mutagenic effect of these agents. The induction of ouabain or 6-thioguanine resistance after ENNG, EtMes, or x-ray treatment was dose-dependent and similar in both cell types. As previously described (34), no induction of ouabain resistance was observed after treatment with x-rays. Treatment with MNNG also resulted in a dose-dependent increase in the mutation frequencies, but in this case the VR-43 cells yielded lower mutation frequencies than the wild-type V79 cells (Fig. 2). However, when analyzed at equicytotoxic doses of MNNG, the VR-43 cells were found to be more mutable than the V79 cells. At doses yielding 37% survivors, the VR-43 cells gave 2-fold higher mutation frequencies than the V79 cells (Fig. 3). No such differences could be observed after treatment with x-rays or the ethylating agents. Based on these results, we suggest that the VR-43 cells may represent a cell type with an enhanced ability to cope with DNA damage, caused by methylating agents, that is responsible for cytotoxicity but to a lesser degree with DNA damage that is responsible for mutagenicity.

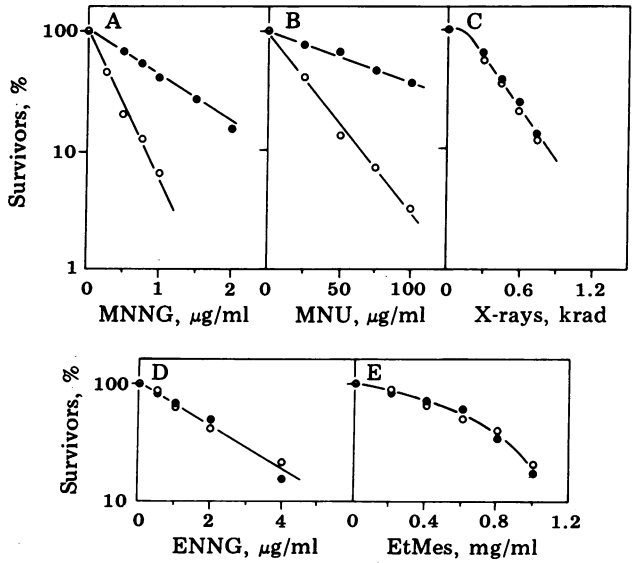

FIG. 1. Susceptibility of V79 (○) and VR-43 (●) cells to MNNG (*A*), MNU (*B*), x-rays (*C*), ENNG (*D*), and EtMes (*E*). The mean (± SD) cloning efficiencies of the V79 and VR-43 cells in the different experiments were 75 ± 15% and 80 ± 11%, respectively. The cloning efficiencies of the control cells in each experiment were considered as 100% for the determination of the percentage surviving cells after mutagen treatment.

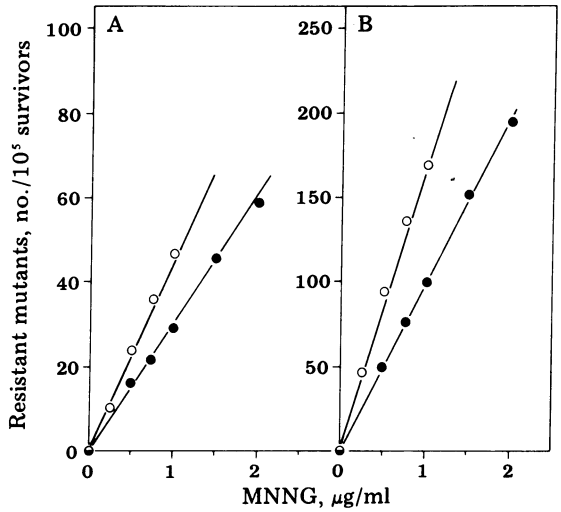

FIG. 2. Induction of ouabain-resistant (*A*) and 6-thioguanine-resistant (*B*) mutants in V79 (○) and VR-43 (●) cells by different doses of MNNG.

Induction of Postreplication Repair After Treatment with the Mutagenic Agents. Both VR-43 and the wild-type V79 cells exhibited a similar binding of [*methyl*-^{14}C]MNNG to DNA when exposed to 0.1 and 1 μg/ml of the mutagen for up to 3 hr. In view of this, we analyzed both cell types for their ability to perform postreplication repair after MNNG treatment. The increase in the molecular weight of the daughter strands of the DNA was determined by sedimentation in alkaline sucrose gradients. For comparison we also analyzed DNA from cells pretreated with ENNG and EtMes, agents that gave a similar biological response in both cell types. After testing different doses of the alkylating agents, we chose 2 μg of MNNG per ml, 40 μg of ENNG per ml, and 8 mg of EtMes per ml for our postreplication DNA repair studies. All of these doses induced similar initial DNA damage as determined by a reduction in the weight average molecular weight of the DNA from 1.5 × 10^8 in controls to about 0.6–0.8 × 10^8 in mutagen-treated cells.

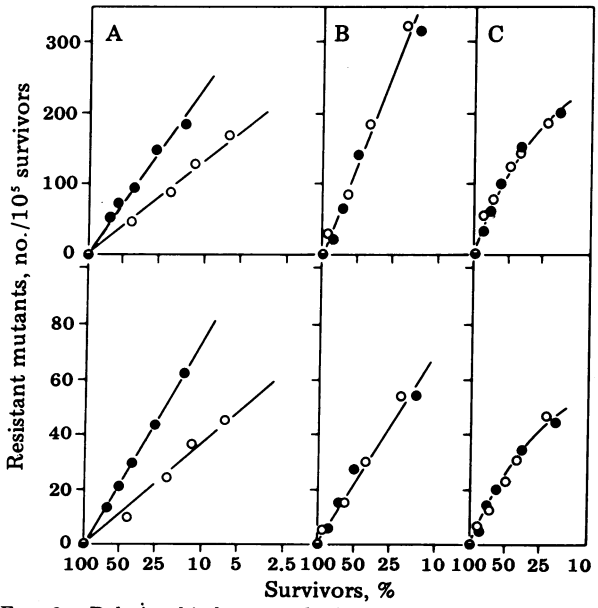

FIG. 3. Relationship between the frequency of ouabain-resistant (*Lower*) or 6-thioguanine-resistant (*Upper*) mutants induced by MNNG (*A*), ENNG (*B*), and EtMes (*C*) and the susceptibility of the V79 (○) and VR-43 (●) cells to the cytotoxic effect of these mutagens. Cytotoxicity was expressed as percentage of cell survival (see Fig. 1).

At different times after treatment with MNNG, the VR-43 cells showed a greater rate of postreplication repair than did the V79 cells. After 1 and 2 hr of chase, VR-43 cells had molecular weights of the newly synthesized DNA of 1.7 × 10^8 and 2.0 × 10^8, respectively, compared with 1.2 × 10^8 and 1.4 × 10^8, respectively, in the V79 cells (Fig. 4). After 4 hr of chase, both cell types yielded DNA with a similar molecular weight, of about 2 × 10^8. Unlike treatment with MNNG, treatment with either ENNG or EtMes resulted in a similar increase in the molecular weight of the newly synthesized DNA. After 4 hr of chase, the newly synthesized DNA in ENNG- and EtMes-treated cells had molecular weights of 1.6 × 10^8 and 2 × 10^8, respectively. These results indicate that, after MNNG treatment, VR-43 cells exhibited an enhanced rate of postreplication repair relative to the wild-type V79 cells, although eventually a similar weight average molecular weight was observed. No such enhanced rates in DNA repair were observed after treatment with ENNG or EtMes.

To determine whether similar differences could also be observed in excision repair processes, in both cell types we measured the elimination of DNA adduct after treatment for 3 hr with 1 μg of [*methyl*-^{14}C]MNNG per ml. After 2 days, about 50% of the adducts were removed from the DNA of both cell types, as estimated from the ratio between the ^{14}C-labeled adduct and [^{3}H]thymidine-labeled parental DNA (unpublished observation). These results suggest to us that the enhanced cell survival and mutagenicity at equicytotoxic doses in the VR-43 cells are presumably associated with enhanced postreplication repair and not merely with a change in the overall excision of methylated DNA, although we cannot rule out differences in the excision of specific DNA adducts (35).

DISCUSSION

For specific DNA repair processes to be linked with mutagenesis and cell recovery in mammalian cells, there is a need for a variety of DNA repair mutants in addition to the few now known—e.g., xeroderma pigmentosum cells (13–17). Of interest are those mutants that exhibit an enhanced repair after carcinogen/mutagen insult. Our present isolated VR-43 cells express characteristics of such mutants, as shown by the fact that after treatment with MNNG but not with ENNG or EtMes these cells exhibited an enhanced postreplication repair compared with the wild-type V79 cells (Fig. 4). After treatment with MNNG and MNU, they also yielded, on a dose basis, increased cell survival and reduced mutation frequencies for ouabain or 6-thioguanine resistance (Figs. 1 and 2). However, when analyzed at equicytotoxic doses, the VR-43 cells expressed higher mutation frequencies for the two independent genetic markers (Fig. 3). No difference in cell susceptibility to the cytotoxic or mutagenic effect was observed after treatment with ENNG, EtMes, or x-rays. Based on these experiments, we can propose that, after treatment of these and presumably other mammalian cells with some methylating agents, postreplication repair can cope with DNA lesions responsible for cytotoxicity and, to a lesser degree, with lesions responsible for mutagenicity.

The mechanisms underlying postreplication processes are not clear. Rupp and Howard-Flanders' model originally proposed for prokaryotes (36) suggests that unexcised lesions in parental DNA interrupt DNA chain growth, which then bypasses the damage site, leaving a gap opposite the damage that can be filled by a presumed error-free recombinant exchange (36) or by other mechanisms including the error-prone *de novo* synthesis pathway (37–41). We do not know the mechanism

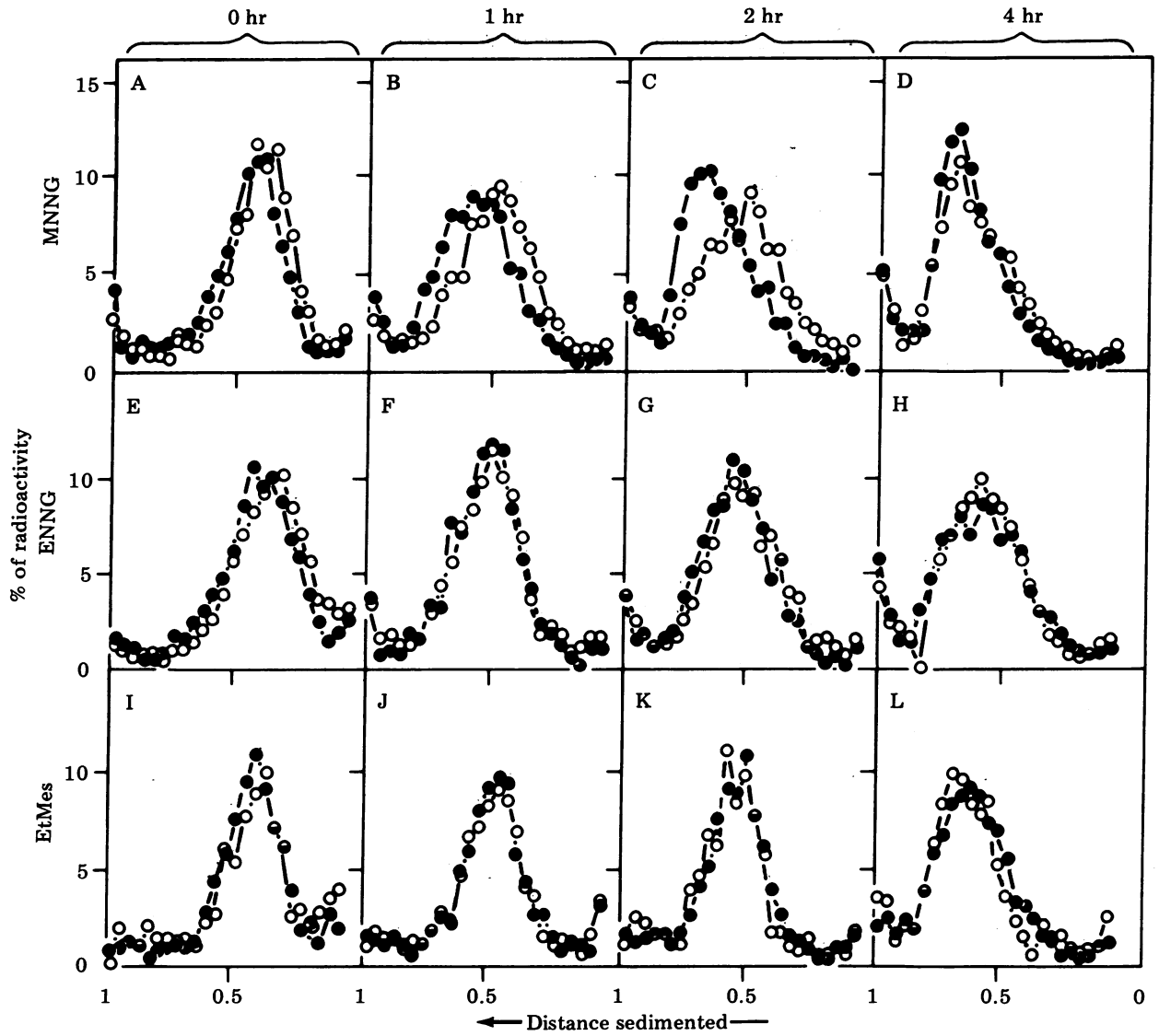

FIG. 4. Alkaline sucrose gradient profiles of DNA of V79 (○) and VR-43 (●) cells treated with 2 μg of MNNG (*A–D*) or 40 μg of ENNG (*E–H*) per ml for 20 min or 8 mg of EtMes (*I–L*) per ml for 60 min. V79 and VR-43 cells were pulse-labeled for 25 min with [^{14}C]thymidine and [^{3}H]thymidine, respectively. Then the cells were chased with unlabeled thymidine and deoxycytidine for 0, 1, 2, and 4 hr. The cells were centrifuged at 25,000 rpm for 120 min in an SW 50.1 rotor. After each chase, the molecular weight of the newly synthesized DNA from V79 cells treated with MNNG was 0.7, 1.2, 1.4, and 2×10^8; from VR-43 cells it was 0.8, 1.7, 2, and 2×10^8. With ENNG the molecular weights of the DNA from both cell types were 0.7, 1.3, 1.4, and 1.6×10^8; after EtMes treatment they were 0.7, 1, 1.4, and 2×10^8. The molecular weight of the DNA in the control was 1.5×10^8 at 0 hr and 3.4×10^8 after 1, 2, and 4 hr.

whereby enhanced postreplication repair is associated with enhanced cell survival and mutagenesis at equicytotoxic doses in these cells. In bacteria, MNNG can induce an adaptive response characterized by enhanced cell survival but, unlike the SOS repair (37, 38), this enhanced cell survival is characterized by a dramatic reduction in mutagenesis (42, 43). Our VR-43 cells presumably are not locked in such an adaptive state because in contrast to the adaptive bacteria, they are highly susceptible to the mutagenic effect of MNNG. There is a possibility that our VR-43 cells may have an altered DNA polymerase or poofreading exonuclease that poorly discriminates methylated from unmethylated bases. In such a situation, replication of methylated DNA goes on with less interruption but with a reduced fidelity.

More knowledge on the linkage between the various types of DNA repair processes, cell survival, and mutagenesis after mutagen/carcinogen insult in these and other cell types (26, 44–46) may help our understanding of the mechanism of mutagenesis and perhaps carcinogenesis.

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