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Genetics

Measurement of mutagenesis in mammalian cells

(environmental screening/carcinogenesis/genetic disease)

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ABSTRACT **A method using mammalian cells *in vitro* for detection and quantitation of mutagenic actions that appears to be useful for screening for carcinogenesis and genetic damage by environmental agents is presented. The method involves use of stable human–Chinese hamster ovary hybrid cells that have retained a single human chromosome not necessary for cell reproduction. Forward mutations are detected in genes necessary for production of specific human cell surface antigens. Such mutants form colonies in the presence of specific antisera and complement that destroy the unmutagenized cells. Use of the method is illustrated for the action of x-irradiation, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and caffeine. The method appears to be unique in that it permits assessment of lesions that cause loss of all or most of the chromosome as well as various localized gene mutations. The former action is particularly important because of the major involvement of chromosomal lesions in an extremely important class of human genetic disease.**

A convincing body of evidence has now accumulated demonstrating that many cases of cancer are caused by environmental agents (1, 2). It has also become clear that many if not most of the external agents that cause malignancy do so by production of changes in the genome of the affected somatic cells (2–4). As a result of these two advances, various systems have been proposed for use in screening environmental agents. These offer promise for monitoring the environment to decrease harmful exposure in human populations. The work of Ames in calling attention to the need for such monitoring systems and in providing one that is simple and effective is especially noteworthy (5) although various other tests have also been proposed (6–12).

All of the *in vitro* mutagenesis tests known to us emphasize measurement of single gene mutations in specific biological systems (13). Human cells, however, are prone to chromosomal defects such as aneuploidy breaks, and translocations. Such lesions could be missed in any screening test that utilizes marker genes carried on chromosomes that also contain large numbers of other genes needed for reproduction. Multigene deletions or complete loss of such chromosomes would cause failure of the affected cell to form a colony and would, therefore, not be recorded.

Chromosomal errors are characteristic of human neoplasia. Most malignancies are known to be aneuploid (14); conditions such as chronic myelogenous leukemia have been shown to be associated with a specific deletion and subsequent translocation (15); and the high incidence of malignancy in patients with trisomy 21 is well known. Moreover, the problem involved in such chromosomal anomalies has great importance for human disease in addition to cancer. The single most immediate cause of newly produced (not inherited) genetic disease in man is chromosomal damage, of which loss of all or a significant portion of a chromosome may be the most important event. Loss of a portion of a specific chromosome may be followed by secondary translocations that can cause further problems. Such chromosomal abnormalities produce serious disease in approximately 0.5–1% of all live human births and a significant proportion of fetal wastage through spontaneous abortion (16). Epidemiological evidence has demonstrated a high probability that chromosomal nondisjunction is strongly influenced by environmental agents (17) and experimental studies *in vitro* have shown that nondisjunction can be caused in mammalian cells by agents not in themselves known to be mutagenic at the single gene level (18). It follows that a screening test capable of detecting chromosomal loss and deletions as well as single gene mutations is necessary and that such a test may help prevent exposure to agents important in both cancer and genetic disease. Such a procedure is described here.

MATERIALS AND METHODS

Cells. Cells of the A_L hybrid, formed by fusion of human amniotic fluid fibroblasts and the *gly*$^-$*A* mutant of the Chinese hamster ovary CHO-K1 cell, were used. Their preparation and their properties have been described (19–24). This cell contains the standard chromosomes of the CHO-K1 cell plus human chromosome 11 on which have been identified and regionally mapped genes for cell surface antigens a_1, a_2, and a_3 and the gene for lactic dehydrogenase A (*LDH-A*). The a_1, a_2, and a_3 loci cause the formation of specific cell surface antigens that render the cell sensitive to killing by different specific antisera in the presence of complement. Fig. 1 presents the mapping information so far available. Of particular importance is the fact that markers have been identified on both chromosomal arms. The subclone used in these experiments has been in continuous culture for 5 years without apparent change in phenotype. Cells were cultivated in medium F-12 (25) supplemented with 8% fetal calf serum (FC8).

Serological Reagents. Rabbit antiserum prepared against human erythrocytes as described (24) contains activity against a_1 but not against a_2 or a_3 antigen. Antiserum active against the a_2 but not the a_1 antigen is prepared by exhaustive absorption, with human erythrocytes, of rabbit antiserum prepared against HeLa cells (24). Antiserum from sheep immunized with human erythrocytes was used as a source of antibodies against a_1 and a_3; it had no appreciable activity against a_2 (24). Normal rabbit serum was used as a source of complement. All complement preparations were tested before use by a described procedure (19, 24). Those displaying nonspecific toxicity (i.e., greater than 20% killing in a concentration of 2%) were rejected.

Treatment with Suspected Mutagens. This paper describes studies on the effects of x-irradiation, *N*-methyl-*N'*-nitro-*N*-

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Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; D_0, mean lethal dose.

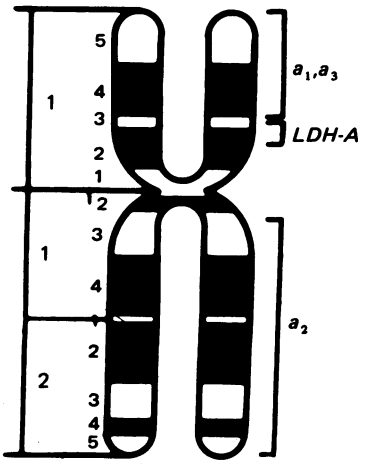

FIG. 1. Schematic representation of human chromosome 11, showing the location of the human genes utilized in this study.

nitrosoguanidine (MNNG; Aldrich), and caffeine (Sigma). A single-cell survival curve was determined for each agent under exactly the same conditions as when the agent was tested for mutagenesis. In the case of x-irradiation, cells were exposed to 230-kv x-rays [84 rad/min (1 rad = 0.01 gray)] (26), at either 37°C or room temperature; there was no detectable difference in the survival curves or mutation frequencies obtained at the two temperatures. Scoring of surviving colonies and determination of the mean lethal dose (D_0) were carried out as described (27). The values of D_0 and the mutational frequencies observed were constant regardless of whether the radiation was delivered to cells attached to a plastic surface or contained in liquid suspension.

For each dose of radiation, the number of exposed cells was adjusted to produce approximately 10^4 survivors per dish after treatment as calculated from the x-ray survival curve. For determination of mutagenesis, three separate tubes containing 10^6 cells per ml were irradiated and aliquots calculated to contain 10^4 surviving cells were removed and inoculated in replicate into 60-mm petri dishes.

Experiments measuring mutational effectiveness of chemical agents were carried out in similar fashion. Cells were exposed on plates, in at least quadruplicate, to selected concentrations of chemical agents for periods of 16–20 hr at 37°C. The plates were rinsed, fresh medium was added, and cell survival and specific mutants were determined as described (27).

Scoring of Mutants That Have Lost the a_1^+ Marker. It is necessary to permit surviving cells to recover from the temporary growth lag produced by agents such as radiation and to multiply to the point at which the progeny of the mutated cells no longer contain lethal amounts of the surface antigen. If the challenge with antibody is made too early, mutants will be killed. If made too late, colonies of unmutated cells may not be completely destroyed by the antibody/complement mixture. Therefore, after mutagenesis the cells were incubated in growth medium for 8–14 days to permit restitution of growth and expression of the induced mutations. During this period the cells were subdivided as often as necessary. New plates were seeded with approximately 2×10^5 cells, a number large enough to minimize possible distorting effects that could result from random fluctuations in the number of mutants present in small inocula (28). Cells from each mutagenesis vessel were kept in separate dishes during subculture and subsequent challenge with antiserum. At the end of this growth period, aliquots were challenged with appropriate antiserum and complement and the ratio of resistant to sensitive forms was determined. This procedure assumes that, on the average, the growth of antibody-sensitive and -resistant mutants will be equal.

The cell cultures to be challenged were gently trypsinized, rinsed with 0.025% trypsin at room temperature, and resuspended in growth medium; their number was determined in a particle counter. Aliquots containing 5.0×10^4 cells were removed from each population and dispensed into each of five 60-mm dishes in a total of 2.5 ml of growth medium. The dishes were incubated for 4 hr to allow cell attachment to the surface and recovery from trypsinization, after which 0.2% anti-a_1 antiserum and 2% of freshly thawed complement were added. All operations were carried out at 37°C and the plates were incubated for 6–8 days, after which they were fixed, stained, and scored for surviving colonies. Controls included identical sets of plates with appropriate numbers of cells containing antiserum alone, complement alone, or neither agent. No diminution in plating efficiency was ever observed in the antiserum alone. Occasionally, small reductions were observed in the plates with complement alone, in which case appropriate corrections were made in the colony counts. The ratio of the number of colonies appearing on the plate to the total number of cells plated, corrected for any nonspecific killing due to complement alone, was taken as the mutation frequency resulting from the given treatment. A sample set of results is presented in Table 1.

To confirm that each of the scored colonies had indeed lost the a_1^+ phenotype, randomly chosen colonies were picked from each challenged plate, grown up to approximately 10^6 cells in standard growth medium, and retested by inoculation of 300 cells per dish in triplicate together with the appropriate antiserum plus complement. Control plates without complement and with complement alone were also prepared, and colonies were counted in both test and control plates after standard incubation. The efficiency of recovery of mutants was measured by reconstruction experiments in which cells of mutant phenotype $a_1^-a_2^+a_3^+$ or $a_1^-a_2^+a_3^-$ were added to a wild-type population in known proportions. The resulting mixture was then challenged with anti-a_1 antiserum plus complement under standard conditions. In such experiments, the mean (± SEM) number of resistant mutant colonies recovered was 82 ± 8% of the theoretical value.

The presence or absence of human lactate dehydrogenase A (EC 1.1.1.27) was determined by polyacrylamide gel electrophoresis as described (21).

Because the markers studied include genes on both arms of the chromosome, the complete absence of all four markers was taken as an index of loss of all or most of chromosome 11 (19, 20). This criterion appears to be a better indication of the loss of all or most of chromosome 11 than is cytogenetic analysis because the latter could fail to register the presence of small portions of chromosome 11 translocated to Chinese hamster chromosomes.

RESULTS

Survival Curves. The single-cell survival curves for A_L hybrid cells exposed to graded doses of the agents studied are presented in Fig. 2. D_0 and extrapolation number, as previously defined (27), calculated from these curves are presented in Table 2.

Yield of Mutants. Fig. 3 shows the yield of a_1^- mutants obtained with each agent. The averaged results of six or more independent experiments with each agent in which the frequency of a_1^- mutants was determined for a series of doses are presented. The doses are expressed in terms of D_0 units, a convenient expression of the number of cell lethal events which

Table 1. Results obtained from experiments measuring the fraction of a_1^- mutants in the surviving populations after exposure to graded doses of x-rays

Dose, rads	Surviving colony-forming cells tested,* no. × 10^{-6}	Plates scored, no.	a_1^- clones counted, no.	a_1^- mutants per 10^4 survivors†
0	4.1	126	3,047	7.5 ± 2.8
150	2.6	80	6,422	24.7 ± 4.2
300	3.5	180	15,060	42.9 ± 9.4
450	1.4	42	6,714	49.2 ± 8.5
600	0.78	24	5,124	65.7 ± 6.2

* With antiserum and complement.
† Mean ± SEM.

permits comparison between the different agents used (27). Each point in each experiment was determined at least in quadruplicate. The results of the separate experiments for each agent were in excellent agreement, and the averaged standard deviation for each treatment point on each curve was 16.5% of the mean although the standard deviation of the untreated samples tended to be higher. The number of plates scored for a_1^- mutants for each treatment dose ranged from 24 to >120. Several thousand surviving clones were counted at each dose for each agent.

In order to check that the colonies surviving the challenge with a mutagenic agent were truly a_1^-, 144 random colonies were picked—52 from unirradiated and 92 from x-irradiated plates. These colonies were grown into new cultures and their sensitivity was measured under standard challenge conditions with anti-a_1 antiserum and complement. None of these 144 clones was killed under conditions that routinely killed 100% of all a_1^+ cells. Similar results were obtained for mutants obtained after treatment with MNNG.

The data of Fig. 3 reveal that, when compared for mutagenicity at the a_1 locus, MNNG is a powerful mutagenic agent in lethal dose units; x-rays are demonstrably less effective, and caffeine reveals no mutagenicity. In the case of the first two agents, mutagenicity is clearly demonstrable at doses less than 1 D_0; for the third agent, mutagenicity is absent even at 4-fold higher values. Values for D_0 calculated from the slopes of the linear part of each curve are shown in Table 2.

Test for Induced Loss of All or Most of Chromosome 11. The a_1^- mutant clones can be picked and analyzed for retention of each of the other three markers, and the frequency of each combination of marker loss can be determined exactly. This procedure is lengthy, especially because at particular doses some of the possible phenotypes are relatively rare so that it would be necessary to analyze large numbers of clones to secure meaningful values. However, a faster proximate analysis can be carried out which yields a measure of those mutational events that cause loss of all or most of chromosome 11 as opposed to those that produce more localized changes in the genome.

We therefore resolved the a_1^- mutants into two classes: (*i*) mutations that cause loss of a_1^+ but retain at least one of the other three markers (called "limited marker loss" mutations); and (*ii*) mutagenic events causing loss of all four markers. Because they include markers on both chromosome arms, these latter events are associated with loss of all or most of chromosome 11.

A representative exposure for each of the agents was selected and the a_1^- mutants resulting were identified, picked, and grown into new cultures that were tested for the presence of the three other markers. The dose used for each agent was selected to be sufficiently low so as to lie in the linear region of the mutant yield curve of Fig. 3 but high enough to produce sufficient a_1^- mutants for a satisfactory analysis. The values chosen for x-irradiation and MNNG were D/D_0 = 2.6 and 1.0, respectively.

The results shown in Table 3 reveal different patterns in the mutagenic actions of x-rays and MNNG: the mutation frequency for the loss of the a_1 locus alone was about 2%, with the former and approximately 28% with the latter. Similarly, a much higher fraction of the a_1^- forms from x-irradiation lost all or most of chromosome 11 than was the case with MNNG.

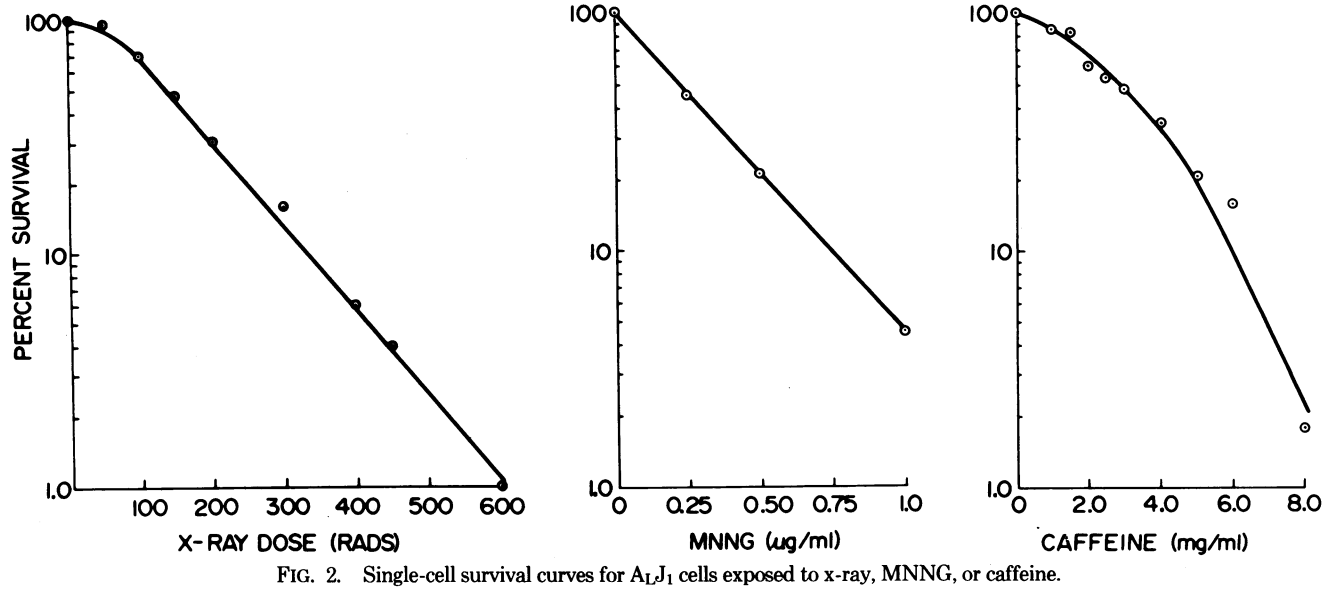

FIG. 2. Single-cell survival curves for A_1J_1 cells exposed to x-ray, MNNG, or caffeine.

Table 2. Survival parameters for A_L cells and production of a_1^- mutants as a function of D_0

Agent	Extrapolation number*	D_0	Mutants/D_0/ 10^4 survivors†
X-ray	1.8	116 rads	11
MNNG	1.0	0.3 μg/ml	66
Caffeine	9.5	1.3 mg/ml	0

* From Fig. 2.
† From Fig. 3, slope of linear part of each curve.

If only point mutations were scored, it would be difficult to establish the mutagenic action of x-radiation. In the experiments summarized in Table 3, the limited marker loss for x-irradiation was 66%, in contrast to 93% for MNNG; the proportion of total marker loss was 34% for the former and 7% for the latter.

The relative fractions of the different patterns of marker loss in Table 3 are shown as absolute mutation frequencies in Table 4 (by combining these data with the mutation yields derived from Fig. 3).

These data show how loss of all or most of chromosome 11 and loss of specific markers can be measured quantitatively. They also reveal the different patterns of action of the mutagens used. When expressed in terms of number of mutants per 10^4 cells per D_0, MNNG was 8 times more potent than x-rays in causing limited marker loss but roughly similar in its ability to cause loss of all or most of the chromosome. Caffeine, shown to be a mutagen in bacteria (29), was without measurable effect in our mammalian cells, a result that had been demonstrated before (27, 30–32).

Table 4 demonstrates that most of the lesions produced by both x-rays and MNNG appear to involve appreciably large portions of the chromosome, so that a test that could only reveal point mutations would fail to register most of the genetically damaging events. Indeed, as noted above, in the case of x-irradiation, only 2% of a_1^- mutants revealed loss in no other of the markers studied here, and a considerable fraction of these might still be multigene deletions.

DISCUSSION

This study indicates how deletions and losses of an entire chromosome can be detected by the present approach, along with more localized genetic events. Because deletions and nondisjunction are processes that appear frequently in human disease and because nondisjunction can be caused by agents, such as Colcemid, that distort mitosis by weakening the microtubular structure (18), the ability to detect such effects of environmental agents would be a promising tool in preventive medicine. This technique also appears adaptable to the detection of agents that may inhibit repair of genetic damage. Used in conjunction with a standard mutagen, such agents may increase the yield of genetic lesions. Caffeine appears to be such an agent (33) and, although it may be innocuous by itself, its use may well be ill-advised in situations in which exposure to otherwise tolerable mutagenic action cannot be avoided.

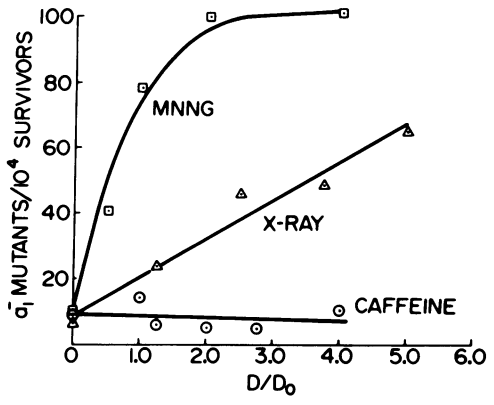

FIG. 3. Production of a_1^- mutants by MNNG, x-ray, and caffeine.

Table 3. Frequency of occurrence of different subclasses of a_1^- clones obtained spontaneously and from treatment with x-irradiation or MNNG

Markers lost	Untreated control No.	%	X-ray* No.	%	MNNG† No.	%
Only a_1	13	16	1	2	8	28
a_1 and up to 2 others	44	56	30	64	19	65
All 4	22	28	16	34	2	7
Total	79	100	47	100	29	100

The sum of the values of the first two lines corresponds to the class of limited marker loss; the third line is the class of complete marker loss. The spontaneous mutants were much more rare and were collected from a large series of experiments.
* $D/D_0 = 2.6$.
† $D/D_0 = 1.0$.

The method described here should be expected to provide a higher mutagenic yield than produced by methods that measure only highly localized events. Although it is difficult to compare mutant yields reported by other laboratories because of the differences in the cells used and in conditions of exposure, the current method appears to yield mutation efficiencies for x-rays that are 20–50 times greater than those reported previously (27, 34–37).

The ability to resolve mutagenic events into different processes may help define the mode of action of different mutagens. As more genes are identified on the marker chromosome, the resolving power will increase. Other hybrids containing different human chromosomes (20) are available for use in this approach.

In its present form, complete analysis of a single agent requires approximately 5 weeks. However, only a fraction of a laboratory worker's time is required during this interval, so that a single person could analyze quantitatively the mutagenicity of 5–10 agents during this period. It is hoped that, with increasing experience, techniques will be found to shorten the time required.

The method described here utilizes the following principles for monitoring of environmental agents. (*i*) Mutagenesis is assayed by scoring marker genes on a chromosome that is unnecessary for cell division. Therefore, genetic damages like the production of large deletions are counted; these would be missed if the chromosome carrying the scored genes contained many loci vital for reproduction. (*ii*) Human genes contained

Table 4. Net frequency of mutants resulting from exposure to x-rays, MNNG, or caffeine*

Mutants	No. mutants/10^4 surviving cells/D_0 X-ray	MNNG	Caffeine
All a_1^-	11.1	68	0
Limited marker loss	8.1	66	0
Complete marker loss	3	2	0

The average frequency of the appropriate class of mutant obtained from unexposed controls was subtracted from the value for the yield for each agent.
* D/D_0: x-ray, 2.6; MNNG, 1.0; caffeine, 1–4.

on a human chromosome are scored, so that the results conceivably might be more relevant to human situations than when cells from other test organisms are used. (*iii*) Forward mutation to loss of gene function is measured, so that a defect anywhere within the genes under study will be detected.

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