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Genetics

Measurement of low levels of x-ray mutagenesis in relation to human disease

(mutagenesis measurement/lethal mutations/human chromosome 11/genetic disease/cancer)

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ABSTRACT **We previously demonstrated that conventional methods for measurement of mutagenesis in mammalian cells are subject to serious error that causes underestimation of environmental contributions to cancer and genetic disease. This error has been corrected by use of somatic cell hybrids containing a single human chromosome on which the marker genes are carried and by using doses of mutagenic agents so low that little cell killing occurs. This method permits direct measurement of the effects of low doses of radiation and other mutagens without resort to the controversial extrapolation procedure customarily used to estimate effects of doses in the neighborhood of actual human exposures. The new data demonstrate that the true mutagenesis efficiency at the low doses of ionizing radiation that approximate human exposures is more than 200 times greater than those obtained with conventional methods. This methodology also permits evaluation of localized mutations, large and small chromosomal deletions, and nondisjunctional processes and can be used for mutagens that need metabolic activation as well as for cooperatively acting agents. The two opposing classical views that in mammalian cells extrapolation to low doses of x-radiation is linear, on the one hand, or involves a threshold, on the other, are both demonstrated to be incorrect at least for the conditions here considered. The actual curve exhibits a downward concavity so that the mutational efficiency is maximal at low doses. These data may have important implications for human health.**

Diseases due to mutation in germ and somatic cells are now recognized to contribute substantially to the burden of human illness. Effective means to evaluate the magnitude of this problem and to control or ameliorate its effects require the ability to measure accurately the nature and amount of mutagenesis produced in the mammalian genome by exposure to various doses of any agent. A comprehensive program should include accurate assessment of effects of background radiation and spontaneous mutagen production in the body under conditions presumably normal, as well as the influence of environmental mutagens.

A variety of different approaches to measurement of genetic insults has been proposed (refs. 1–5, for example). Perhaps the most widely used is the pioneering method of Ames *et al.* (6, 7), which measures the fraction of the surviving bacterial population that has been mutated in the histidine locus. Various adaptations of this procedure have been developed for use with mammalian cells in culture (1–5). In earlier publications we demonstrated that use of such procedures in mammalian cells can produce serious errors in quantitative assessment of the amount of mutagenesis that has occurred, because of the effect of cell killing (8–11). Some of these considerations have more recently also been discussed by others (12, 13). The most important deficiencies of this approach are as follows:

(*i*) Extensive cell killing usually occurs in the test cell population. We demonstrated earlier that mammalian cells are enormously more sensitive than bacteria to killing by mutagens such as x-rays and we and others have shown that much or most of the killing exhibited by at least some mutagenic agents is due to lethal mutations (8–16). Regardless of whether these are single or cumulative, they must be taken into account when evaluating the capacity of any agent to cause genomic damage.

(*ii*) In every application of the mutagenic procedure known to us the test genetic marker is contained on a chromosome that also contains many other genes needed for cell reproduction. Therefore, although the method may be suitable for detection of point mutations, it would appear seriously to discriminate against deletions and against nondisjunctional processes in which a pair of cells is produced, one member of which has an extra chromosome and the other a missing chromosome. Since the latter often constitutes a lethal situation, whereas the former would still contain the original marker, this kind of genetic event would fail to be perceived. Nondisjunction and deletion are associated with very important classes of human disease (17).

(*iii*) The standard procedure as used with mammalian cells has been employed most often to measure actions due to relatively large, acute doses of mutagens [600–2000 rads (1 rad = 0.01 gray) of x-rays, for example], which represent relatively rare human exposures. Assessment of the effects of low doses, which are the common human experience, is then usually carried out by means of extrapolation, the validity of which has been debated for decades (18). In the present, still primitive state of knowledge, it would appear essential not only to be able to measure accurately the total number of mutational events of all kinds resulting from exposure to a given situation but also to resolve this total into the individual types of genetic lesions that are important in human disease. Such information would seem important not only in achieving measures to counteract the biological effects of each type of genetic insult but also to secure more penetrating theoretical understanding of the molecular processes involved in mutation, repair, reproductive lag, and metabolic consequences. It is obvious that realistic evaluation of the effects of mutagenesis on human disease requires much more sensitive measurements if one is to rely on direct laboratory measurements rather than on the incredibly more difficult and tragic epidemiological data to evaluate environmental risks. The well-known history of events in the asbestos industry illustrates this situation.

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Abbreviations: CHO, Chinese hamster ovary; B[*a*]P, benzo[*a*]pyrene; D_0, mean lethal dose; MNNG, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine; EtMes, ethyl methanesulfonate; HGPRT, hypoxanthine (guanine) phosphoribosyltransferase.

Effective disease prevention would appear to require direct access to the low-dose range in order to be able to screen effectively for agents that can prevent the consequences of exposure to mutagens. If it should turn out that appreciable amounts of human disease indeed result from exposures to low levels of mutagens, the search for antimutagens must be conducted at these very levels. It may well be difficult or impossible to achieve, by methods compatible with health, sufficiently high concentrations within the cell of agents needed to counteract the genetic insult of the high doses of mutagens that are most frequently measured by standard tests.

In 1979 (8) we proposed an approach for detection of mutagenesis and described measurements designed to begin correction of the deficiencies of current methodologies. The ultimate goal of these studies is to develop a system that (*i*) removes the distortion due to cell killing in mammalian cell mutagenesis measurements, (*ii*) permits direct measurement at low doses of each mutagen so that it becomes unnecessary to resort to controversial extrapolations that have plagued this field for decades, (*iii*) permits assessment of each of the different kinds of genetic changes known to be associated with significant human disease, (*iv*) allows more effective screening for antimutagens useful in preventing human disease, and (*v*) offers rapid, convenient, and accurate experimental procedures for achievement of these goals. The present paper demonstrates recent developments in this program. Most of the experiments described here involve use of x-rays as a convenient, model, mutational agent.

MATERIALS AND METHODS

The A_L-J1 hybrid used in these experiments contains a standard set of CHO-K1 chromosomes (CHO, Chinese hamster ovary) and human chromosome number 11 as described (8, 19, 20). The a_1 antigenic marker (also referred to as SA11-1 and S1) maps to the short arm at 11p13; the a_2 marker is on the long arm of chromosome 11 (20). Complement, as normal rabbit serum, was obtained from Dutchland Laboratory (Denver, PA). Lethal polyclonal and monoclonal antibodies against the a_1 marker and polyclonal antibodies against a_2 and a_3 were prepared as described (19, 21). The growth medium was F12 (22) supplemented with 5–8% fetal calf serum. Chemical mutagenesis was carried out by means of a standard 16-hr exposure procedure; x-irradiation was delivered at 230 kV at 84 rad/min at room temperature; UV-irradiation was carried out by exposure of cells suspended in growth medium to a germicidal lamp delivering predominately 253.7-nm light at a measured dose rate of ≈0.3 J/m^2 per sec (23). The S9 preparation of microsomes (Litton Bionetics) was obtained from araclor-induced rats and was used according to the activation protocol for benzopyrene (B[*a*]P) of Machanoff *et al.* (24). The dose of mutagen is expressed in absolute units and also in terms of D_0, the mean lethal dose. The latter unit provides a standard for comparison of mutagenesis by physical or chemical agents over a wide range of doses and experimental conditions (23).

Determination of the fraction of mutants in a surviving population was carried out by our standard procedure in which complement and antiserum were added in concentrations sufficient to kill the cells retaining the surface antigenic marker (8). Briefly, 5×10^4 A_L-J1 cells were inoculated into 2.5 ml of growth medium in 60-mm dishes and incubated ≈4 hr for attachment, at which time antiserum and complement, 0.2% and 2.0%, respectively, were added and incubation was resumed for ≈7 days for colony formation. Testing confirmed that at least 99% of the colonies that arise represent mutants. Appropriate controls were included to correct for any killing caused solely by antiserum alone or complement alone. The frequency of mutants is expressed as number of mutants per 10^5 survivors by using the plates that received only complement to normalize for any cell killing due to complement alone. The background mutation frequency in the cultures selected initially was in the range of about $1–10 \times 10^{-5}$ but rose at a rate of 1.5×10^{-6} per cell per generation with continued cultivation to a value around 10^{-3}, at which point it remained constant. After mutagenesis, populations were subcultured for 5–21 days.

B[*a*]P was obtained from Aldrich Chem (Metuchen, NJ); *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), ethyl methanesulfonate (EtMes), and 6-thioguanine, from Sigma.

RESULTS

Dynamic Considerations. Our previous results (8–10) demonstrated that when marker genes are contained on a chromosome unnecessary for cell reproduction, an increased mutational yield is obtained. This comes about because hits in such marker genes are less likely to be lost through cell death than if the marker genes are contained in a chromosome that also contains many other genes necessary for reproduction.

This principle has been retained in the current experiments. However, with increasing dose, eventually a point will be reached at which damage to the diploid CHO chromosomes becomes sufficiently great so that cells in which mutations on the human chromosomes have lodged will be killed and fail to be scored as mutants. This effect will increase with dose. However, if meaningful measurements can be secured at doses so low as to avoid appreciable cell killing, the results obtained should be free from distortions occasioned by the killing process. Fig. 1 presents the survival curve for the A_L hybrid used in these studies. It is apparent that at doses of <100 rads cell killing is reduced so that below this dose range the desired conditions should be approximated.

Experimental Results. *Measurements at low doses of x-radiation.* Experiments were carried out to determine whether mutagenesis could reliably be measured in doses of <100 rads. The data are shown in Table 1. They demonstrate unequivocal mutagenesis for doses as low as 25 rads. These data were obtained with a single marker, a_1.

Comparison of extended curves for mutagenesis as a function of x-ray dose obtained by conventional method and by our approach. Prediction can be made about the shape of the curve to be expected as one extends the mutagenesis measurements to higher doses. Referring to Fig. 1, the

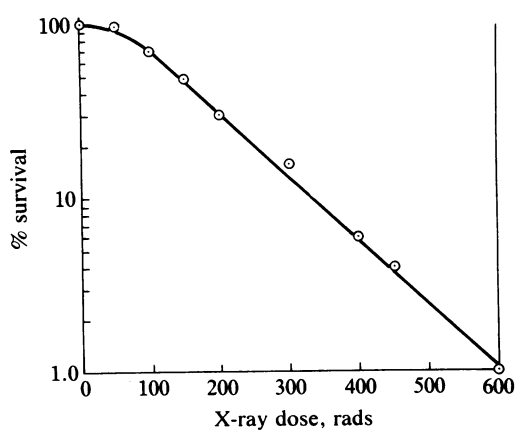

FIG. 1. Single cell survival curve of the A_L hybrid treated with single acute doses of x-irradiation. It is apparent that at doses of 600 rads or more, 99% or more of the cell population is killed, a huge number compared to the number of mutants scored. The initial shoulder in the curve represents a region in which cell killing is minimal.

Table 1. Yield of total a_1 mutants in a population of A_L-J1 hybrid cells exposed to x-radiation in the doses shown

Dose, rads	Average number of a_1 mutants per 10^5 survivors
0	116 ± 50
25	217 ± 90
30	260 ± 80
42	264 ± 70
50	272 ± 120
100	332 ± 150
150	374 ± 140

At least 20 plates, each containing 5×10^4 cells, were exposed to each dose and tested with antiserum and complement. Data are presented as mean ± SEM.

survival curve of the A_L hybrid, an initial shoulder is followed by an exponential fall in the number of survivors. A certain fraction of the killed cells will also have incurred mutation among the markers of the human chromosome. Therefore, their contribution to the total mutagenesis score will be lost. We would predict that with increasing dose the mutagenesis obtained should fall off as increasing cell killing causes failure to record mutagenic events in the marker genes. In Fig. 2 are summarized the combined results of all experiments carried out by our hybrid method using the a_1 marker (upper curve). These are compared with results reported by Hsie *et al.* (25), who used the conventional method in which loss of the Chinese hamster hypoxanthine (guanine) phosphoribosyltransferase (*HGPRT*) gene was scored in x-irradiated CHO cells (25, 26) (lower curve). Background mutation frequencies have been subtracted in both cases. The data of the top curve indicate that the predicted behavior is obtained. With increasing dose, cell death, which can presumably result from damage to any of several CHO chromosomes, outstrips the specific mutations being scored on the human chromosome. The resulting curve bends toward the horizontal as the dose is increased over the range considered so that the maximum mutational efficiency occurs at the lowest doses.

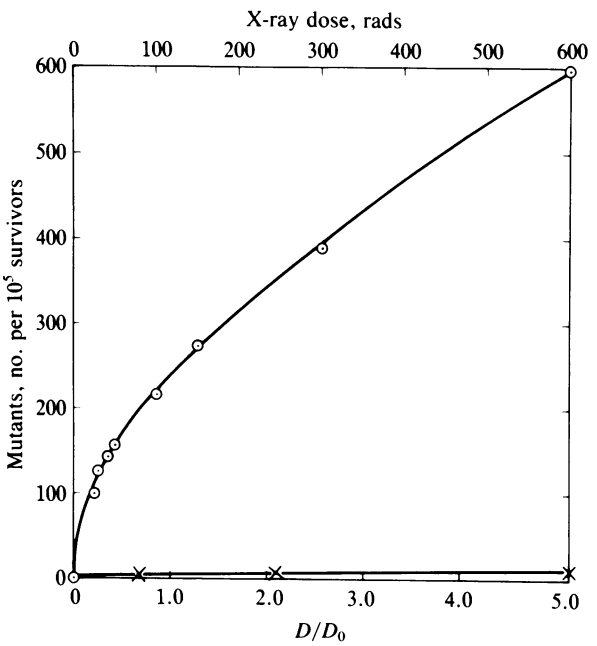

FIG. 2. Upper curve: the mutant yield obtained in our laboratory for loss of the human a_1 gene in x-irradiated A_L-J1 hybrid cells for all doses studied. The slope is greatest at the lowest doses (0.5 D_0 values). The average slope between the doses of 0 and 55 rad is 3.45 mutants per 10^5 survivors per rad, or 400 mutants per 10^5 survivors per D_0. The value of D_0 is 116 rads. Lower curve: recalculated data of Hsie *et al.* (25, 26) for mutant yield for loss of the *HGPRT* gene in CHO cells after x-irradiation with doses of 50–800 rads (0.35–5.7 D_0). The straight line fit to these data has a slope of 0.01 mutant per 10^5 survivors per rad (2 mutants per 10^5 survivors per D_0 when D_0 is estimated to be 140 rads). Thus, the maximum mutant yield for the data in the upper curve is ≈200 times greater than that in the lower.

Comparison of this behavior with that obtained by the conventional HGPRT methodology reveals that (*i*) the latter yields an apparent straight line passing through the origin for the x-ray mutation yield, (*ii*) most of the experimental points lie in the region of significant cell killing, and (*iii*) the slope of the straight line obtained is far less than that of any part of the upper curve and is less by a factor of at least 200 than the limiting slope achieved in the neighborhood of 0–50 rads. The data of Hsie *et al.* (25) and of Tindall and Hsie (26) were selected for this comparison because they were obtained in a system that is well defined and widely employed and they are reasonably representative of reported values. Examples of mutant yields for x-rays (mutants per 10^5 survivors per rad) reported in the literature are 0.0001 in cultured human fibroblasts (27), 0.0069 in human lymphoblasts (28), 0.042 in hamster V79 cells (29), 0.01 for CHO/HPRT (25, 26), 0.1 in pSV2qpt-transformed CHO (26), as opposed to 3.45 in A_L-J1 here described. Values for radiation-induced mutations in other systems are presented in ref. 30.

Preliminary studies with other mutagens. Fig. 3 presents data obtained with a chemical mutagen requiring activation in order to be effective. The mutant score obtained with the use of the carcinogen B[*a*]P alone is contrasted with the results obtained when a microsomal preparation is added to the cell culture. Ames *et al.* (6) have demonstrated that microsomal activation of potential mutagens significantly increases the yield of mutations for compounds such as B[*a*]P.

Caffeine, an agent that is not mutagenic by itself in mammalian cells (8) and that has been postulated to prevent mutational repair (31), was found to double the mutagenic yield of x-rays on the a_1 marker in the A_L-J1 cell (data not shown).

A number of other mutagens has so far been tested and the numbers of mutants obtained have been compared with those resulting from conventional tests, which do not use markers on a chromosome unnecessary for cell reproduction. The

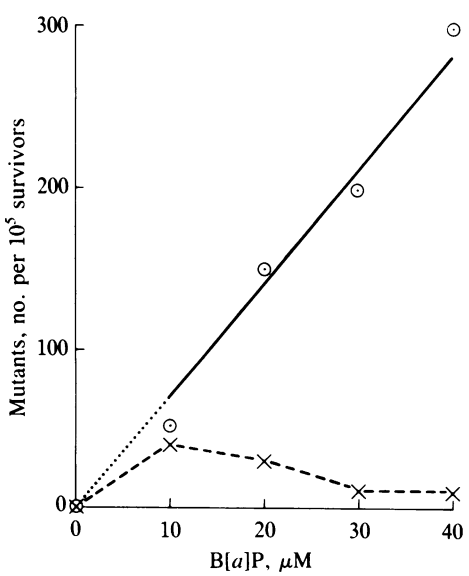

FIG. 3. Mutant yield obtained by the cell hybrid method for B[*a*]P exposures, without activation (lower curve) and with activation (upper curve). Dose is expressed in μM because the D_0 value so far obtained is only approximate.

results shown in Table 2 reveal that in every case a substantially greater yield is obtained by the test using the hybrid cell.

Analysis of the nature of the mutations produced. It is important to be able to ascertain the nature as well as the number of mutations produced by any agent, since in many cases the nature of the disease produced, the mechanism of the repair processes involved, and the measures required to prevent mutation may depend in an important fashion on the nature of the lesion. Previously we demonstrated (8–10) that our method lends itself to differential determination of the sizes of the lesions involved by use of multiple markers on the human chromosome. Loss of a single marker only represents a localized lesion; loss of two closely linked markers was taken to represent a small deletion; loss of poorly linked markers on the same chromosome arm represents a large deletion; loss of all of the markers, including markers on both chromosome arms, represents loss of all or most of the chromosome. The data of Table 3 show such a differential analysis based on lesion size for five agents used in this study. They demonstrate the wide range of differences in action among these agents, varying from 51% of large lesions for UV-irradiation to 98% for x-radiation and activated B[*a*]P. Some of these data were reported previously (8).

Table 2. Comparison of the mutational yield obtained for a series of agents using the hybrid methodology with those using standard procedures

Agent	D_0 value	No. of mutants per 10^5 surviving cells per D_0	
		A_L method	Standard method
X-ray	116 rads	400	2
UV	4 J/m^2	44	8
EtMes	0.8 mM	200	20
MNNG	0.2 μM	650	100
B[*a*]P + S9	$\approx$10 μM	65	15

A dose of 0.5 D_0 was employed for x-ray and MNNG; 2 D_0 was used for the other agents.

DISCUSSION

The present data demonstrate that the use of mammalian genetic markers carried on a chromosome unnecessary for cell reproduction increased the yield of mutants produced by a variety of different mutagens at doses corresponding to 1–3 D_0 values by factors varying between 4 to 22. In the case of x-rays, the measurements were also extended down to doses so low that cell killing becomes quite small. Under these conditions, a yield of mutagenesis >200 times greater than that reported by studies utilizing conventional measurements with mammalian cells was achieved. Extension of studies to very low doses with a variety of other mutagens is necessary.

Perhaps the chief significance of these results lies in the fact that the great bulk of human exposures lies in the range of very low doses. Most previous measurements of acute mutagenesis yields of x-rays have been made in the region of very high doses, such as 6–15 cell lethal doses. Such measurements have required extrapolation to the low-dose region for interpretation of magnitude of human health risks. The present data appear to resolve in unexpected fashion one of the classical controversies of mammalian genetics having to do with whether the extrapolation of mutation yield to very low doses of ionizing radiation would yield a straight line or a threshold in which the mutation efficiency drops toward zero at low doses.

In contrast to both of these expectations the actually observed mutational efficiency at low doses is considerably higher than that observed at higher doses. The reason for this discrepancy appears to lie in the heretofore neglected role of cell death, which eliminates from the scoring process cells that have developed or accumulated lethal mutational insults. These considerations require new examination of the effects of a wide variety of doses and dose rates in mammalian systems (34).

An important additional aspect of the ability to carry out measurements in the low-dose region of mutagen action is the resulting elimination of most of the mutagenesis-induced lag, which can introduce delays of large magnitudes before cells resume reproduction (14, 32).

The present approach makes possible separate evaluation of cell killing and mutagenesis over a wide range of exposure to mutagens. It may well permit new understanding of the action of mutagens during fetal development, especially during the first 3 months of pregnancy, which appear to be of such vital importance in the developmental process.

A hypothetical objection might be raised to our interpretation of these experiments to the effect that cells that we are using may be abnormally sensitive to mutagenesis, perhaps by lacking repair mechanisms. However, in earlier papers we demonstrated that cellular repair is active in these cells and, indeed, is responsible for the initial shoulder of the survival curve in Fig. 1 with respect to mutagenesis by agents as diverse as x-radiation, UV light, and certain chemical mutagens (31). Moreover, the fact that experiments on our cells using the *HGPRT* locus yield the same low values of mutagenesis reported by others confirms that our cells do exhibit standard mutagenesis and repair behavior.

Our system can be applied to measurement of the effects of agents that require activation and to combinations of agents. It also promises to yield more effective study of agents that affect repair processes since compounds can be screened for such actions in cell populations to which a standard dose of mutagens is administered along with test compounds before scoring the mutation yield. Measurement of the mutagenic action of colchicine derivatives, compounds not usually regarded as mutagens, will be described elsewhere. But perhaps the most important application of the

Table 3. Analysis of the size of the genetic lesions produced by various agents acting in a single treatment on the A_L-J1 hybrid

	X-ray		UV		MNNG		EtMes		B[*a*]P		Untreated control	
Genetic lesion	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Localized event*	4	4	63	48	8	28	41	49	1	2	13	16
Complex event†	99	96	68	52	21	72	42	51	48	98	66	84
Total	103	100	131	100	29	100	83	100	49	100	79	100

Four markers were scored in each clone. a_1, a_3, and lactate dehydrogenase A (LDH-A) are contained on the short arm of chromosome 11, whereas a_2 is on the long arm. Cells were treated with each agent in a single dose to yield 10–50% survivors, from which clones were picked and analyzed.

*Loss of only a_1.

†Loss of a_1 and up to three other markers.

developments described here may be the ability to screen compounds and agents for prevention of damage at the cellular level at low doses of mutagens. Tests that can only detect high doses may fail to screen effectively for antimutagenic actions that can be effective only at the low doses that constitute common human experiences.

This approach should also expedite molecular understanding of the processes underlying mutational damage and its repair. High doses of mutagens produce primary genetic damage, which is succeeded by more complex secondary processes. The ability to study accurately effects of low doses should simplify interpretation of these dynamics.

It is of interest that Muller *et al.* (35) 30 years ago found a flattening of the mutation–dosage curve in offspring of irradiated *Drosophila* males, although at doses considerably higher than those used here. Their result was also interpreted as a lethal effect.

Further steps planned in these developments should include elimination of the bulk of background mutations, as described in preliminary publications (33), increasing the sensitivity by increase in the number of markers utilized so that the target size is increased, addition of translocations to the scored mutants, analysis of repeated doses and dose rate effects like those reported by Little and Thilly (13, 28), and increase in rapidity, convenience, and economy of the procedure. It appears feasible to reach a sensitivity capable of direct detection of mutagenic effects in the neighborhood of background radiation and spontaneous cellular metabolic processes.

Availability of methods for measurement of mutagenesis at considerably lower doses of mutagens now makes worthwhile epidemiological studies to determine more precisely the relationship between human disease and various doses of mutagenic agents and combinations of agents that can affect mutational response. Germ cell mutational dynamics as well as somatic cell consequences for disease must be explored. Although the magnitude of the studies required is not trivial, the possibility of achieving significant prevention of somatic cell diseases such as cancer and germ cell pathologies such as those represented by the large numbers of inherited diseases that afflict our society would appear easily to justify concerted efforts in these directions.

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