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PROBLEMS AND SOLUTIONS IN THE ESTIMATION OF GENETIC RISKS FROM RADIATION AND CHEMICALS

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ABSTRACT

Extensive investigations with mice on the effects of various physical and biological factors, such as dose rate, sex and cell stage, on radiation-induced mutation have provided an evaluation of the genetics hazards of radiation in man. The mutational results obtained in both sexes with progressive lowering of the radiation dose rate have permitted estimation of the mutation frequency expected under the low-level radiation conditions of most human exposure. Supplementing the studies on mutation frequency are investigations on the phenotypic effects of mutations in mice, particularly anatomical disorders of the skeleton, which allow an estimation of the degree of human handicap associated with the occurrence of parallel defects in man. -- Estimation of the genetic risk from chemical mutagens is much more difficult, and the research is much less advanced. Results on transmitted mutations in mice indicate a poor correlation with mutation induction in non-mammalian organisms. On the one hand, mice show little or no mutagenic response to several compounds that are highly mutagenic in other systems. On the other hand, recent results with ethylnitrosourea show that a single injection of 6 mg per mouse of this compound induces a mutation rate 75,000 times greater than that considered as a maximum permissible level of risk from a whole year of exposure to radiation. Further investigation in mice is obviously needed, not

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only on the screening for mutagenicity of other chemicals, but also on the nature of the mutagenic action of ethylnitrosourea.

INTRODUCTION

The title of this conference, "Measurement of Risks," indicates an emphasis, not on the end product of the measurement, the actual risks, but on the measurement process itself. Accordingly, this paper on genetic risks focuses on the methods of measuring, the rationale for their choice, problems and solutions in interpreting the results, gaps in our knowledge, and future possibilities for better estimation of risks. Special attention is given to the suggestion, by the organizers of the conference, to "examine both the logical soundness of the inferences of risk and the validity of the experimental evidence of damage, with examples drawn from environmental hazards of toxic chemicals and ionizing radiation."

The experimental results discussed here come primarily from work with mice, and, since these are so much more extensive for radiation than for chemical exposures, the measurement of genetic risks from radiation is treated first. Additional information is available in excellent reviews by Searle (35) and Selby (38).

RADIATION

For two reasons, measurement of the risk from major chromosomal aberrations is not discussed here. First, the presentation at this conference by Bender covers part of this subject. Second, I agree with the consensus of the current National Academy of Sciences Committee on the Biological Effects of Ionizing Radiation (45) that the radiation hazard from this class of genetic effects is probably small compared with that from gene mutations and small deficiencies. This, of course, does not detract from the great importance of using chromosomal aberrations in somatic cells of human beings to monitor human exposure.

This paper is also limited to measurement of risks in the first-generation offspring of irradiated parents. The paper by Crow at this conference deals with estimates for later generations.

In spite of extensive studies, attempts to detect radiation-induced transmitted genetic damage in humans have not, so far, been conclusive, although there is a suggestion of some damage in the children of irradiated fathers in the Hiroshima-Nagasaki surveys (12). Estimates of genetic risk are consequently still based on results from experimental organisms. Until 1950, measurements of mutation rate used in the evaluation of human hazards came mainly from Drosophila. Since then, the data accumulated on the mouse have

been the major basis for risk estimation. Radiation-induced mutation frequencies in the mouse were found to be much higher than those observed for Drosophila (17). Furthermore, several of what were thought to be basic principles of radiation genetics derived from the Drosophila work turned out not to apply to the mouse germ-cell stages of primary importance in risk estimation (23). However, although the mouse results presumably carry us much closer to a reliable prediction of mutagenic effects in man, extrapolation of these experimental findings to humans is still one of the problems in risk estimation. This is discussed later.

The questions that are important to answer by the measurement of radiation-induced genetic damage fall into two main groups: - (i) What are the factors affecting mutation rate and how do they affect it? (ii) What is the nature and extent of the phenotypic disorders caused by a given mutation rate? Much has been discovered in answer to the first question. The second question has proved more difficult, but two approaches to it have, in recent years, provided risk estimation committees with useful material.

Factors Affecting Mutation Rate

In order to investigate the effects that various physical and biological factors might have on mutation frequency, we developed the specific-locus method in the mouse (16). We started building the stocks of mice for it in 1947. If, at that time, anyone had predicted that 33 years later neither we nor anyone else would have devised a better method for its purpose, I would not have believed him. Yet we are still using it. It detects gene mutations and deficiencies. These are the two subgroups of radiation-induced mutational damage that comprise the major part of the genetic hazard from radiation. The phenotypic expression of the homozygotes of the mutations scored by the specific-locus method ranges all the way from lethality in early embryonic stage, through lethality at weaning age, to minor effects intermediate in expression between wild type and the viable alleles in the test stock used in the method.

Male

The rationale for the first use of the specific-locus method was not only to obtain, for the first time, a reliable estimate of radiation-induced gene mutation rate in the mouse, but also to have a rate that might be meaningfully compared with that in Drosophila. For a reason that will become apparent later, it was desirable to make this species comparison on mutations induced in the spermatogonial stage, and since there were no data on specific-locus mutations induced in this stage in Drosophila, we sponsored such a study

by Alexander (2) in our own laboratory. The mean mutation rate per locus for 7 loci in the mouse came out about 15 times higher than that for 8 loci in Drosophila (17), and this finding naturally had an impact on the setting of standards for permissible levels of radiation. If equal weight is given to a later, much smaller, study of 5 additional loci in the mouse (8) the mouse to Drosophila ratio is about 10.

Another of the early studies with the specific-locus method in the mouse was a comparison of mutation frequencies in spermatogonial and postspermatogonial stages. For high-dose-rate irradiation, the mutation rate from postspermatogonial stages was twice that from the stem-cell spermatogonia (30). However, because human germ cells spend only about 3 months of the average 30-year generation time (or 1/120) in postspermatogonial stages it was concluded that, from then on, it would be most relevant for hazard estimation to focus on the collection of data from irradiated spermatogonia. This conclusion is still valid after a later finding that there is a dose-rate effect in spermatogonia and none in postspermatogonial stages (33). This result indicates that, under most conditions of human radiation exposure, the mutation rate in spermatogonia may be only about 1/6 of that in postspermatogonial stages. Even with this much differential, however, the limited exposure time for the postspermatogonial stages would result in a mutational damage in these stages that would be only about 1/20 (i.e. 6 x 1/120) of that incurred in spermatogonia per human generation.

In the early days of the mouse work it was discovered that the mutation frequency in spermatogonia following high doses of acute irradiation was not linearly related to dose, but actually showed a marked decrease at 1000 R compared with 600 R (18, 34). This raised many questions that were investigated by further experiments. It immediately suggested differential response among the spermatogonia, both to killing and mutation induction. Results from experiments with fractionated doses supported this view (34). In terms of hazards, it was important to find out at what lower dose levels the humping of the dose-response curve might still exist. Data at 300 R showed no significant departure from a linear fit with those at 600 R. However, the evidence of differential sensitivity among the spermatogonia, along with the finding of extensive spermatogonial killing at high doses (13), led directly to studies to find out what might happen if the dose rate were lowered. Extensive Drosophila results indicated that there would be no effect. A marked effect was found in the mouse, however, in spermatogonia, but not in spermatozoa (33). Because the Drosophila data had come from spermatozoa, it was widely believed that Drosophila spermatogonia might show a dose-rate effect like that in the mouse. H. J. Muller immediately started testing this possibility for sex-linked mutations in Drosophila. For various technical reasons, he chose oogonia rather than spermatogonia. He ended this work very

disappointed that, despite intensive investigation, he was not able to show to his own satisfaction a clear-cut effect of dose rate, and he concluded that mice and flies are simply different. He generously congratulated us on finding a basic principle important for risk estimation that had been missed in Drosophila studies. Abrahamson and Meyer (1) have recently reanalysed Muller's data and have concluded that his treatment of them was "possibly in some ways wrong" and that there is a dose-rate effect. If their interpretation is correct, (and it would have been interesting to have had Muller's own evaluation of it), then, after 18 years, Drosophila results are finally brought in line with those in the mouse. However, any effect so far detected in Drosophila is small, and we still await a dose-rate study on specific-locus mutations in Drosophila.

The finding of a dose-rate effect, originally with results from 0.001 and 0.009 R/min compared with those from 90 R/min, led to investigations at other dose rates (20) and to the conclusion that below 0.8 R/min, even down to 0.0007 R/min in recent work (28), there is no further reduction in mutation frequency. Thus, there appears to be no threshold dose rate in the male. Accordingly, risk estimates for the offspring of irradiated males are based on a linear fit to the data obtained at dose rates of 0.8 R/min and below.

An obvious prediction based on the dose-rate effect was that small doses of high-dose-rate irradiation, or large doses delivered in small fractions, would give mutation frequencies per R approaching the lower response at low dose rates. This has proved to be the case (10). Therefore, under almost all conditions of human exposure, that is, low dose rates or small doses at high dose rates, the mutational risk is now estimated from the experimental data at low dose rates.

It was discovered that the distribution of mutations among the seven loci used in the specific-locus test was not significantly different at high and low dose rates. This indicates that there is no qualitative difference in the array of mutations obtained at the different dose rates. In other words, the reduction in mutation frequency at low dose rates is not the result of elimination of a particular class of mutational events, but simply a consequence of a lower probability of each event occurring (21). This conclusion is strengthened by the fact that distribution among the loci and other qualitative characteristics are capable of being changed. Thus, they are affected by factors such as radiation quality (neutron compared with X irradiation) and cell stage (spermatozoa and oocytes compared with spermatogonia).

Another factor that has been explored in the male is the possible effect of the interval between irradiation and fertilization. The rationale for this study was the possibility that the mutated stem-cell spermatogonia might be selectively reduced in number over

the breeding period of approximately two years following exposure. No statistically significant effect has been found.

Age of the male at irradiation has also been studied for its possible effect on mutation frequency. No difference between the mutational sensitivity of young and old sexually mature males has been detected. Mutation frequencies of irradiated immature males of various ages and of males in fetal stages at the time of irradiation have also been studied (36, 37). In general, any departure from the mutation frequency observed following irradiation of adults is a decrease.

With regard to the extrapolation of the mouse results in spermatogonia to the risk in man, the germ-cell stages and the process of spermatogenesis appear to be so similar in the two species that the only obvious major question is whether their mutational sensitivity is similar. Probably the most satisfactory answer to this is that the finding of no clear-cut genetic effect in the offspring of exposed males in Hiroshima and Nagasaki, (merely the suggestion of an effect on the borderline of statistical significance), indicates that the human male cannot be much more mutagenically sensitive to radiation than the male mouse, and may be less so (12, 44).

Female

Use of the specific-locus method to measure the relative influence of the various factors affecting mutation rate has revealed sex and cell stage to be dramatically important variables. In fact, the largest difference in effect observed anywhere occurs between different phases within one prophase stage of one cell type, the primary oocyte. Before presenting this, it may be helpful to outline the general aspects of the problem of relating the experimental results in the female to risk estimation.

The female germ cells in both the mouse and human go through the early stages of prophase of the first meiotic division in the fetus. At about the time of birth in the mouse, and before birth in the human, the primary oocytes go into an arrested diplotene stage and remain in this state until they prepare to take part in one of the successive estrus or menstrual cycles by starting on the path of maturation toward ovulation. This process takes approximately two months in the mouse and possibly as long as a year in humans. Even if it takes as long as a year, it is clear that in the average 30-year generation most of the radiation exposure will be accumulated by the arrested oocytes, not by the maturing ones. The arrested oocyte stage in the mouse has accordingly been extensively investigated in our laboratory. The results were unexpected and remarkable. In more than a quarter

of a million offspring scored for specific-locus mutations following X, γ, or neutron irradiation of arrested oocytes in their mothers with a variety of doses and dose rates, only 3 mutations have been observed (22). This is actually slightly, but not, of course, significantly, below both of two estimates of the control, or spontaneous, mutation rate (25).

These results on the arrested oocyte indicate the possibility that the only genetic risk in the irradiation of women may reside in the exposure of the maturing and mature oocytes, even though the duration of these stages is short relative to the 30 years of a generation. The mutational response of these stages is, therefore, obviously worth considering. There was also another reason for examining mutagenicity in these stages. The arrested oocyte in the mouse is quite sensitive to killing by high-dose-rate irradiation, while the human arrested oocyte appears to be much more resistant. Furthermore, there are differences between the two species in the cytological appearance of oocytes in this stage. Therefore, it was desirable to look at the mutational response of other oocyte stages in the mouse which might parallel the human more closely in one or both of these two characteristics.

In extreme contrast to the mutational insensitivity of the arrested oocytes, the mutation frequency at high doses and dose rates in the maturing and mature oocytes of the mouse turns out to be high, higher than that in spermatogonia. However, the dose-rate effect is much greater than in spermatogonia and does not reach its lower limit of effectiveness at 0.8 R/min. The mutation frequency continues to drop as the dose rate is lowered to 0.009 R/min (25), and at this dose rate, which is the lowest dose rate tested in females, the mutation rate is not significantly above the control spontaneous rate, except when compared with the lower of two estimates of the spontaneous rate, in which case a one-tailed statistical test gave $0.05 > P > 0.01$. A similar low mutational response is obtained when high-dose-rate irradiation is given in small fractions. Lyon and Phillips (9) found only one specific-locus mutation in 35,875 offspring following exposure of maturing and mature oocytes to an effective weighted mean dose of approximately 200 R of X rays delivered in 20 fractions over either 5 days or 4 weeks. The number of spontaneous mutations expected in this many offspring is 0.5 or 1.4, depending on which estimate of the spontaneous rate is used.

The cytological appearance of maturing and mature oocytes and their sensitivity to killing by radiation appear to be similar in mice and humans, as well as in other mammalian species studied. If, on this basis, the mouse mutation results can be used for human risk estimation, it is clear that low-level irradiation of maturing and mature oocytes would, at most, present only a very small hazard relative to that from irradiation of spermatogonia, because of the

low mutation frequency and the relatively short duration of these oocyte stages (25). The possibility of a threshold dose or dose rate is not excluded, even at the experimental levels used, which are much higher than most conditions of human exposure.

Because the maturing and mature oocytes of the mouse are much more resistant to killing than are the arrested oocytes, the possibility of using them as a model for the human arrested oocyte, which is also resistant to killing, has been considered. However, sensitivity to killing and to mutation induction show no consistent correlation, either negative or positive, among the various oocyte stages (25, 3). Therefore, to expect a similarity in mutational response solely on the basis of a similarity in sensitivity to cell killing does not seem to be well founded.

A possibly better model for estimating the mutational sensitivity of the human arrested oocyte is the mouse oocyte near the time of birth. This cell is quite resistant to killing, and its chromosomes are thought to bear a closer resemblance to those of the human arrested oocyte than do the chromosomes of the mouse arrested oocyte. In a recent investigation by Selby et al. (42), mice 18 1/2 days pregnant were given 300 R of 0.8 R/min gamma irradiation, and their daughters were mated in a specific-locus test. In the 37,218 offspring produced by those daughters, only one mutation was observed. In this size of sample, 0.5 or 1.5 spontaneous mutations would be expected depending on which estimate of the spontaneous rate is used.

In conclusion, although there are problems in trying to match oocyte stages in mice and humans, the low mutational sensitivity of all mouse oocyte stages to low-level irradiation provides reasonable confidence that radiation-induced mutation frequency in the human oocyte will be less than that in spermatogonia, probably much less, and possibly near zero.

Support for the view that this conclusion, based on the mouse results, does not underestimate the human risk comes from the Hiroshima and Nagasaki studies (12). The estimated doubling dose of low-level radiation for possible mutational damage resulting in death during the first 17 years after live birth of offspring of irradiated mothers is at least 1000 rem. Furthermore, while there is some evidence, on the borderline of statistical significance, for an effect in the children of irradiated fathers, there is no suggestion of any effect from maternal exposure.

Comparison with Measurement of Somatic Risks

A few words about the comparison between the measurement of genetic and somatic radiation risks seem in order. The two types

of damage are often not separated in discussions of the effects of conditions such as very low levels of radiation. It is sometimes assumed that the violent controversy over the shapes of the dose response curves for cancers at low levels of radiation, and the pessimism about ever settling it, apply as well to genetic damage. I would argue that this is not the case. I believe we have fairly reliable answers for genetic effects of radiation in both sexes.

In the male, the marked effect of dose rate in spermatogonia over the range of 90 to 0.8 R/min, and the absence of any further reduction in mutation frequency as the dose rate is lowered to 0.0007 R/min (at which dose rate the effect is still highly signif-cantly above the control) strongly suggest that the response will not change at even lower dose rates. In short, it would seem valid, and not an overestimation of genetic risk from irradiation of the male, to assume that there is no threshold dose rate, and that the response is linear with dose at all dose rates below the low ones for which we already have experimental data.

In the female, there is no evidence of mutation induction in arrested oocytes even with acute irradiation, and the sensitivity of other oocyte stages is so low at the lowest dose rates tested that the damage, at most, is small compared to that in the male. So, for mutation induction in both sexes, I think we have answers from experimental data as to what to expect at very low doses and dose rates, and there is nothing from the human data to contradict these estimates.

This view differs from that of Weinberg (46) who chose the estimation of the genetic effects of low-level radiation as a prime example of what he calls, "trans-science," that is, a problem "which cannot be answered by science." I agree, of course, with his statement that the number of mice that would have to be raised and examined to determine the mutation frequency induced by a yearly dose of 170 millirem is impossibly large. However, I think our indirect approach, by measuring the effect of successively lower dose rates, has brought the problem within the realm of science. The constancy of mutational response in spermatogonia over the more than 1000-fold drop in radiation dose rate from 0.8 R/min to 0.0007 R/min, coupled with the fact that, at 0.0007 R/min, the ionization tracks passing through the gene, with its limited target size, are presumably sparsely distributed in time, indicates that there is scientific validity in extrapolating these results to the lower dose rates involved in human hazards.

The problem of estimating the risk of cancer and other somatic effects from low-level radiation is much more difficult. From the kinetic and operational point of view, there are only two classes of genetic defect to measure: gene mutations and small deficiencies, on the one hand, and major chromosomal aberrations on the other.

Furthermore, we are concerned with only two organs, the testis and ovary, and with only a limited number of cell types within each. Contrast this with the myriad of somatic effects and the probably large number of possible kinds of kinetic response and it is obvious that the dose-response problem is much more complicated for somatic than for genetic effects. It is possible, nevertheless, that the approach of determining the effects of successively lower radiation dose rates on some critically important cancers could make the estimation of risk from them at human dose-rate levels reliable, and thereby remove the estimation from the realm of trans-science.

When it comes to measuring the phenotypic expression of the mutations, however, the variety of possible important medical disorders from mutations is enormous. It must be much greater than the number of important somatic effects. Here, geneticists still have much to do, as is demonstrated in the next section of this paper.

Nature and Frequency of Genetic Disorders

In addition to the information on mutation rate and how it is affected by dose rate, cell stage, and all the factors discussed earlier, we also need, for adequate risk estimation, some knowledge of the nature and extent of the physiological detriment or anatomical disorders caused by mutations.

Here I shall limit my discussion to direct measures of damage, leaving a treatment of the doubling-dose method to Crow's presentation at this conference.

One approach has been to look for effects on such vital statistics as early mortality, growth, and lifespan in the descendants of irradiated populations. This approach has not produced any clear-cut positive evidence of genetic damage in the Hiroshima and Nagasaki studies. Similar investigations in experimental mammals have been generally inconclusive. A few have given apparently positive effects, some of which were not, however, reproducible; and most have yielded only equivocal results.

As an example, I published one report indicating a shortening of life in the offspring of male mice exposed to neutron radiation from an atomic bomb (19). Spalding (43) tried to confirm this with a laboratory neutron source and found no effect. I could point out that he irradiated a different strain of mice and a different germ-cell stage, and that the mean lifetime in his controls was much shorter than in mine, indicating a less viable strain or a less favorable environment -- either of which might have accounted for the greater variation than in my experiment, and consequently have made it more difficult to detect an effect. But, without further

replications, one cannot feel convinced that my results were unequivocally positive. Even if they actually were, the fact that the conditions of another experiment had obscured the effect would still demonstrate the difficulty of using F_1 lifespan as an end point.

Long before the Spalding report appeared, I had decided on the basis of my own experience that vital statistics, such as lifespan, have so much natural variability and are so easily affected by numerous factors, many of which are not under control, that a small increment of damage due to mutation is not easily detectable. Furthermore, even if a clear-cut positive effect on a vital statistic, such as longevity could be demonstrated in the mouse, how would one translate this into human detriment? Therefore, I decided to determine whether it would be possible to score radiation-induced mutations affecting one of the major body systems in the mammal. Both Dr. Liane Russell and I had had experience in observing skeletal defects in mice, and in 1960 we collaborated with Dr. Ehling in setting up an attempt to detect skeletal variants in the offspring of male mice exposed to X irradiation.

Ehling's experiments were successful (4), and I urged the use of his findings by committees involved in risk estimation. The results were not generally accepted for this purpose, mainly because the animals were killed for observation of their skeletons, and there was, therefore, no unequivocal proof, by breeding tests, that the defective animals were true mutants. I have discussed elsewhere (26) why I thought the evidence for mutational origin was adequate. In any case, the point is now moot because the reluctance to use skeletal results has been dispelled by the work of Selby and Selby (39, 40, 41). They performed an extensive investigation similar to that of Ehling, but they raised offspring from all animals that were to be killed for skeletal examination, thereby permitting proof by further breeding tests that the skeletal defects scored by them were true mutations.

The skeletal findings have now been used by risk estimation committees such as the United Nations Scientific Committee on the Effects of Atomic Radiation (44) and the U. S. Committee on the Biological Effects of Ionizing Radiation (45). In order to convert this information on one body system into an estimate of the total damage in all systems, use was made of McKusick's (11) tabulation of monogenic disorders in man. The proportion of clinically important autosomal dominants that involve at least one part of the skeleton has been used, with some modifications for relative ease of detection of skeletal variants and for pleitropy, to derive a factor by which the skeletal defects should be multiplied to estimate the number of disorers in all body systems. Some of the mouse skeletal abnormalities are minor, and a consultation between Selby and McKusick has provided an estimate of what proportion of the mutational effects in the mouse would probably impose no real harm

if they occurred in humans. About half were in this category. The parallelism between mouse and human skeletal mutations is often striking, as, for example in the case of cleidocranial dysplasia, a syndrome marked by absence of clavicles and by skull defects.

The validity of the method for extrapolating from one class of defects to estimate disorders in all body systems has been strengthened by recent work of Kratochvilova and Ehling (7). They measured the frequency of radiation-induced mutations that cause cataracts in the lenses of mice. Even though this is a much more restricted class of damage than that of the whole skeleton, application of the results to estimate, by the use of McKusick's list, the total disorders in all systems yielded an answer similar to that obtained from the skeletal data.

Much more information is obviously needed on the nature and frequency of genetic disorders that have their parallels in man, but the skeletal and cataract studies have pioneered an extremely important aspect of risk estimation. For important groups of disorders, they provide estimates of the mutation rates, information on the nature of the mutational events (gene or chromosomal), and data on penetrance, expressivity, etc. Most important, they furnish a detailed description of the phenotypic effects of the mutations which can be examined by human geneticists for an estimation of the degree of human handicap associated with the occurrence of parallel defects in man.

CHEMICALS

Here again the discussion is limited to results obtained with the specific-locus method, namely, presumed gene mutations and small chromosomal deficiencies. The possibility should not be overlooked, however, that some chemicals may turn out to have their major, or even sole, genetic effect by inducing major chromosomal aberrations. There is, in fact, already some evidence for this, but, so far, the chromosomal damage measured has not resulted from exposure of spermatogonia or arrested oocytes, the cell stages of primary importance in human genetic hazards.

In the specific-locus tests on the mouse completed so far, the most striking feature is that most chemicals have either induced no mutations in spermatogonia or have not increased the mutation frequency in this germ-cell stage significantly above the spontaneous mutation rate (5, 15). Among the compounds showing no mutagenic effect in mouse spermatogonia are several that are well-known potent mutagens in other organisms. One example is ethyl methanesulfonate (EMS) which is highly mutagenic in many organisms, including Drosophila. In this case, and probably in several others, the lack of mutagenic effect in mouse spermatogonia cannot be attributed to

failure of the chemical or its active metabolite to reach the testis. Thus, EMS does induce some mutations in postspermatogonial stages in the mouse.

Until recently, only three chemicals, out of more than 20 tested by the specific-locus method, have given a clear-cut positive mutagenic effect in mouse spermatogonia. These are triethylenemelamine, mitomycin C, and procarbazine (Natulan). At sublethal doses, the most mutagenic of these is procarbazine, but the most effective dose of this compound (6) produced only approximately one-third as many mutations as had been obtained with a sublethal, 600-R, dose of acute X-irradiation.

An impression was growing that perhaps no chemical could break through the mammalian body's defense barriers, or circumvent its genetic repair capabilities, to produce more than a moderate mutagenic effect in spermatogonia. Recent results with N-ethyl-N-nitrosourea (ENU) (32) refute this view. In comparison with other chemicals that have shown a positive mutagenic effect in the mouse, ENU may be classed as a supermutagen. Thus, the highest reported mutation frequency obtained from a single dose of any other chemical is 16 mutations in 45,413 offspring produced by an injected dose of 600 mg/kg of procarbazine (6). In our laboratory, the current mutation frequency from ENU at 250 mg/kg is 160 mutations in 29,577 offspring. This represents an induced mutation rate (experimental minus control) which is 18 times higher than that from procarbazine at 600 mg/kg, even though the ENU dose is slightly less than the molar equivalent of the procarbazine dose.

The supermutagenicity of ENU is also illustrated by the fact that the induced mutation frequency cited above is 6 times the mutation frequency induced by 600 R, the most effective single acute dose of X-irradiation; and 18 times as effective as 600 R of chronic γ irradiation. The results with ENU greatly increase our concern over the potential human genetic risk from chemicals, and it is appropriate at this point to review what general conclusions can be reached from the data presently available on all chemicals investigated in mice.

I have pointed out elsewhere (24, 26) that the problem of chemical mutagenesis in mammals is exceedingly complex and that there have been dangerous tendencies to oversimplify it. Perhaps the most important general conclusion is that results in other organisms are not reliably predictive of what to expect in mammals. This applies even to eukaryotes as high in the evolutionary development of their chromosome structure as Drosophila. For example, diethylnitrosamine, which is a potent mutagen in Drosophila, gives no elevation above the control mutation frequency in one of the most extensive specific-locus tests conducted in the mouse (29). Many examples could be cited of the failure of the Ames Salmonella

test to predict the mutagenic effect of a chemical in mice. The most striking is procarbazine, which is negative in the Ames test, but was, until the effect of ENU was discovered, the most powerful mutagen known in mouse spermatogonia. ENU is mutagenic in many organisms including Drosophila and Salmonella, but in Salmonella it is a weak mutagen compared with extremely potent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), whereas, in the mouse MNNG has given zero mutations in 8302 offspring (5). The only short-term test which has, so far, given good correlation with mutagenicity in mouse spermatogonia is the in vivo somatic mutation method (spot test) developed by L. B. Russell (14).

Even when mutagenicity of a particular chemical has been demonstrated in the mouse, whether by the specific-locus or any other method, a vast array of complexities must still be explored. As with radiation, the effects of dose, dose fractionation, sex, cell stage, etc. must be determined along with additional variables encountered with chemicals, such as route of administration, and factors affecting variation in pharmacodynamics. Furthermore, as is already clear, the determination of the effects of the above factors on mutagenesis for one chemical is not necessarily predictive of what will happen with another chemical that might have given a similar response in the initial mouse test. Thus, procarbazine has a similar mutagenic effect in spermatogonial and post-spermatogonial stages (6), but mitomycin C and ENU, both of which are mutagenic in spermatogonia, have little or no effect on post-spermatogonial stages (5, 31). Completing the contrast, EMS, which is mutagenic in postspermatogonial stages has, so far, produced no mutations in spermatogonia (5).

It is clear, even from the limited number of examples of complexities within the mouse cited above, that, although mutagenesis studies on mammalian cells in culture are useful for investigating basic mechanisms under the conditions of the tests, nevertheless they are unlikely to be reliably predictive of the mutagenic events that occur in the various germ-cell stages and which are transmitted to descendent generations. Even the in vivo somatic mutation (spot) test, which has already been cited as giving good correlation with mutagenesis in spermatogonia, must be interpreted with caution in risk estimation, because not all the mutational events detected by it are necessarily of the kind that would survive passage through gametogenesis and fertilization to final expression in the offspring. It is, therefore, obvious that there is a critical need for comprehensive studies on transmitted mutations in the mouse for an array of model compounds that show any mutagenicity in this organism. By "comprehensive" is meant investigation into the effects of dose, dose fractionation, sex, cell stage, and all the other factors known or expected to affect mutation frequency.

Results along these lines have already been obtained by Ehling and coworkers on mitomycin C and procarbazine (5, 6), but progress is slow, compared to that possible with radiation studies, owing to the lower mutagenicity of these compounds relative to that from X rays. In contrast, the high mutagenicity of ENU offers an excellent opportunity for an in-depth study of this compound. Such a study is being conducted in our laboratory and is making rapid progress. Reference has already been made to the report on preliminary results showing that ENU, like mitomycin C, is much less mutagenic in postspermatogonial stages than in spermatogonia (31). The same report also cites preliminary data indicating low mutagenicity in treated females, and, in contrast to the effect of radiation, this low response applies to mature and maturing oocytes as well as to arrested oocytes. Another recent finding (27) is that of a marked variation in response in replicate experiments using the same dose. The variation may be due to age of the males at the time of injection, a factor that has not been evaluated in other chemical mutagenesis studies. Data are also rapidly accumulating on the dose-response curve, on the distribution of mutations among the seven loci, on the viability of the mutations in homozygous condition, and on the proportion of mutations that are intermediate in expression between that of the test allele and wild type.

As with radiation, the second part of the information needed for estimation of genetic risk of a chemical is the nature and extent of the phenotypic disorders caused by the induced mutations. It is obvious that, here again, we need mammalian information, and virtually none is yet available. We do not know whether the disorders will be similar in expression and severity to those induced by radiation, or whether there will be marked differences in the effects of different chemicals. ENU again offers an opportunity, and Selby, in our laboratory, is now looking for skeletal disorders in the offspring of mice injected with ENU.

There are two unrelated arguments which the results on ENU have already settled, at least to my satisfaction. These were circulating among discussion groups, although they may not have appeared in formal publications. The first was the view that the series of negative results obtained with the mouse specific-locus method on compounds that were potent mutagens in other organisms raised the question of whether the method was failing to detect the kind of mutational events induced by chemicals. The incredibly high mutation frequency obtained with ENU vindicates the method, especially in view of our finding, to be reported elsewhere in detail, that a high proportion of the mutations detected are minor changes intermediate in expression between that of the test allele and wild type.

The second argument has to do with the approach I have used for estimating an upper limit of risk on the basis of a negative

finding in the mouse. The most extreme example of this was based on an observation of zero mutations in 314 offspring of male mice exposed to large doses of 5-chlorouracil in their drinking water. Taking the upper 95% confidence limit, namely 3.3, of the observed zero number of mutations, it was calculated, on the basis of the relative dose x exposure time for 5-chlorouracil in human drinking water and in the mouse experiment, that the genetic risk in humans would not, with 95% confidence, exceed 0.02% of the spontaneous mutation rate (26). Some have objected to the conclusion on the grounds that, with the specific-locus method, it was absurd to expect anything other than zero mutations in the small sample of 314 offspring. This objection seemed irrelevant to me. If the concentration of 5-chlorouracil in human drinking water had really been potent enough to induce a mutation rate as high as, or higher than, 0.02% of the spontaneous rate, then, accepting the assumptions involved, the much higher concentration in the mouse experiment would have induced some mutations in 314 offspring, provided the mice could survive this concentration, which, in fact, they did with no signs if ill health. In any case, the objection is no longer valid. One of our experiments with ENU produced 78 mutations in 12,054 offspring (27), a rate of slightly more than 2 per 314 offspring, thereby showing that this is not an absurd possibility for the specific-locus method.

It seems appropriate to end this paper by reemphasizing the mutagenic potency of ENU. The mutation frequency cited in the above paragraph, obtained from a single injection of 6 mg of ENU per mouse, is 75,000 times greater than that considered as a maximum permissible level of risk from a whole year of exposure to radiation. Fortunately, ENU is apparently not encountered outside the controlled conditions of the laboratory, but its powerful mutagenic effect in mice demonstrates that we can no longer regard the mammalian body as resistant to all chemical mutagens. It is sobering to reflect on the possibility that there may be other chemicals with similar mutagenic potency to which man is exposed. Further scientific investigation in mammals is obviously needed, and this should involve not only the screening for mutagenicity among other chemicals, but also continued studies on the various important questions still to be answered with ENU.

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