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Search for Mutations Altering Protein Charge and/or Function in Children of Atomic Bomb Survivors: Final Report

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Summary

A sample of (1) children whose parents had been proximally exposed (i.e., <2,000 m from the hypocenter) at the time of the atomic bombings of Hiroshima and Nagasaki and (2) a suitable comparison group have been examined for the occurrence of mutations altering the electrophoretic mobility or activity of a series of 30 proteins. The examination of the equivalent of 667,404 locus products in the children of proximally exposed persons yielded three mutations altering electrophoretic mobility; the corresponding figure for the comparison group was three mutations in 466,881 tests. The examination of a subset of 60,529 locus products for loss of enzyme activity in the children of proximally exposed persons yielded one mutation; no mutations were encountered in 61,741 determinations on the children of the comparison group. When these two series are compared, the mutation rate observed in the children of proximally exposed persons is thus 0.60×10^{-5}/locus/generation, with 95% confidence intervals between 0.2 and 1.5×10^{-5}, and that in the comparison children is 0.64×10^{-5}/locus/generation, with 95% intervals between 0.1 and 1.9×10^{-5}. The average conjoint gonad doses for the proximally exposed parents are estimated to be 0.437 Gy of gamma radiation and 0.002 Gy of neutron radiation. If a relative biological effectiveness of 20 is assigned to the neutron radiation, the combined total gonad dose for the parents becomes 0.477 Sv. (Organ absorbed doses are expressed in gray [1 Gy = 100 rad]; where dose is a mixture of gamma and neutron radiation, it is necessary because of the differing relative biological effectiveness of gamma and neutron radiation to express the combined gamma-neutron gonad exposures in sieverts [1 Sv = 100 rem]).

Introduction

Planning for a study on the genetic effects of the atomic bombs was initiated in 1946, and data collection began in 1948. The details of the program that evolved over the years have been presented on several occasions (see, e.g., Neel and Schull 1956; Neel et al. 1974; Schull et al. 1981, 1982; Satoh et al. 1982). In 1972 a pilot study was undertaken of the feasibility of incorporating into the program a search for mutations resulting in an altered electrophoretic mobility of a carefully chosen battery of proteins (Neel et al. 1978); on the basis of the results of the pilot study, a full-scale investigation of this phenomenon was added to the other ongoing studies in 1975. A preliminary report of this study was presented in 1980 (Neel et al. 1980). In 1979 the biochemical approach to the genetic effects of the bombs was expanded with the introduction of a search for mutations resulting in approximately half-normal values for a selected series of enzymes, i.e., a search for mutations resulting from any one of several routes in gene inactivation or loss. A preliminary report has also been issued on the results of this approach (Satoh et al. 1983). Both of these studies have now been terminated. In the present report the findings will be summarized and related to the revised dosage schedules which are just becoming available for the survivors of Hiroshima and Nagasaki, because of a reappraisal of the yields and transmission of radiation

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from the atomic bombs (Radiation Effects Research Foundation [RERF] 1987).

The Protocol

The Indicators

The classical experimental studies on the genetic effects of radiation were conducted in the era before biochemical indicators were conveniently available. When the present study was conceived, in 1970, there were no published data on the production by radiation of germinal mutations resulting in transmitted electrophoretic variants in the mouse, the usual human surrogate in these matters. The present study was thus launched because, on theoretical grounds, it was difficult to believe that some fraction of the mutations resulting from radiation of germ-line cells would not be characterized by nucleotide substitutions. The probability of spontaneous loss of the purine moiety of a nucleotide in a mammalian cell has been estimated at 3×10^{-11}/s (Lindahl 1977). This low probability suggests a remarkable stability for any single nucleotide, but, because there are so many nucleotides in a mammalian nucleus, by this same calculation a mammalian cell should lose by spontaneous hydrolysis approximately 10,000 purines (and 500 pyrimidines) from its DNA during a 20-h generation period. Failure to repair each of these losses exactly would result in a mutation. Radiation might be expected not only to accelerate this spontaneous disintegration of nucleotides, with additional mutations as a result of misrepair, but to impair the repair process as well.

Furthermore, the policy of the genetic studies in Japan has been to examine as many valid indicators of the potential genetic effects of the bombs as possible. "Dogma" has had it that, in contrast to the results with Neurospora (Malling and de Serres 1973), the germinal mutations produced by the radiation of mammals are very predominantly deletions and other major chromosomal events. These should manifest themselves as children with congenital defects and/or decreased life spans. A search for such defects and for impaired survival was a major aspect of the early studies of the genetic effects of the bombs (Neel and Schull 1956). This dogma has been based primarily on the well-documented clastogenic effects of radiation and on the high frequency with which radiation-induced mutations are lethal when homozygous. A further, more recent argument has been that, in a variety of mammalian cell systems, ionizing radiation does not induce mutations to ouabain resistance, a type of mutation believed, on theoretical grounds, to arise from base-pair substitutions (see references in Liber et al. 1987). In our opinion, even if this viewpoint were essentially correct, it would have been a mistake, in any effort at a comprehensive study of the genetic effects of atomic bombs, to have concentrated only on phenotypes reflecting endpoints known to be especially sensitive to radiation.

There was a further point of principle for undertaking a study directed toward indicators reflecting mutations in single nucleotides. Studies of chemical mutagenesis—conducted some decades after the bulk of the studies on radiation mutagenesis—do demonstrate that the production of mutations resulting in electromorphs (as well as nulls) is an important component of the spectrum of mutations induced in mammalian systems by a variety of chemical mutagens. Lewis and Johnson (1986), in a summary of germinal mutations induced in the mouse by such agents as ethylene oxide, procarbazine, ethylnitrosourea, and triethylene-melamine, find that, in a system in which both loss of protein and/or enzyme activity and a change in electrophoretic mobility could be studied, 17 of the induced mutations were of the former type and 12 were of the latter type. Thus far, only a single spontaneous mutation has been observed in the controls of these experiments; it was an electromorph. In the ultimate, our ability to evaluate the relative genetic risks of the exposure of human populations to radiation and to chemical agents requires comparable data for the two types of mutagens.

Since the present study was initiated, two kinds of data directly relevant to the potential role of electrophoresis in evaluating X-ray-induced mutagenesis in humans have begun to become available. The first entails the molecular basis of the chromosomal lesions that occur spontaneously or are induced by X-rays; the second entails some empirical observations on the offspring of irradiated mice. With respect to the first development, we note that some 20%–40% of the spontaneous germinal and somatic cell mutations at the hypoxanthine phosphoribosyltransferase, adenine phosphoribosyltransferase, thymidine kinase, factor VIII, and Duchenne muscular dystrophy loci can be shown with the Southern blot technique to involve readily detectable genomic alterations, whereas the proportion of such readily detected lesions among X-ray-induced mutations at these loci in somatic cells is more like 50%–60%, the results dif-

fering by investigator and locus (see, e.g., Albertini et al. 1985; Stout and Caskey 1985; Turner et al. 1985; Grosovsky et al. 1986; Liber et al. 1986; van Ommen et al. 1986; Wilson et al. 1986; Yandell et al. 1986; Ashman and Davidson 1987; Gibbs and Caskey 1987; Monaco et al. 1987; Youssoufian et al. 1987). The relevance of these data in the context of electrophoretic variants must be interpreted with caution. On the one hand, the Southern blotting technique will not reliably detect small duplications or deletions (i.e., $\leq$100 bp) in restriction fragments of the usual 5–10-kb size such as were employed in the above-referenced studies; but such small lesions would result in the absence of an electrophoretically detectable gene product. Furthermore, as the analysis of the thalassemias shows so well (for review, see Antonarakis et al. 1985), even when the genome is intact, point mutations resulting in nucleotide substitutions may result in the absence of an electrophoretically detectable gene product. *On the other hand, the screening systems for mutations affecting these loci is highly selective, whether in the intact human or in cell lines,* depending on total or near-total loss of gene function. The mutations being detected must for the most part correspond to the thalassemias among the hemoglobin mutants. Thus, nucleotide substitutions resulting in mild or no loss of gene-product function, substitutions that might alter electrophoretic mobility, would not be detected in these systems. The recent extension of studies of this type to the sequencing of mutant loci failing to exhibit abnormalities on Southern blotting analysis should greatly clarify this question of the molecular basis for the mutations at these loci (see Grosovsky et al. 1988) but still will not compensate for the biased sampling of mutations resulting from the clinical or biochemical selective sieve.

Evidence of a different sort on the nature of X-ray-induced mutations comes from the studies of Liber et al. (1986). As noted, failure of X-rays to produce ouabain resistance in cultured cells has been seen as evidence that X-rays do not produce point mutations. However, Liber et al. (1986) find that at two other loci where the mutations selected should, for physiological reasons, be "point" (nucleotide-substitution) mutations, X-rays do produce mutations; these are a locus that codes for a mRNA synthesis factor (mutations selected through resistance to 5,6-dichlororibofuranosylbenzimidazole) and a locus that codes for tubulin (mutations selected as resistance to podophyllotoxin).

Recently, studies have also been initiated on the molecular basis of X-ray-induced mutations in mice (Rinchik et al. 1986; Russell 1986). These important studies are revealing predominantly deletion-type mutations, but to some extent this is inherent in the choice of some of the mutations studied (e.g., simultaneous involvement of the dilute and short-ear traits). Furthermore, it is by no means clear that the phenotypic screening involved in the specific locus system in mice (Russell 1951) would detect most nucleotide substitutions.

With respect to the second development mentioned above, since this investigation was launched the results of two studies searching for X-ray-induced electrophoretic variants in mice have been reported. Russell et al. (1976) examined the hemoglobin and albumin of 8621 F_1 of mice receiving 3–6 gray (Gy) of X-rays. The fact that the mice were heterozygous at both the hemoglobin loci, *Hba* and *Hbb,* (leading to a diffuse hemoglobin band) facilitated the recognition of quantitative variants. Three offspring exhibited hemoglobin bands of diminished intensity, attributed to simple loss-of-gene-activity mutants. One offspring had a hemoglobin band of increased activity, shown to be due to a duplication. Finally, one offspring exhibited a diminished hemoglobin band best explained on the basis of nondisjunctional events involving the chromosomes 7 of both P_1. No albumin variants were encountered. Malling and Valcovic (1977) irradiated the males of a cross between two lines heteromorphic with respect to the electrophoretic mobility of at least eight enzymes and the β-hemoglobin chain. The resultant F_1, as heterozygotes, should permit the recognition of both loss-of-activity and electromorphic mutations. Two doses of 5 Gy at a 24-h interval were employed. Four mutations in "somewhat over 2600 animals" were encountered. Two of the mutations involved loss of enzyme activity; two involved loss of hemoglobin production. Thus, none of nine X-ray-induced mutations encountered in the two studies was expressed as an electrophoretic variant. Although the data base is scanty, these findings are to be contrasted with those of the previously quoted studies, which had indicated that ~40% of the X-ray-induced mutations in somatic cell systems highly selective for reduced enzyme activity are not accompanied by alterations detectable on Southern blots. Note also the manner in which the data from the hemoglobin loci dominate these early findings.

Thus, the recent data on the molecular basis of

spontaneous and X-ray-induced mutations and the extent to which electrophoretically detectable variants are produced by X-rays are not as helpful in the present context as is desirable. The results (not reviewed in detail) appear to differ significantly both (1) from investigator to investigator working on a given locus and (2) from locus to locus. Given the prevailing thinking among radiobiologists, we may say that a surprising frequency of spontaneous mutations are being found to be deletions—and that an equally surprising frequency of X-ray-induced mutations, all in somatic cell systems, are not accompanied by evidences of DNA damage detectable with Southern blotting techniques. As already noted, this latter finding of course does not preclude small lesions incompatible with either mRNA synthesis or functional mRNA. There is thus a very limited basis for predicting what proportion of X-ray-induced mutations should manifest as electrophoretic variants; the arguments for proceeding with a study like this remain today much as they were 15 years ago.

As indicated in the Introduction, the second indicator of induced mutation employed in this study was the finding in a child of a half-normal level of any of a series of nine enzymes, when both parents exhibited normal levels for that enzyme. These enzymes had all been selected for study on the basis of exhibiting low coefficients of variation for their activity levels (Mohrenweiser 1981). Such a finding would result either from a gene-inactivation mutation of some type or from a chromosomal deletion. The preceding discussion should have justified the search for mutations of this type (see also Satoh et al. 1983). In principle, one-dimensional electrophoresis utilizing either protein or enzyme-activity stains should be useful for the detection of such variants, but in practice it has not appeared feasible, for any substantial subset of these indicators, to detect with the requisite accuracy samples characterized by levels 50% of normal. Unfortunately, the search, by other techniques, for variants characterized by half-normal enzyme levels is still relatively labor-intensive, so that this series of observations is limited.

We note, finally, that the previous studies of the genetic effects of the atomic bombs have been directed primarily toward what could be termed the "dominant" component of any genetic damage. There has in the past been much conjecture concerning a less apparent "recessive" component, expected to become manifest in later generations. The two indicators of the present study are an important aspect of any recessive component.

The Study Cohorts

The subjects of this study were drawn from two cohorts established in the course of the so-called F_1 Mortality Study of the RERF (see Neel et al. 1974; Schull et al. 1982). The composition of the original cohorts, consisting of children born between 1946 and 1959 (see Kato et al. 1966), has been updated on several occasions. Cohort 1 now consists of all the children born alive in Hiroshima and Nagasaki between May 1946 and December 1980 to parents in the various RERF study populations, one or both of whom had been "proximally exposed," i.e., <2,000 m from the hypocenter at the time of the Hiroshima or Nagasaki bombings (ATB). Cohort 2 consists of a sample of children born during the same period to "distally exposed" parents, i.e., (1) both parents >2,500 m from the hypocenter ATB, (2) one parent >2,500 m from the hypocenter and the other not in either city ATB, or, for a small fraction, (3) neither parent in either city ATB. It is assumed that the genes contributed by the fathers of these children were all in the spermatogonial stage at the time of the bombings.

Because the number of children born to distally exposed parents is much greater than the number born to proximally exposed parents, cohort 2 has been reduced to a manageable size for this study by selecting at random from the children of the distally exposed a subset matching cohort 1 as to sex and year of birth. At the time this study was designed, individuals >2,500 m from the hypocenter ATB were thought to have received no or negligible (<0.01 Gy) radiation ATB. With the current revision of the distance-dose curve (see below), it now appears that, if in the open, persons in the 2,500–3,000-m ring from the hypocenter ATB in Nagasaki may have received ≤0.02 Gy. Children born to parents 2,001–2,499 m from the hypocenter ATB have been excluded from the study because of the difficulty of evaluating the (small) doses received at this distance. In both of these cohorts, ~34% of the children are siblings to some members of the remaining 66%. Each child constitutes an independent test for mutation, however, and no correction of the data for the occurrence of siblings is indicated. The status of the cohorts is updated on a 4-year cycle.

The collection of the necessary blood samples extended from 1975 until 1985. The ongoing mortality study mentioned above supplied considerable information on those members of the cohorts who had died or left the city. Specially trained RERF personnel attempted to contact and explain the study to those

Table 1

Outcome of Effort to Conduct Family Studies (FS) on Individuals Exhibiting Rare Electrophoretic Variants

Exposure Category	Outcome of FS					
	Both P_1 Alive; FS Completed	Both P_1 Alive, Decline Study	One P_1 Unavailable,[a] the Other Cooperative	Both P_1 Deceased	Other[b]	Total
Parents proximally exposed	567 (81.0%)	45 (6.4%)	71 (10.1%)	4 (0.6%)	13 (1.9%)	700
Parents distally exposed	397 (74.5%)	44 (8.3%)	81 (15.2%)	0	11 (2.1%)	533
Total	964 (78.2%)	89 (7.2%)	152 (12.3%)	4 (0.3%)	24 (1.9%)	1233

[a] Includes those deceased, unwilling to participate in study, having moved from city, seriously ill, etc.
[b] Includes a miscellany of reasons for noncompletion of FS.

members of the cohorts who the records indicated were, at time of last contact, alive and residing in the city. No effort was made to obtain the necessary follow-up on, or blood samples from, children until they had attained the age of 13, so that children born subsequent to 1971 (~5% of the potential total sample) are not represented in the samples.

Whenever a "rare variant" (see below) was encountered in a child, examinations of both parents were necessary, to determine whether it represented an inherited variant or the result of a new mutation; and an effort was made to contact the parents and enlist their cooperation. As the study progressed, it became apparent that many parents of "children" exhibiting rare variants were either deceased or not in either city. (A "child" who entered the cohort in its early years could at the time of contact be as old as 37, and his/her parents, if alive, could be in their 70s.) Accordingly, during the latter two-thirds of the study, preference was given to obtaining specimens from children both of whose parents were known, on the basis of records available to the RERF, to be alive. The fact that both parents were alive of course did not ensure that they would wish to be examined if a family study seemed indicated.

Among a total of 16,702 children born to proximally exposed parents who were contacted for study, 13,052 (78.1%) agreed. For the children of the distally exposed, the corresponding numbers were 13,993 and 10,609 (75.8%). Table 1 presents an analysis of the availability of the parents in the event that a family study was necessary because a rare electrophoretic variant was detected in a child. When we consider below the results of the study (tables 2, 3), it will be apparent that there are substantially more locus tests on the children of proximally exposed parents than on the children of distally exposed parents. This is in part because the cohort of children of the distally exposed parents was somewhat smaller than the cohort having proximally exposed parents, but to a lesser extent it is because the children of the distally exposed parents (and the parents themselves) were aware from various sources of information that they were at low genetic risk and so were somewhat less inclined to participate in the study (table 1). Even so, overall the cooperation of the available F_1 in the studies was 77.1%, and that of their parents, when both were available, was 91.5%, levels to be considered exceptional in view of the fact that a venapuncture was involved. An additional reason for the difference in the number of determinations in the two series was a redefinition of the location of the hypocenter in Nagasaki during the course of the study (a redefinition that transferred some subjects from the distally to the proximally exposed category). Since, however, the biochemical indicators on which the study was based are not apparent to the subjects, and since this unequal participation was maintained throughout the study, we see no way in which this unequal participation could introduce bias into the study.

Biochemical Procedures

Although at the outset ammonium potassium oxalate was used as anticoagulant for the various blood samples, the majority of the samples were drawn into vacutainers in which formula A ACD solution was the anticoagulant. Most determinations were on samples that had been temporarily stored at −70 C or in liquid nitrogen. The processing of the samples for electrophoresis and for the enzyme activity measurements, as well as the techniques employed, have been

described by Ferrell et al. (1977), Ueda et al. (1977), Tanis et al. (1978), and Satoh et al. (1977, 1983). The 30 proteins examined for variants by starch-gel electrophoresis are listed in table 2; the nine proteins (enzymes) examined for activity levels are listed in table 3. The calculation of the number of gene products examined must take into consideration gene duplication (the α-hemoglobin locus) and multiple polypeptide subunits (i.e., lactate dehydrogenase, the hemoglobins). In earlier publications, we also treated the three esterase A1 isozymes as dimers of a shared polypeptide combined with three different independently coded gene products. Because all of the 34 variants of this isozyme encountered in this study appear to involve all three isozyme bands (K. Goriki, personal communication), we now feel it more appropriate to consider these isozymes as products of a single locus. The number of different gene products examined thus becomes 33. In employing electrophoretic and activity variants for studies of mutation under the present protocol, we make the implicit assumption that whatever negative selection (if any) may have operated on variants of the type under consideration since birth is equally distributed across the two classes of children.

A rare variant was defined as one that was represented by a gene frequency <.01. In nine of these systems, previously described genetic polymorphisms were encountered. Family studies were not performed on individuals apparently heterozygous or homozygous for one of these polymorphisms. It is, however, possible that mutation could result in a variant phenotypically indistinguishable from an established polymorphism; our protocol would not detect such "mimics." We have estimated that failure to perform family studies on all individuals exhibiting polymorphisms—an overwhelming task, given the personnel available—should result in an underestimate of the mutation rate of ~10% (Neel et al. 1983).

Special Studies of Putative Mutations

For the 1,280 determinations (tables 2, 3) in which a rare electrophoretic or activity variant was encountered, an effort was made to obtain blood samples from both parents to determine whether the variant was hereditary (nonmutational) or newly arisen. On the relatively few occasions when (1) neither parent exhibited the variant and (2) there was no information that the child was not the biological offspring of these parents, genetic typings appropriate for detecting discrepancies between legal and biological parentage were performed. These included A and B of the ABO system; M, N, S, and s of the MNSs system; C, D, E, c, and e of the RH system, Fy^a of the Duffy system; and the α_1-antitrypsin types. Among the 30 proteins studied for the occurrence of mutation, polymorphisms useful for the detection of parentage discrepancies occur in the following: HP, ACP1, 6PGD, PGM1, PGM3, ADA, ESD, GPT, and GOT1. In addition, major-histocompatibility-complex typings were obtained, involving 12 antigens of the A complex, 32 of the B complex, and four of the C complex. If the putative mutation involved one of the proteins included in the test battery, the findings with reference to that protein were excluded from the calculation of exclusion probabilities.

Dosimetry

Not until 1965 were the factors influencing the amount of radiation that was received by the survivors of the atomic bombings thought to be sufficiently well understood that individual dose estimates, termed *T*entative 1965 Doses (T65), could be assigned to survivors (Milton and Shohoji 1968; Auxier 1975; Hashizume and Maruyama 1975). At this time the T65 dose estimates suggested that the proximally exposed included some individuals who, because of felicitous shielding, had apparently received no gonadal radiation. A subsequent *D*ose *R*evision resulted in T65DR estimates. Unfortunately, in the late 1970s serious questions concerning the accuracy of these estimates emerged (for review, see Kerr 1981). A 5-year reevaluation has now terminated, with agreement on a new (and presumably final) approach to the problem of assigning individual doses (Radiation Effects Research Foundation 1987). The most salient feature of this reevaluation has been with respect to the bomb detonated over Hiroshima, for which a sharp reduction in the estimated neutron yield is coupled with a less marked increase, in parts of the city, in the estimated gamma yield. In addition, however, the estimates of the shielding from radiation provided by the roof tiles of Japanese homes and by concrete walls have been revised, as well as the estimates of the attenuation, because of the shielding effect of intervening tissues, in the amount of radiation reaching the gonad. The previous estimates of individual doses were thought to be characterized by a possible error, in either direction, of ~30% (Jablon 1971); presumably the same error applies to the new dose estimates. Gilbert (1984) has developed reasons

why doses might be systematically overestimated. We will calculate the gonadal doses sustained by the parents of the children included in the present study by using the new formulas, which result in what are termed *D*osimetry *S*ystem 1986 (DS86) doses. In the calculation of DS86 doses, any gonad radiation exposure to a survivor that is calculated to be >4 Gy is arbitrarily rounded down to 4; a midline dose >4 Gy is currently assumed to be lethal. This practice may underestimate the exposure of a few exceptionally hardy individuals. On the other hand, it does to some extent offset the source of bias identified by Gilbert (1984).

Unfortunately, at this writing DS86 doses are available only for those parents of the children under study who were in the open or in Japanese-style houses ATB. There remain a substantial number of parents who were shielded from the effects of the bombs in more complex ways, such as by their presence in concrete buildings or bomb shelters, and although estimates of the attenuating effect of this shielding are available in the T65DR schedule, they will only become available for DS86 over the course of the next several years. The estimation of radiation exposure for this group will be discussed below.

Because of the differing biological effectiveness of gamma and neutron radiation, the total dose must be expressed in sieverts (Sv). A critical factor in arriving at the Sv dose is the relative biological effectiveness (RBE) assigned to neutrons. The RBE of the latter increases as dose per unit time decreases. Primarily on the basis of the data of Grahn and his collaborators (Garriott and Grahn 1982; Grahn et al. 1983, 1984) on cytogenetic endpoints and the induction of dominant lethals and reciprocal translocations in mice (see also Sinclair 1985; International Commission on Radiation Units and Measurement 1986), we shall use an RBE of 20 for the relatively low neutron radiation doses to survivors' gonads. By presenting the estimated neutron exposures separately, we make it possible for others to analyze the data by using a different RBE.

Results

The findings with reference to electrophoresis are presented in table 2, and those with reference to enzyme activity are presented in table 3. Table 2 presents only the results of the 767,665 locus tests performed by electrophoresis on children of proximally exposed persons; the data on the children of distally exposed persons have been presented elsewhere (Neel et al. 1986). Table 3 presents the results of the 126,460 locus tests performed for quantitative variation on the children of both proximally and distally exposed persons. As noted earlier, it was not always possible to test both parents of a child with a rare variant. Tables 2 and 3 indicate, by system, the number of times that both parents of a child with a variant could be examined. Since it is only when both parents of a child with a variant have been tested that a rigorous treatment of mutation becomes possible, for each system we have estimated—by multiplying the fraction of variants for which family studies are complete (table 2, col. 7 ÷ col. 6) by the total number of determinations (table 2, col. 3)—the number of alleles that have been effectively screened for mutation. With this convention, all of the determinations of a polypeptide for which no variants have been encountered are credited as contributing to locus tests.

Among the children of proximally exposed persons, there were five with an electrophoretic variant not observed in either parent; but for two of these children, the test battery described earlier indicated a discrepancy between legal and biological parentage. We are thus left with three putative mutations among 667,404 equivalent locus tests, of the following description:

1. A slowly migrating variant of glutamate-pyruvate transaminase (GPT) was detected in a female child of proximally exposed Hiroshima parents. This enzyme is a dimer. The abnormal phenotype consisted of three bands, which are interpreted as corresponding to the normal homodimer; a heterodimer of one normal and one mutant polypeptide; and the homodimer of the mutant polypeptide. The phenotype is similar to that of a rare hereditary variant of this enzyme, encountered in Nagasaki, which we have termed 6NG1. The mobility of the mutant homodimer is identical with that of the homodimer of 6NG1, but the staining intensity of the former is weaker than that of the latter. The variant will be described in greater detail elsewhere (C. Satoh, unpublished data). Neither the parents nor a younger sister of the proposita showed the abnormal phenotype. There was no parentage exclusion with the complete battery of tests (the mother's gonad exposure was 0.03 Gy of γ; the father was not exposed).

2. A slowly migrating variant of phosphoglucomutase-2 (PGM2) was encountered in a male child of parents both of whom had been proximally exposed

Table 2

Results from Examinations for Occurrence of Mutations Altering Electrophoretic Mobility: Data from Children of Proximally Exposed Parents

Protein (E.C. No.), Locus Symbol	Total Loci Screened	Rare Variants		Variants (Both Parents Examined)	Equivalent Locus Tests	Exceptional Children
		No. of Types	Total			
Haptoglobin, *HP*	25,734	9	29	25	22,184	1[a]
Transferrin, *TF*	26,096	12	132	108	21,351	
Ceruloplasmin, *CP*	26,068	5	17	13	19,934	
Albumin, *ALB*	26,102	4	34	27	20,728	
Hemoglobin Al, *HBA1 + HBA2*	52,168	2	7	7	52,168	
HBB	26,084	3	4	2	13,042	
Hemoglobin A2, *HBD*	26,088	0	0	0	26,088	
Adenosine deaminase (3.5.4.4), *ADA*	26,092	2	7	3	11,182	
6-Phosphogluconate dehydrogenase (1.1.1.44), *PGD*	26,054	7	11	10	23,685	
Adenylate kinase-1 (2.7.4.3), *AK1*	25,164	0	0	0	25,164	
PGM1 (2.7.5.1), *PGM1*	25,046	13	78	61	19,587	
PGM2 (2.7.5.1), *PGM2*	25,064	4	9	8	22,279	1
Phosphoglucomutase-3 (2.7.5.1), *PGM3*	5,962	0	0	0	5,962	
Acid phosphatase-1 (3.1.3.2), *ACP1*	25,014	1	1	1	25,014	
TPI (5.3.1.1), *TPI*	21,916	2	2	2	21,916	
NP (2.4.2.1), *NP*	24,160	4	24	20	20,133	1
Esterase A1 (3.1.1.1), *ESA1*	23,776	5	14	14	23,776	
Esterase B (3.1.1.1), *ESB*	22,878	0	0	0	22,878	
Esterase D (3.1.1.1), *ESD*	24,126	1	1	1	24,126	
Peptidase A (3.4.11.–), *PEPA*	26,002	1	18	15	21,668	
Peptidase B (3.4.11.–), *PEPB*	26,104	2	14	12	22,375	
Glucosephosphate isomerase (5.3.1.9), *GPI*	26,100	7	132	105	20,761	1[a]
Isocitrate dehydrogenase-1 (1.1.1.42), *IDH1*	26,050	4	25	20	20,840	
Lactate dehydrogenase (1.1.1.27), *LDHA*	26,068	1	1	1	26,068	
LDHB	26,068	3	4	3	19,551	
Malate dehydrogenase-1 (1.1.1.37), *MDH1*	26,098	2	3	3	26,098	
Carbonic anhydrase-1 (4.2.1.1), *CA1*	25,958	3	17	15	22,904	
Carbonic anhydrase-2 (4.2.1.1), *CA2*	26,060	0	0	0	26,060	
Glucose-6-phosphate dehydrogenase (1.1.1.49), *G6PD:*						
Male	2,488	3	5	3	1,493	
Female	5,774	3	9	6	3,849	
Glutamate-oxaloacetate transaminase-1 (2.6.1.1), *GOT1*	17,292	3	53	42	13,703	
GPT (2.6.1.2), *GPT1*	17,282	6	49	40	14,108	1
Phosphoglycerate kinase-1 (2.7.2.3), *PGK1:*						
Male	2,163	0	0	0	2,163	
Female	4,566	0	0	0	4,566	
Total	767,665		700	567	667,404	5

[a] Parentage exclusion.

in Nagasaki. The abnormal phenotype consisted of the three bands customarily associated with the PGM2 1 phenotype and a variant band that migrated identically or slightly cathodally to the *d*-band of PGM1. This mutant has been designated PGM2 9NG2 (Satoh et al. 1984). Only two other variants of PGM2 have been encountered. On starch-gel electrophoresis, the mutant variant migrates slightly anodal to the band of one hereditary variant, PGM2 9NG1, but to the same position as that of the other variant, PGM2 9HR1. However, on thin-layer polyacrylamide-gel electrofocusing, the phenotypes of PGM2 9NG1, PGM2 9NG2, and PGM2 9HR1 are clearly distinguishable. Neither the parents nor two siblings exhibited an abnormal phenotype. No parentage discrepancy was revealed by the complete battery of tests (the mother's gonad exposure was 0.74 Gy of γ; the father's gonad exposure was 0.05 Gy of γ).

3. A rapidly migrating variant of nucleoside phosphorylase (NP) was detected in a male child of proximally exposed Nagasaki parents. The abnormal phenotype consisted of both a set of bands associated with the NP 1 phenotype and a set of rapidly migrating bands that exhibited a mobility similar to that of the bands associated with the NP 2 phenotype, a he-

Table 3

Results of Examining Nine Erythrocyte Enzymes for Occurrence of Mutations Resulting in Loss of Activity in (*a*) Children Born to Parents Proximally Exposed to the Atomic Bombs and (*b*) a Suitable Comparison Group

Enzyme (E.C. No.), Locus Symbol	Children of Proximally Exposed Parents				Children of Distally Exposed Parents			
		Variants Confirmed				Variants Confirmed		
	Total Loci Screened	Total	Both Parents Examined	Equivalent-Locus Tests	Total Loci Screened	Total	Both Parents Examined	Equivalent-Locus Tests
6-Phosphogluconate dehydrogenase (1.1.1.44), *PGD*	9,598	0	0	9,598	9,890	4	4	9,890
Adenylate kinase-1 (2.7.4.3), *AK1*	9,520	3	3	9,520	9,626	1	1	9,626
Triosephosphate isomerase (5.3.1.1), *TPI*	6,774	6	6	6,774	6,370	3	3	6,370
Glucosephosphate isomerase (5.3.1.9), *GPI*	9,978	11	9	8,164	10,052	3	3	10,052
Lactate dehydrogenase (1.1.1.27), *LDHB*	9,624	2	2	9,624	9,820	0	0	9,820
Glutamate-oxaloacetate transaminase-1 (2.6.1.1), *GOT1*	4,876	2	2	4,876	5,544	7	4	3,168
Phosphoglycerate kinase-1 (2.7.2.3), *PGK1:*								
Male	2,371	0	0	2,371	2,363	3	3	2,363
Female	4,810	0	0	4,810	4,996	0	0	4,996
Glyceralde-3-phosphate dehydrogenase (1.2.1.12), *GAPD*	560	1	1	560	0	722	0	722
Hexokinase (2.7.1.1), *HK*	4,232	1	1	4,232	4,734	0	0	4,734
Total	62,343	26	24	60,529	64,117	21	18	61,741

reditary variant. The variant will be described in detail elsewhere (C. Satoh, unpublished data). Neither of the parents exhibited the abnormal bands. There was no parentage exclusion with the full battery of tests (the mother's gonad exposure was 0 Gy; the father's gonad exposure was 0 Gy). (In this instance, although both parents had been <2,000 m from the hypocenter ATB, they had been so shielded that no radiation is thought to have reached their gonads.)

As shown in table 4, the estimated mutation rate on the basis of these findings is 0.45×10^{-5}/locus/generation, with the 95% confidence interval, calculated on the assumption that the number of mutations corresponds to a Poisson variant, being between 0.1×10^{-5} and 1.3×10^{-5}. The mutation rate in the children of the distally exposed (control) cohort, as based on three mutations in 539,170 effective locus tests, was previously reported to be 0.56×10^{-5}/locus/generation, with 95% confidence intervals between 0.1×10^{-5} and 1.6×10^{-5} (Neel et al. 1986). As a result of altering our treatment of the number of loci contributing to the ESA1 isozymes (see above) and, with continuing study, the reclassification of several previously ambiguous findings into true rare variants, we now estimate the number of locus tests in the control series as being 466,881 (see table 4) and the mutation rate as being 0.64×10^{-5}/locus/generation, with 95% confidence intervals between 0.1×10^{-5} and 1.9×10^{-5}.

The findings regarding enzyme activity will be presented in detail elsewhere (C. Satoh, unpublished data). As shown in table 4, there was one mutation in 60,529 tests conducted on the children of proximally exposed persons and no mutation in 61,741 tests on the children of distally exposed persons. The apparent mutant of triosephosphate isomerase (TPI) was encountered in a female child of Nagasaki parents. The TPI activity of the propositus was 65% of normal, 3.9 SDs below the mean. The mother, father, and a younger brother exhibited 92%, 100%, and 104% of normal activity, respectively. The electrophoretic patterns of all of them were normal. There was no parentage discrepancy with the complete battery of tests (the mother was not exposed; the father's gonad exposure was 0.03 Gy of γ.)

We wish to combine the results of these two approaches. In principle, this requires that the proportionate contribution of distally (or proximally) exposed persons be the same for the two data sets. For reasons already noted, in the electrophoretic data there is an excessive representation of determinations on the children of proximally exposed persons; these children are 58.7% of the total. On the other hand, since the data on activity variants are derived from a

Table 4

Summary of Data Concerning Mutation in the Two Series

Parameter	Proximally Exposed Parents	Distally Exposed Parents	Total
A. Electromorphs			
Children examined	13,052	10,609	23,661
Electrophoretic tests	347,040	288,984	636,024
Rare variants[a]	700	533	1,233
Variants (both parents examined)	567 (81%)	397 (74%)	964 (78%)
Equivalent-locus tests	667,404	466,881	
Exceptional children	5	6	11
Mutations	3	3	6
Mutation rate/locus/generation	0.45×10^{-5}	0.64×10^{-5}	0.53×10^{-5}
95% Confidence limits:			
Lower limit	0.09×10^{-5}	0.13×10^{-5}	0.19×10^{-5}
Upper limit	1.3×10^{-5}	1.88×10^{-5}	1.15×10^{-5}
B. Enzyme-Deficiency Variants			
Children examined	4,989	5,026	10,015
Tests	32,357	33,240	65,597
Deficiency variants (≤66% of normal)	26	21	47
Variants (both parents examined)	24	18	42
Equivalent-locus tests	60,529	61,741	122,270
Mutation	1	0	1
Mutation rate/locus/generation	1.65×10^{-5}	0	0.82×10^{-5}
95% Confidence limits:			
Lower limit	0.04×10^{-5}	0	0.02×10^{-5}
Upper limit	9.20×10^{-5}	4.85×10^{-5}	4.56×10^{-5}

[a] Allele frequency <.005.

subset of the persons contributing to the electrophoretic data, it was easier to regulate the numbers to the desired 1:1 ratio; now the children of proximally exposed persons contribute 49.5% of the data. This disparity would, under most circumstances, require some adjustment, but with the "positive events" so few in number, such an adjustment seems an overrefinement of the data, and we shall simply add the numerators of the enzyme activity series (1,0) to those of the electrophoretic series (3,3). This results in a mutation frequency of 4 in 667,404 locus tests conducted on the children of proximally exposed persons and of 3 in 466,881 locus tests among the children of distally exposed persons; expressed in conventional fashion, these are rates of 0.60 and 0.64×10^{-5}/locus/generation, respectively. The 95% confidence interval for the former rate is $0.2–1.5 \times 10^{-5}$, and that for the latter is $0.1–1.9 \times 10^{-5}$. It should be emphasized that these rates are not normative values in any respect. A normative value for nucleotide-substitutions-plus-deficiency-states would require (1) equal numbers of the two types of observations and (2) correction for the fact that only approximately one-third to one-half of all nucleotide substitutions result in variants detectable by electrophoresis.

Technically, those mutation rates must be corrected for the possibility that they are inflated by undetected parentage discrepancies, i.e., that the variant allele was contributed to the child by someone other than the father (nonmaternity is relatively much less likely). The probability of this event depends on three parameters. The first is the frequency of nonpaternity in this series. We are indebted to Dr. Howard Hamilton (personal communication) for data on ABO blood group typings in 3,295 mother-father-child trios drawn from this study, typings that enable us to calculate that the frequency of extramarital conceptions in this series is .0045. The second is the probability that the true father of the child transmitted a rare allele, of the type on which this study of mutation was based. In the total data set of the children of

both the proximally and distally exposed persons, this allele frequency is ~.001. The use of this frequency is a conservative procedure, in that all except one of the variants in this series that appear to be new mutations are unique in their electrophoretic mobility; that is, the pool of possible fathers who could contribute this allele is even smaller than the overall frequency of rare variants would suggest. The third parameter is the probability of *not* detecting that a child was conceived extramaritally. On the basis of the blood and enzyme typing described earlier, employing the probabilities supplied by Chakraborty et al. (1974), supplemented when necessary by our own calculations, we find this probability to be .141. On the basis of data in Ito et al. (1985), it can be calculated that with the battery of HLA typings that we have employed the frequency of failing to detect an extramarital conception would be .057. With the combination of serological, enzymatic, and HLA typing employed, the probability of not detecting an extramarital conception becomes .0085. The a priori probability that an apparent mutant in this series is the result of an extramarital relationship is .0045 $\times$ .001 $\times$ .0085, or $\sim 4 \times 10^{-8}$. We note that the frequency with which our procedures detected discrepancies between legal and biological parentage was 2/663,494 locus tests, or 3.0×10^{-6}, with 95% confidence intervals between 0.3×10^{-6} and 10.0×10^{-6}. On the basis of the above parameters, the theoretical expectation is .0045 $\times$ .001 $\times$ .9915, or 4.5×10^{-6}. There is thus satisfactory agreement between observation and expectation.

We come now to the assignment of gonadal doses. Recall that for a child to be classified as born of proximally exposed parents, only one of the parents was required to be <2,000 m from the hypocenter ATB. In fact, of the parents of the 13,044 children in the proximally exposed–parent category, 37.0% were either >2,500 m from the hypocenter ATB or not in the city ATB—and so received no radiation. Of the remaining parents, 75.7% can be assigned doses under the new DS86 schedule, but for the remaining 24.3% the shielding data are still inadequate for the application of the DS86 procedures. This is due to the individual's presence ATB in a concrete building, a factory, or other structure more complex than a Japanese home. For this group we have adopted, for the time being, a hybrid procedure for assigning dose category, a procedure that calculates organ radiation dose on the basis of the DS86 Kerma in air and tissue transmission factors but that uses the T65 DR physical shielding factors. Combining the data from this procedure with the data from the DS86 procedure, we estimate doses in Gy for the proximally exposed parents as follows: Hiroshima fathers, .204 γ and .002 μ; Hiroshima mothers, .231 γ and .001 μ; Nagasaki fathers, .216 γ and .001 μ; and Nagasaki mothers, .223 γ and .0001 μ. Since, as noted, for a child to be classified as born to proximally exposed parents, only one parent need have been <2,000 m from the hypocenter ATB, in the calculation of this average some parents are included who were distally exposed—or even not in the city—ATB. Assigning, as discussed earlier, an RBE of 20 to the neutron component of these exposures, we find that the average radiation doses to gonads become the following: Hiroshima parents, 0.495 Sv; and Nagasaki parents, 0.459 Sv. For the cities combined, the estimated conjoint parental exposure is 0.477 Sv. The average gametic dose—the measure in which the genetic effects of radiation is usually expressed—would of course be half this, or 0.239.

Because of the complexity of the dosimetry problem, no attempt has been made to calculate standard statistical errors to attach to the doses. Especially critical is the evaluation of the neutron component of the exposures, as well as the evaluation of the radiation attenuation provided by complex shielding. Efforts to refine these estimates will undoubtedly continue over the next several years and will be taken into consideration when, later, we attempt to use these data and other endpoints to develop an estimate of the doubling dose of radiation for humans. It is important from the standpoint of radiation biology to emphasize that 48.4% of this dose is derived from paternal exposure and 51.6% from maternal exposure.

Discussion

As we have emphasized in the past, the studies in Hiroshima and Nagasaki are not designed to test the hypothesis that radiation produced mutations in the survivors of the atomic bombings. Radiation has produced mutations in every properly investigated plant or animal species to which it has been applied, and it can scarcely be doubted that this is true of the situation under study. Our challenge, rather, is to treat the results of each of the various studies that have sought to elucidate the genetic effects of the bombs as an approximation of the true effect, ultimately combin-

ing all these findings to derive a best estimate of the genetic doubling dose of radiation for humans.

With respect to the indicators employed in this study, the two rates—that is, the rate in the children of proximally exposed persons and that in the children of distally exposed persons—are essentially identical. However, the errors to be attached to the two estimates are such that it cannot be excluded with any degree of confidence that the rates in the two series actually differ by a factor of 2. Any attempt at estimating a genetic doubling dose of radiation will be delayed until there has been a final analysis of the data on those other six indicators of a possible genetic effect—i.e., untoward pregnancy outcomes, survival through childhood, sex-chromosome aneuploids and balanced structural rearrangements of chromosomes, sex ratio, and childhood malignancies in the F_1—that lend themselves to the doubling-dose approach. An estimate based on a combination of these seven sources of information will of course have greater stability than an estimate based on any one of them.

Given the fact that the mutation rates are so similar in the two series, we are entitled to suspect that the mutations in the children of proximally exposed persons are "spontaneous" rather than radiation related. One indication of this is that the conjoint parental exposure in the parents of these four mutants averages 0.213 Sv, below the mean for the proximally exposed group—rather than above the mean, as would be expected if the mutations were radiation induced. Given the assumption that the mutations encountered in the children of proximally exposed persons are really spontaneous in nature, we can pool these mutations with the mutations in the children of distally exposed (i.e., nonradiated) persons (Neel et al. 1986), to ask the question, Are mutations more common in the systems exhibiting the larger number of electrophoretic variants? Dividing in half the number of polypeptides being scored, into those showing the greater number of different variants per locus and those showing the lesser number, we find that four of the mutations occurred among the former group and two among the latter group. The single activity mutant also involved a more variable system. At this point we can only record the finding for future reference.

We note, finally, that during the 10-year duration of this study, a particular effort was made to hold techniques constant. Were one launching such a study today, the electrophoretic technology employed would undoubtedly differ significantly (see discussion in Neel et al., in press). Furthermore, one would have to ask whether, in biochemically oriented studies of mutation, this technology is about to be superseded by the use of two-dimensional PAGE coupled with computer algorithms for gel analysis (Neel et al. 1984; Skolnick and Neel 1986) or by the emerging DNA techniques (Delehanty et al. 1986; U.S. Congress, Office of Technology Assessment 1986).

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