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SEARCH FOR MUTATIONS ALTERING PROTEIN CHARGE AND/OR FUNCTION IN CHILDREN OF ATOMIC BOMB SURVIVORS: FINAL REPORT

原爆被爆者の子供における蛋白質の電荷又は機能を変化させる突然変異の検出：最終報告

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原爆被爆者の子供における蛋白質の電荷又は機能を変化させる突然変異の検出：最終報告§

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

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要　約

広島・長崎の近距離被爆者（爆心から2,000 m以内で被爆した者）の子供と適切な対照群について，30種類の蛋白質の電気泳動上の移動度又は活性を変化させる突然変異の発生を調べた．近距離被爆者の子供の667,404遺伝子座の産物を調べたところ，電気泳動上の移動度を変化させる突然変異を3例検出した．対照群では，466,881遺伝子座テストを行い，3例の突然変異を検出した．酵素活性欠損に関しては，近距離被爆の子供の60,529遺伝子座産物を調べ，1例の突然変異を検出した．対照群の子供には61,741回のテストを行ったが，突然変異は検出されなかった．両検定をあわせると，近距離被爆者の子供の突然変異率は，0.60×10^{-5}/遺伝子座/世代であり，95％信頼区間は0.2から1.5×10^{-5}であった．また，対照群の突然変異率は0.64×10^{-5}/遺伝子座/世代，95％信頼区間は0.1から1.9×10^{-5}であった．近距離被爆者である両親の平均相加生殖腺線量はガンマ線が0.437 Gy，中性子線が0.002 Gyと推定される．中性子線の生物学的効果比を20とすると，両親の相加総生殖腺線量は0.477 Svとなる．

§本報告にはこの要約以外に訳文はない．

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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 children whose parents were proximally exposed at the time of the atomic bombings of Hiroshima and Nagasaki (i.e., within 2,000 m of the hypocenter) and 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. Combining these two series, the mutation rate observed in the children of proximally exposed is thus 0.60×10^{-5}/locus/ generation, with 95% confidence intervals between 0.2 and 1.5 $\times 10^{-5}$, and in the comparison children, 0.64×10^{-5}/locus/generation, with 95% intervals between 0.1 and 1.9×10^{-5}. The average conjoint gonad doses of the proximally exposed parents are estimated to be 0.437 Gy of gamma radiation and 0.002 Gy of neutron radiation. Assigning a relative biological effectiveness of 20 to the neutron radiation, the combined total gonad dose of the parents becomes 0.477 Sv.

Introduction

Planning for a study on the genetic effects of the A-bombs was initiated in 1946, and data collection began in 1948. The details of the program that

[§]**The complete text of this report will not be available in Japanese.**

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evolved over the years have been presented on several occasions.[1–5] In 1972 a pilot study 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 was undertaken[6]; 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.[7] 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 of a selected series of enzymes, i.e., a search for mutations resulting by any one of several routes in gene inactivation or loss. A preliminary report has also been issued on the results of this approach.[8] Both of these studies have now been terminated. The findings will be summarized in this report and related to the revised dosage schedules which are just becoming available for the survivors of Hiroshima and Nagasaki.[9]

Materials and Methods

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. This 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}/sec by Lindahl.[10] 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 about 10,000 purines (and 500 pyrimidines) from its DNA during a 20-hour 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 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,[11] 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 impaired survival was a major aspect of the early studies of the genetics effects of the bombs.[1] This dogma has been based primarily on the well-documented clastogenic effects of radiation and 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 (references in Liber et al[12]). 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 A-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 towards 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,[13] in a summary of germinal mutations induced in the mouse by such agents as ethylene oxide, procarbazine, ethylnitrosourea, and triethylenemelamine, find that in a system where 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 of the latter. 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, some empirical observations on the offspring of irradiated mice. With respect to the first development, we note that some 20% to 40% of the spontaneous germinal and somatic cell mutations at the hypoxanthine phosphoribosyltransferase, adenine phosphoribosyltransferase, thymidine kinase, factor VIII, and Duchenne's muscular dystrophy loci can be shown with these 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% to 60%, the results differing by investigator and locus.[12, 14–24] 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, of 100 base pairs or less, in restriction fragments of the usual 5 to 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,[25] 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 are 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, which 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[26]) 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.[12] As noted, failure of X rays to produce ouabain-resistance in cultured cells has been seen as evidence that they do not produce point mutations. However, Liber et al[12] 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 which codes for an mRNA synthesis factor (mutations selected through resistance to 5, 6-dichlororibofuranosylbenzimidazole) and a locus coding for tubulin (mutations selected as resistance to podophyllotoxin).

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[27] examined the hemoglobin and albumin of 8,621 F_1 of mice receiving 3–6 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 by nondisjunctional events involving the chromosomes 7 of both P_1. No albumin variants were encountered. Malling and Valcovic[28] irradiated the male mice 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-hour interval were employed. Four mutations in "somewhat over 2,600 animals" were encountered. Two of the mutations involved loss of enzyme activity; two, 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 the previously quoted studies, indicating that about 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" from investigator to investigator working on a given locus, and 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 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 mRNA synthesis or with 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.[29] Such a finding would result either from a gene inactivation mutation of some type or a chromosomal deletion. The preceding discussion should have justified the search for mutations of this type (see also Satoh et al[8]). 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 for variants characterized by half-normal enzyme levels by other techniques 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 A-bombs have been directed primarily towards 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, 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 RERF F_1 Mortality Study.[2,4] The original cohorts, consisting of children born between 1946 and 1959,[30] have 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 were "proximally exposed," i.e., within 2,000 m of 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 beyond 2,500 m from the hypocenter ATB, 2) one beyond 2,500 m and the other not in either city ATB, or, for a small fraction, 3) neither parent in the city ATB. Because the number of children born to distally exposed parents is much greater than the number born to proximally exposed, 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 beyond 2,500 m from the hypocenter 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 to 3,000 m ring from the hypocenter may have received as much as 0.02 Gy in Nagasaki. Children born to parents between 2,001 and 2,499 m have been excluded from the study because of the difficulty of evaluating the (small) doses received at this distance. In both of these cohorts, about 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 cohorts are updated on a four-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 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 (about 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 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

Table 1. The outcome of the effort to conduct family studies on individuals exhibiting rare electrophoretic variants

Exposure category	Outcome of family studies (FS)					
	Both P alive, FS completed	Both P alive, decline study	One P unavailable,[a] other cooperative	Both P 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, moved from city, seriously ill, etc.

[b]Includes a miscellany of reasons for noncompletion of family studies.

P: parent(s)

child. When we consider the results of the study (Tables 2 and 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. This is in part because the cohort of children of the distally exposed was somewhat smaller than the cohort having proximally exposed parents, but to a lesser extent because the children of the distally exposed (and their parents) 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 of their parents when both were available, was 91.5%, levels to be considered exceptional in view of the fact that a venipuncture 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 (which transferred some subjects from the distally to 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 and the techniques employed have been described by Ferrell et al,[31] Ueda et al,[32] Tanis et al,[33] and Satoh et al.[8,34] 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

Table 2. Results from examinations for the occurrence of mutations altering electrophoretic mobility: Data from children of the proximally exposed parents

Protein	EC No.	Locus symbol	Total loci screened	Rare variants: No. of types	Rare variants: Total	Variants, both parents examined	Equivalent locus tests	Exceptional children
Haptoglobin		HP	25,734	9	29	25	22,184	1*
Transferrin		TF	26,096	12	132	108	21,351	
Ceruloplasmin		CP	26,078	5	17	13	19,934	
Albumin		ALB	26,102	4	34	27	20,728	
Hemoglobin A1		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	6PGD	26,054	7	11	10	23,685	
Adenylate kinase 1	2.7.4.3	AK1	25,164	0	0	0	25,164	
Phosphoglucomutase 1	2.7.5.1	PGM1	25,046	13	78	61	19,587	
Phosphoglucomutase 2	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	
Triosephosphate isomerase	5.3.1.1	TPI	21,916	2	2	2	21,916	
Nucleoside phosphorylase	2.4.2.1	NP	24,160	4	24	0	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	
Peptidate B	3.4.11.-	PEPB	26,104	2	14	12	22,375	
Glucophosphate isomerase	5.3.1.9	GPI	26,100	7	132	105	20,761	1*
Isocitrate dehydrogenase	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	1.1.1.37	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	1.1.1.49	G6PD M	2,488	3	5	3	1,493	
dehydrogenase		F	5,774	3	9	6	3,849	
Glutamate-oxaloacetate transaminase (soluble)	2.6.1.1	GOT1	17,292	3	53	42	13,703	
Glutamate-pyruvate transaminasae	2.6.1.2	GPT	17,282	6	49	40	14,108	1
Phosphoglycerate kinase-1	2.7.2.3	PGK1 M	2,163	0	0	0	2,163	
		F	4,566	0	0	0	4,566	
Total			767,665		700	567	667,404	5

*Parentage exclusion

M - male, F - female

Table 3. Results of examining nine erythrocyte enzymes for the occurrence of mutations resulting in the loss of activity in children born to parents proximally exposed to the atomic bombs and a suitable comparison group

Enzyme	EC No.	Children of the proximally exposed parents					Children of the distally expose parents			
		Locus symbol	Total loci screened	Variants confirmed		Equivalent locus tests	Total loci screened	Variants confirmed		Equivalent locus tests
				Total	Both parents examined			Total	Both parents examined	
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 M	2,371	0	0	2,371	2,363	3	3	2,363
		F	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	722	0	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

M-male, F-female

consideration gene duplication (the α-hemoglobin locus) and multiple polypeptide subunits (LDH, 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. On the basis that 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 <0.01. In nine of these systems, previously described genetic polymorphisms were encountered. Family studies were not performed on individiuals 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, and 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 about 10%.[35]

Special studies of putative mutations

For the 1,280 determinations (Tables 2 and 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 neither parent exhibited the variant, and there was no information that the child was not the biological offspring of these parents, genetic typings appropriate to 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 4 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 A-bombings thought to be sufficiently well understood that individual dose estimates, termed Tentative 1965 Doses (T65),

could be assigned to survivors.[36–38] 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 dose revision resulted in T65DR estimates. Unfortunately, in the late 1970s serious questions concerning the accuracy of these estimates emerged.[39] A five-year reevaluation has now terminated with agreement on a new (and presumably final) approach to the problem of assigning individual doses.[9] 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-ray yield. In addition, however, the estimates of the shielding from radiation provided by the roof tiles of Japanese houses 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 about 30%[40]; presumably the same error applies to the new doses. Gilbert[41] has developed reasons why doses might be systematically overestimated. We will calculate the gonadal doses sustained by the parents of the children included in this study by the new formulas, which result in what are termed Dosimetry System 1986 (DS86) doses. In the calculation of DS86 doses, any whole-body radiation exposure to a survivor which is calculated to be greater than 4 Gy is arbitrarily rounded down to 4 Gy, as incompatible with survival; 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.[41]

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 availalbe for DS86 over the course of the next several years. The estimation of radiation exposure for this group will be discussed later.

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 collaborators[42–44] on cytogenetic endpoints and the induction of dominant lethals and reciprocal translocations in mice,[45,46] we shall use an RBE of 20 for the relatively low gonadal doses from neutrons experienced by survivors. 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 with reference to enzyme activity in Table 3. Table 2 presents only the results of the 767,665 locus tests performed by electrophoresis on children of proximally exposed; the data on the children of distally exposed have been presented elsewhere.[47] Table 3 presents the results of the 126,460 locus tests performed for quantitative variation on the children of both proximally and distally exposed. 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, we have estimated for each system the number of alleles that have been effectively screened for mutation by multiplying the fraction of variants for which family studies are complete (Table 2, column 7 ÷ column 6) by the total number of determinations (column 3). 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 the 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 (Satoh, unpublished data). Neither of 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 were proximally exposed in Nagasaki. The abnormal phenotype consisted of the three bands customarily associated with the PGM2 1 phenotype and a variant band which migrated identically or slightly cathodally to the *d*-band of PGM1. This mutant has been designated PGM2 9NG2.[48] 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 of 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 a set of bands associated with the NP 1 phenotype and a set of rapidly migrating bands which exhibited a mobility similar to that of the bands associated with the NP 2 phenotype, an hereditary variant. The variant will be described in detail elsewhere (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 were within 2,000 m of the hypocenter, they had been so shielded that no radiation is thought to have reached the 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, 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[47] to be 0.56×10^{-5}/locus/generation, with 95% confidence intervals of 0.1×10^{-5} and 1.6×10^{-5}. 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 466,881 (see Table 4), and the mutation rate as 0.64×10^{-5}/locus/generation, with 95% confidence intervals of 0.1×10^{-5} and 1.9×10^{-5}.

The findings regarding enzyme activity will be presented in detail elsewhere (Satoh, unpublished data). As shown in Table 4, there was one mutation in 60,529 tests conducted on the children of proximally exposed, and no mutations in 61,741 tests on the children of distally exposed. 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 standard diviations below the mean. The mother, father, and a younger brother exhibited 92%, 100%, and 104% of normal activity, respectively. The electrophoretic pattern of all of them was 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 the 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

Table 4. Summary of the data concerning mutation in the two series

	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*	700	533	1,233
Variants, both parents examined	567 (81%)	397 (74%)	964 (78%)
Equivalent locus tests	663,494	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	1.32×10^{-5}	1.88×10^{-5}	1.16×10^{-5}
	B. Enzyme deficiency variants		
Children examined	4,989	5,026	10,015
Electrophoretic tests	32,357	33,240	65,597
Deficiency variants ($\leq$66% of normal)	26	21	47
Variants, both parents examined	24	18	42
Equivalent locus tests	60,529	61,741	122,270
Mutations	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}

*Allele frequency <0.005

the children of the proximally exposed persons; these are 58.7% of the total. On the other hand, since the data on activity variants are derived from a 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 $\times 10^{-5}$/locus/generation, respectively. The 95% confidence interval for the former rate is 0.2–1.5 $\times 10^{-5}$ and for the latter, 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 equal numbers of the two types of observations and correction for the fact that only approximately 1/3 to 1/2 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 B. 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 0.0045. 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 from the children of both the proximally and distally exposed persons, this allele frequency is approximately 0.001. 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; i.e., 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,[49] supplemented where necessary by our own calculations, we find this probability to be 0.141. From data in Ito et al[50] it can be calculated that the battery of HLA typings we have employed would fail to detect an extramarital conception with a frequency of 0.057. With the combination of serological, enzymatic, and HLA typing employed, the probability of not detecting an extramarital conception becomes 0.0085. The a priori probability that an apparent mutant in this series is the result of an extramarital relationship is $0.0045 \times 0.001 \times 0.0085$, or approximately 4×10^{-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 of 0.3×10^{-6} and 10.0×10^{-6}. On the basis of the above parameters, the theoretical expectation is $0.0045 \times 0.001 \times 0.9915$, 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 within 2,000 m of the hypocenter ATB. In fact, of the parents of the 13,044 children in the proximally exposed category, 37.0% were either beyond 2,500 m or were not in the city (NIC) 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 presence of the individual ATB in a concrete building, a factory, or other structure more complex

than a Japanese house. For this group we have adopted, for the time being, a "hybrid" procedure for assigning dose which calculates organ dose on the basis of the DS86 kerma in air and tissue transmission factors, but uses the T65DR physical shielding factors. Combining the data from this procedure with the data from the DS86 procedure, we estimate doses in gray for the "proximally exposed" parents as follows: Hiroshima fathers, 0.204 γ and 0.002 neutrons (ν); Hiroshima mothers, 0.231 γ and 0.001 ν; Nagasaki fathers, 0.216 γ and 0.001 ν; Nagasaki mothers, 0.223 γ and 0.0001 ν. Since, as noted, for a child to be classed as born to "proximally exposed" parents, only one parent need have been within 2,000 m of the hypocenter, in the calculation of this average some parents are included who were distally exposed or even NIC ATB. Assigning, as discussed earlier, an RBE of 20 to the neutron component of these exposures, the average gonad doses, expressed in Sv, become: Hiroshima parents, 0.495 Sv; 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 A-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 which have sought to elucidate the genetic effects of the bombs as an approximation of the true effect, ultimately combining 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, in the children of proximally exposed and of distally exposed, 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

the other six indicators of a possible genetic effect which lend themselves to the doubling dose approach: untoward pregnancy outcomes, survival through childhood, sex-chromosome aneuploids and balanced structural rearrangements of chromosomes, sex ratio, and childhood malignancies in the F_1. An estimate based on a combination of these seven sources of information will of course have greater stabililty 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 them with the mutations in the children of the distally exposed (i.e., nonradiated) parents,[47] to ask the question: Are mutations more common in the systems exhibiting the larger number of electrophoretic variants? Dividing the polypeptides being scored into the half showing the greater number of different variants per locus and the half showing the lesser number, we find that four of the mutations occurred among the former and two among the latter. 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 over 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 (discussion in Neel et al,[51] in press). Furthermore, one would have to ask whether this technology is about to be superseded, in biochemically oriented studies of mutation, by the use of two-dimensional polyacrylamide gel electrophoresis coupled with computer algorithms for gel analysis[52,53] or by the emerging DNA techniques.[54,55]

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