Factors that Determine the \textit{in vivo} Dose-Response Relationship for Stable Chromosome Aberrations in A-Bomb Survivors

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An overview is given of the dose-response relationship for stable chromosome aberrations (i.e., translocations and inversions) in the peripheral blood lymphocytes of A-bomb survivors in Hiroshima. Special emphasis is placed on (i) the overdispersion of survivor cases with either unexpectedly high or low aberration frequencies relative to the estimated DS86 kerma values assigned to individual survivors, termed "cytogenetic outliers", and (ii) the correlation of chromosome aberration frequencies with other biological endpoints, such as acute radiation symptoms (severe epilation).

A new molecular biological technique, known as fluorescence in situ hybridization (FISH) with composite, whole-chromosome probes to paint differentially the target chromosomes, has facilitated rapid, efficient, and extensive scoring of translocation-type chromosome aberrations in which the target chromosomes are involved. Using this methodology, the observed findings on translocation frequencies in A-bomb survivors have shown that the frequency of stable chromosome aberrations, which have persisted for years without change in frequency in irradiated persons, is indeed useful as an indicator for biological dosimetry.

INTRODUCTION

Cytogenetic investigations of atomic bomb survivors of Hiroshima and Nagasaki, initiated in the mid-1960's at the Atomic Bomb Casualty Commission (ABCC) and then succeeded to date by RERF, have provided valuable information contributing to basic radiation biology. Main features of a series of the ABCC-RERF studies conducted more than two decades after A-bomb exposure\textsuperscript{1–5} are summarized as follows:

(1) Exchange chromosome aberrations of both symmetrical (stable) and asymmetrical (unstable) types were found to have persisted for decades in the peripheral blood T lymphocytes from A-bomb survivors in both cities.

(2) Among the chromosome aberrations detectable many years after A-bomb radiation
exposure, stable chromosome aberrations (mostly translocations and inversions) were observed predominantly in the proximally exposed. Repeat cytogenetic examinations on the proximally exposed survivors have revealed that the frequencies of cells with stable chromosome aberrations within the same individuals have remained almost unchanged through successive observations. It is thus these cells with stable chromosome aberrations that contribute principally to the dose-response relationship in A-bomb survivors to whom the DS86 kerma estimates have been assigned.

(3) In contrast to the predominance of stable chromosome aberrations in the proximally exposed A-bomb survivors, cells with unstable chromosome aberrations (dicentrics, rings and acentric fragments) were less frequent, constituting less than 10% of the total aberrant cells. This finding implies that, since both stable and unstable exchange events are assumed to occur with equal frequency in irradiated cells, unstable chromosome aberrations would have been preferentially eliminated with time after exposure from the circulating blood lymphocytes.

(4) A difference in aberration frequencies between Hiroshima and Nagasaki was noted previously with the old A-bomb radiation dosimetry system (T65D); for each dose interval, the aberration frequency was consistently higher in Hiroshima than in Nagasaki. The observed inter-city difference has also been confirmed by the re-analysis of the chromosome aberration data using the new dosimetry system (DS86). The difference, however, has become less pronounced with the DS86 system.

In this article, recent progress in cytogenetic studies of A-bomb survivors at Hiroshima RERF will be presented, with special emphasis on the dose-response relationship for stable chromosome aberrations in A-bomb survivors. Factors that might influence the in vivo dose-effect relationship based on the A-bomb survivors' data are considered; e.g., cytogenetic outliers (or "overdispersion" of cases with either unexpectedly high or low aberration frequencies relative to the estimated DS86 kerma values assigned to individual survivors), and correlation of chromosome aberration frequencies with other biological endpoints (somatic mutation frequencies and severe epilation, one of the representative acute radiation symptoms occurring immediately after A-bomb exposure).

MATERIALS AND METHODS

Study Samples

In the early stage of cytogenetic surveys, chromosome analysis was performed on members of the Adult Health Study (AHS) cohort, who have received biennial medical examinations at the Department of Clinical Studies, RERF. They consisted of 582 proximally exposed survivors with DS86 kerma estimates between 0.005 and 3.0 Gy, and 446 distally exposed individuals with 0 Gy dose estimates, the latter being served as controls.

The subjects included in the recent study during the period between 1984 and 1986 were also selected from the participants of the AHS cohort in Hiroshima; comprising 375 subjects, 185 proximally and 190 distally exposed survivors, for whom no previous cytogenetic examination was conducted.
Study subjects who reported either receiving radiotherapy or radioisotope treatments at any time in the past, or diagnosed as having malignant tumors before the present cytogenetic examinations, were grounds for exclusion.

**CYTOGENETIC METHODS**

1) **Culture methods**: Details of cytogenetic methods have been fully described elsewhere\(^2\). A small amount (1-2 ml) of heparinized venous blood was drawn from each of A-bomb survivors at the time of medical examination, and then cultured in the presence of phytohemagglutinin (PHA) for 52 hr, including the last two hours of colchicine treatment. Under these culture conditions, the majority of metaphases were found to be in their first *in vitro* mitosis. In this recent study, time in culture was reduced to 48 hours including the last 2 hours of colchicine treatment. Otherwise there was no basic change between the two procedures in both quality of metaphase spread and metaphase frequency. Cultured lymphocytes were harvested according to the routine method for the preparation of chromosome-spread slides.

Chromosome slides were prepared for (1) *conventional analysis* with ordinary Giemsa stain\(^2\), (2) *G-banding analysis* by digesting the cells on slide with a trypsin solution\(^6\), and (3) *FISH stain* by denaturing the chromosomal DNA and hybridized *in situ* with whole-chromosome probes, and by staining specifically the target chromosomes that are involved in chromosomal exchanges\(^7\).

2) **Conventional and G-banding analysis**: For detection of chromosome aberrations both by conventional and G-banding analyses, 100 well-spread metaphases per case were scored. In order to identify structural alterations of chromosomes on non-banded preparations, the chromosomes were grouped directly under the microscope into A to G groups to see if there is any change in the number of chromosomes for each chromosome group. Any change in the number of chromosomes is a direct reflection of the presence of aberrant chromosomes in a metaphase. All of the cells with either definite or suspected structural rearrangements thus detected were photographed for further detailed karyotype analysis. Cytoscan (I.R.S., U.K.) has been used in our laboratory to expedite the work for detection and documentation of the records (image capture, data-input and retrieval) of all kinds of chromosome aberrations observed.

**Unstable chromosome aberrations** referred to in the study include dicentrics (*polycentrics*), rings, each with an accompanying fragment, and acentric fragments of both terminal and interstitial types. *Minute fragments* and *acentric rings* are considered as *interstitial deletions*, both of which are the products of *intra-arm intra-changes*.

**Stable chromosome aberrations**, the counterpart of unstable aberrations, include reciprocal translocations, inversions of pericentric type, and deletions. *Deletions* considered here are a result of either an *incomplete form of exchanges* or a *terminal* and/or *interstitial deletions*, where the deleted segment is likely to be lost from the cell through subsequent mitosis. This may imply that, a cell with a deleted portion of the chromosome (or a cell with partial monosomy) could have survived *in vivo*. *Paracentric inversions*, the counterpart of acentric rings, can only be identified by detailed examination using G-banding method. Furthermore, *insertions* (*direct
and inverted types produced from three-break rearrangements) cannot be distinguished from the ordinary reciprocal translocations (two-break exchanges) by the conventional method.

It is considered that G-banding analysis can detect all types of induced structural chromosomal rearrangements by virtue of the change in band patterns of chromosomes involved in aberrations. However, the work with this technique is time-consuming, and requires a great deal of experience of the examiners.

iii) Fluorescence in situ hybridization (FISH): The FISH technique can selectively stain specific chromosomes, so that any structural aberrations associated with such target chromosomes can be detected rapidly and efficiently by the application of FISH. In this context, whole chromosome probes are considered especially useful for quick identification of translocations and dicentrics not only in metaphase spreads but also in interphase nuclei. FISH with whole-chromosome probes is used to stain target chromosomes with FITC-avidin so that they appear yellow, while propidium iodide is used to stain non-target chromosomes so that they appear red. Structural rearrangements produced by inter-chromosomal exchanges between target and non-target chromosomes can thus be discriminated easily by bi-color structures. Technical details of FISH have already been described elsewhere.

A pilot study has been designed to compare scoring efficiencies for detection of translocations between FISH, G-banding and conventional methods on 20 Hiroshima A-bomb survivors (0–5 Gy) who were newly selected for this study. The number of cells scored depended on the frequency of translocations involving the target chromosomes, and thus varied from case to case, ranging from 100 to more than 2,000. All of the metaphases were observed under a Nikon fluorescence microscope. Any metaphases carrying at least two bi-color (yellow-red) chromosomes were recorded and photographed.

RESULTS AND DISCUSSION

1) Data on Stable Chromosome Aberrations Using Conventional Analysis.

Previous reports have indicated several important aspects of the dose-response relationship for chromosome aberration frequencies in the peripheral lymphocytes of A-bomb survivors in Hiroshima and Nagasaki. One of the striking features in the findings is that, though there is a dose-dependent increase in aberration frequencies in the proximally exposed survivors, the shapes of dose-response curves seem to differ between the two cities. Dose-response functions are linear-quadratic both in Hiroshima and Nagasaki. The estimated quadratic component is larger in Nagasaki, whereas in Hiroshima the linear term is more pronounced. The difference is statistically significant (p<0.001). Another important feature in association with the dose-response curves is that, in all dose ranges, chromosome aberration frequencies are consistently higher in Hiroshima than in Nagasaki. The reason for the observed inter-city difference remains to be elucidated. One of the possibilities to explain this difference is that the proportion of neutron component relative to the total kerma is still greater in Hiroshima than in Nagasaki even with the new DS86 dosimetry system.

In the early stage of the cytogenetic investigation at ABCC-RERF, scoring efficiency of
stable chromosome aberrations was rather poor and thus inconsistent. The coefficient for the
dose-response function estimated from data obtained in the first four years of cytogenetic
program (1968–1971) was roughly 50% lower than that using data from the later years.
Attempts have been made since early 1970's at RERF to establish standardized microscopic
criteria for detection of stable chromosome aberrations (such as translocations and inversions).
Since then, the efficiency for scoring any aberrations has become greatly improved, and the data
thus obtained have been consistent and reliable to be used for biological dosimetry.

Figure 1 shows our recent data on the frequencies of translocations and inversions (abbrevi-
ated hereafter as “t+inv”) for 184 proximally exposed survivors plotted as a function of the
DS86 kerma in Hiroshima. Because of too many cases plotted in limited space in Figure 1 as
shown by a closed square, a plot of aberration frequencies from survivors in the low dose range
of less than 1 Gy is reproduced on an expanded scale in Figure 2.

As seen in Figure 1, the observed frequencies of translocations and inversions are in

| DS86 KERMA (Gy) | NUMBER OF ABDERATIONS PER CELL |
|-----------------|-------------------------------|
|                 | N = 184                        |

Fig. 1. A plot of the frequency of translocations and inversions as a function of the DS86 kerma
estimates assigned to each of A-bomb survivors in Hiroshima. The solid line shows a
regression through data on 375 proximally and distally exposed survivors. Aberration
data were obtained by the conventional analysis. Double-circles each with an arrow
indicate survivors with either exceptionally high or low aberration frequencies.
accordance with the DS86 kerma estimates assigned to individual survivors. The pattern in the
dose-response relationship for individual frequencies is quite similar to that described in the
previous report\(^8\); i.e., there are constantly a small fraction of cases whose aberration frequen-
cies are grossly discrepant with regard to estimated dose, termed "cytogenetic outliers". As
shown by double-circles in Figure 1, there are two types of outliers: one with high aberration
frequency in the low dose range, and the other with low frequency in the high dose range. The
presence of the outliers is regarded as a contributing factor to the cause of substantial
overdispersion in the distribution of chromosome aberration frequencies noted in all dose
ranges.

There are some confounding factors that may alter the shape of dose-response curves for
the frequency of stable chromosome aberrations. As already mentioned previously, cytogenetic
outliers are considered as the most likely candidates for such factors. Even excluding the
outliers from the the analysis, there still is a striking variability of stable chromosome aberration
frequencies between survivors within a given dose category. Such an overdispersion in aberra-

\[\text{Fig. 2. A plot of the frequency of translocations and inversions as a function of the DS86 kerma}
\text{estimates assigned to 122 proximally exposed survivors with less than 1 Gy. The graph is}
\text{reproduced and magnified from the 1 Gy area as shown by the closed square in Figure 1.}\]
tion frequencies either might be due to random errors in the DS86 estimates assigned to individual survivors, or might suggest differential radiation sensitivity in A-bomb survivors.

While the possibility exists that individuals differ in their radiosensitivity within our A-bomb survivors' cohort, all biological effects of A-bomb radiation studied recently at RERF point to the fact that the contribution of radiation sensitivity to the effects may be small. Consequently, there is a need for further study to investigate whether random errors in DS86 dosimetry is real or not.

Recently Sposto et al. have reported the results of a statistical analysis using data on individual chromosome aberration frequencies in correlation with severe epilation, as one of acute radiation sickness manifested immediately after A-bomb exposure. They have shown that the dose-response relationship for chromosome aberration frequencies using the DS86 assigned dose is significantly steeper in the subgroup of survivors who reported severe epilation after the bomb than in those who did not report severe epilation, almost by a factor of 2. They have further shown that there is substantially more variation between individuals at higher doses in the proportion of stable aberrations than would be expected if there were no heterogeneity in the dose response between individuals. Perhaps the outliers noted would be in extreme cases in this regard.

By undertaking statistical analyses of chromosome aberration and epilation data over a range of dosimetry error models of the form developed by Pierce et al., it is concluded that random dosimetry errors in the range from 45 to 50% of true dose can explain the difference in dose response between severe and non-severe epilation groups. This amount of dosimetry error may also account for the overdispersion in aberration frequencies. The same tendency has also been observed between the occurrence of severe epilation and the subsequent mortality risk of leukemia in the A-bomb survivors.

Figure 2 illustrates the distribution in the frequencies of "t+inv" as a function of DS86 kerma estimates for survivors of less than 1 Gy. It is noteworthy that the distribution of aberration frequencies of individuals in the dose range of 0.2 Gy or less does not differ from that of distally exposed survivors with 0 Gy, as shown by a double circle with a standard deviation. The mean frequency of "t+inv" per cell derived from 36 survivors with less than 0.1 Gy (mean Kerma of 0.05 Gy) is 0.0161 ± 0.0136, while that from 190 distally exposed survivors (0 Gy) is 0.0155 ± 0.0152. Thus no statistically significant difference is demonstrable between the two groups. As seen in Figure 2, the increase in "t+inv" frequencies is noted at 0.3 Gy or more. This result indicates the technical limitation of cytogenetic procedures to detect and quantify previous exposures to low level of radiation in vivo.

A wide variability in the distribution of aberration frequencies is also observable even in the low dose range as shown in Figure 2. It is also known that there is an elevated level in the frequencies of dicentrics and rings observed for both low-dose and distally exposed survivors (data not shown). The increase in both stable and unstable aberration frequencies in these groups might be due in part to the advanced age of the survivors at the time of examination; their mean age at examination is about 60 years, with a range from 40 to 80 years. It is well known that spontaneous aberration frequencies increases with increasing age.

Another possible explanation for the above variations is that aged survivors have received
medical X-ray exposure, especially fluoroscopy of the upper gastro-intestinal region, for diagnostic purposes more frequently than non-exposed people of the same age, because of the better medical care extended to A-bomb survivors. For the survivors in the low dose range, local accumulated doses attributable to medical exposure often were estimated to have exceeded a single acute A-bomb radiation dose in 1945.

2) Data on Translocation Frequency Using FISH Analysis.

It is recognized that fluorescence in situ hybridization (FISH) with whole-chromosome probes, obtained from the Bluescribe libraries, to stain differentially the target chromosomes has been shown to be an effective method for detection of structural aberrations. The feasibility of the FISH technique has been tested in collaboration with Lawrence Livermore National Laboratory for quantification of the frequency of stable rearrangements (primarily translocations) that have persisted for decades in the peripheral lymphocytes of A-bomb survivors. A comparison has also been made for scoring efficiencies of detecting translocation between FISH, G-banding and conventional methods applied to the same A-bomb subjects.

There are two important assumptions necessary for the interpretation of the present findings; one is that the chromosome breaks are initially distributed randomly throughout the chromosomes (or genome), and the other is that the aberrations confer neither selective advantage nor disadvantage on the aberrant cells. It then follows that the frequency of translocations observed for each chromosome type many years after exposure should be proportional either to the relative DNA content of the target chromosomes or to the relative chromosome lengths. These assumptions have been supported in part from the finding on translocation frequencies as determined by the G-banding analysis.

The results of analysis on translocation frequencies derived from 20 Hiroshima A-bomb survivors have been described elsewhere. Mention is simply made that there is an excellent correlation between three techniques employed, in terms of the magnitude of aberration frequencies for the same individuals. Scoring efficiency has been determined by measuring the ratio of translocation frequencies by FISH and conventional analyses to those by G-banding, provided the assumption that virtually all structural rearrangements of stable type can be detected with full scoring efficiency by G-banding analysis. The values thus derived are 0.60 for conventional versus G, and 0.85 for FISH versus G, respectively. The present study has shown that there is a good agreement in translocation frequencies between FISH and G-banding methods (regression coefficient: \( r=0.958, n=20, p<0.001 \)).

In conclusion, a pilot study in collaboration with LLNL has shown that the FISH technique is indeed practical for quick and effective detection of radiation-induced chromosome rearrangements in individuals exposed in vivo to ionizing radiation. This seems especially relevant to establish a better biological dosimetry system with chromosome aberration data. The technique will be well-suited to automation with image-analysis system, so that assessment of the effect of exposure either to low radiation doses or to large population sizes might become more practical.
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