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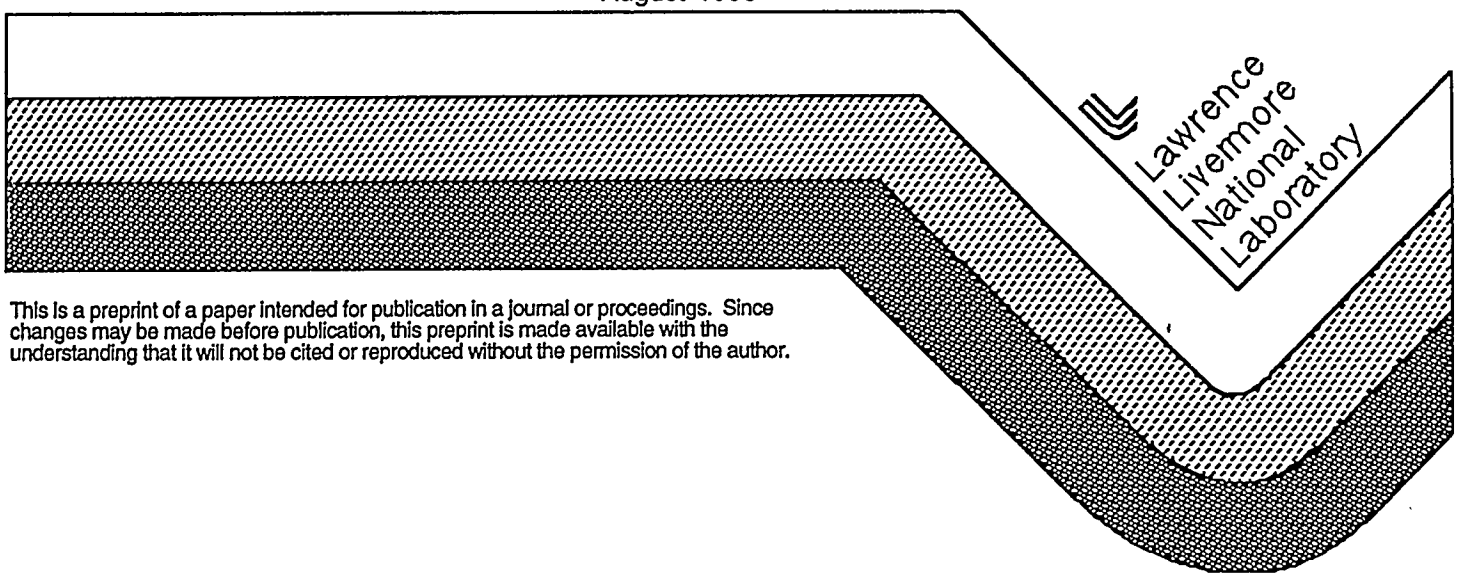

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The Importance of Age and Smoking in Evaluating Adverse Cytogenetic Effects of Exposure to Environmental Agents

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Abstract

Fluorescence *in situ* hybridization with chromosome-specific composite DNA probes ("chromosome painting") is a reliable and efficient method for detecting structural chromosome aberrations. Painting is now being used to quantify chromosome damage in many human populations. In one such study we evaluated 91 unexposed people ranging in age from birth (cord bloods) to 79. We established a baseline frequency of stable aberrations that showed a highly significant curvi-linear increase with age ($p < 0.00001$) that accounted for 70% of the variance between donors. The magnitude of this effect illustrates the importance of understanding the cytogenetic changes that occur with age, which is particularly important for quantifying the effects of prior adverse environmental, occupational, or accidental exposure. In this paper we use the data obtained in our previous study to characterize the distribution of stable aberrations by age and pack-years of cigarette smoking. We also provide estimates of the number of cell equivalents that need to be scored to detect a given increase in aberrations above the background level surveyed in this population.

Introduction

Molecular cytogenetic methods for quantifying chromosome damage have led to significant improvements in the ability to quantify the effects of adverse exposure in humans. Numerous laboratories have shown that chromosome painting is a valid method of quantifying chromosome rearrangements (e.g., 1-7), with the result that painting is now widely used for measuring chromosome damage. Painting is especially useful for evaluating exposures that occurred many years previously because of the speed and accuracy with which stable aberrations (translocations and insertions) can be enumerated. For much the same reason, evaluation of chronic exposures should also be possible with painting.

The most chronic of all exposures are those that last a lifetime. It is reasonable to assume that virtually everyone is exposed on a daily basis to environmental agents that may produce chromosomal damage. Until the advent of rapid methods for evaluating stable cytogenetic damage however, it was not possible to quantify the types of chromosome rearrangements (translocations and insertions) that would be expected to persist through cell division. To determine whether "normal" lifestyle exposures would result in the accumulation of translocations, we previously conducted a study of 91 healthy subjects who reported no significant prior exposures to clastogenic agents. The ages of these subjects ranged from 0 (cord bloods) to 79. We showed that the frequency of stable aberrations in these people increased more than 10-fold with age, and age accounted for 70% of the statistical variation between donors (8-9).

The purpose of the present paper is to present age-stratified maximum likelihood models derived from these data which indicate the number of cell equivalents that must be scored from a putatively exposed individual to determine whether a significant increase in stable aberrations is present. Historically there has been little need for such models because the types of aberrations that were most commonly scored were dicentrics which show little increase with age (8-9). The results shown here can be applied to individuals from populations where physical dosimetry is unknown and the analysis of stable chromosome aberrations is desired.

Materials and Methods

Population studied.

The population has previously been described (8-9). Heparinized blood samples were obtained from 91 healthy people; fourteen samples were from umbilical cords of full-term, healthy babies, and the remaining samples were from adults aged 19 to 79 years. All adults were employees, retirees, or spouses of retirees of the Lawrence Livermore National Laboratory. Each adult completed an extensive questionnaire (available from the authors) inquiring about age and lifestyle factors such as tobacco usage, diet, medical histories and exposure to chemicals or radiation. One parent of each newborn completed the same questionnaire to record *in utero* exposures. All subjects were in normal health for their age with no history of chemo- or radiotherapy. The adverse environmental exposures received by these subjects were judged to be typical and representative of the population as a whole.

Cytogenetic evaluations

Each subject was evaluated for stable chromosome aberrations (translocations and insertions) by simultaneous painting chromosomes 1, 2, and 4. The number of cell equivalents (8) analyzed per subject was at least 1000. All aberrations were recorded according to the PAINT system (10). Reciprocal translocations were thus scored as two separate events, although this is not meant to imply that the derivative chromosomes are mechanistically independent (10).

Statistical methods.

We used both maximum likelihood and minimum chi-square methods to fit a variety of models to our data. Initially we fit a series of linear models with an intercept term, a term for age raised to the power p, where p was varied in integral steps from one to four, and a term for the number of pack-years smoked (set to zero for nonsmokers.) Next we tried an exponential model where the logarithm of the number of stable aberrations was predicted by a linear equation which included terms for age and pack-years. Finally, we considered a biphasic linear model where one slope was used to fit data for ages less than a cutpoint age and another slope was estimated for those over the cutpoint age. This model required estimation of five coefficients: an age zero intercept, a slope for each of two linear portions and the cutpoint age and a coefficient for pack-years.

For the minimum chi-square method each model was fit by minimizing the quantity

$$XSQ = \frac{(X_i - N_i Y_i)^2}{N_i Y_i} \quad , \qquad \text{(equation 1)}$$

where X_i is the observed number of stable aberrations, N_i is the number of cell equivalents scored and Y_i is the number predicted by the model under consideration. Maximum likelihood (ML) fits were based on assuming a Poisson distribution for the number of stable aberrations, X_i. We examined the distribution of Pearson residuals, defined as the square-root of the terms to the right of the equal

sign in equation (1), for each of the fits and used this to determine a reasonable statistical model for the observed data.

This statistical model was then used to predict the number of stable aberrations as a function of the number of cells scored, the age and smoking history of a hypothetical person who would be measured in the future. We also determined an upper 95% confidence bound for the prediction by finding a number which would make the chi-square that is defined in equation (1) to increase to a value equal to k times the upper 5% cutpoint for a chi-square distribution with 1 d.f., where k was determined by the examination of the distribution of Pearson residuals.

To determine the number of cells which must be scored to have a specified power (β) of detecting an increase in the number of stable aberrations (presumably as the result of an exposure) equal to d times the background rate, we used the equation

$$NS = \frac{(Z\sqrt{dk} + \sqrt{kX^2})^2}{Y(d-1)}, \qquad \text{(equation 2)}$$

where Z is the upper $(1-\beta)$% cutpoint for a standard normal distribution, k is the overdispersion factor for the Poisson distributed residuals, X^2 is the upper 5% cutpoint for a chi-square distribution with 1 d.f., d is the background rate multiple, and Y is the predicted background rate of stable aberrations per 100 cells.

Results

A visual examination of a plot of the data (Figure 1) suggested that the number of stable aberrations per 100 cells increased slowly with age until age 45-50 and then increased more rapidly among the subjects in our study. There also appeared to be greater person-to-person variation at older ages than at younger ages. These observations led to consideration of the models described in the statistical methods section of this paper. After fitting all of the models, we found that the model

$$Y = a + b\,(age/100)^3 + c\,(pack\text{-}years), \qquad \text{(equation 3)}$$

with a=0.44 (±0.18), b = 8.06 (±0.70), c = 0.0097 (±0.0042), and Y as the predicted number of stable aberrations per 100 cell equivalents, provided the best fit to our data. The data and the predicted numbers of stable aberrations for nonsmokers and a hypothetical smoker who starts smoking 2 packs a day at age 20 are shown in the Figure 1.

Examination of the Pearson residuals after fitting our data with equation (3) revealed no increase in dispersion with age above that predicted by a Poisson distribution with variance inflated by a factor of 3.6 (i.e., instead of the Poisson variance being equal to its mean, which is increasing with the cube of age, the variance increases by 3.6 times the mean).

The model which assumed that the log of stable aberrations was a linear function of age and pack-years provided a slightly inferior fit to our data. Visual examination of the fit showed that the increase with age was too steep for people under age 50 and too shallow for those over this age. A reasonably close fit to the data was also provided by a model like that given in equation (3) with age squared rather than cubed, but it had the same deficiency as that of the log-linear model.

Figure 1 also shows the upper 95% confidence bounds for the fit provided by equation (3). The compounding effect of smoking 2 packs a day on the upper 95% confidence bound is also shown in the figure.

Tables 1 and 2 show the number of cell equivalents which must be scored to have a specified power of detecting a *d*-fold increase above the background frequency predicted by the fitting equation.

Discussion

Numerous assumptions are implicit when biological dosimeters are used to quantify damage induced many years previously.

(a) *The amount of damage pre-existing in the putatively exposed individual(s) must be known, or at least be estimated from appropriately matched controls.* This is an essential aspect of human exposure studies, because every known genetic endpoint has some (low) frequency of events.

(b) *Clonal expansion and contraction are insignificant.* When proliferation of abnormal cells occurs to a greater or lesser extent than for normal cells, dose calculations will be over- or under-estimated accordingly. Clonal expansion of damaged cells is not expected *a priori* to occur to a different extent than for normal cells, but stochastic processes that appear to produce expanded or contracted clones must be kept in mind. Unfortunately, it is not always possible to determine whether differential cell proliferation has occurred, and as a consequence, clonal expansion is usually ignored, at least for cytogenetic analyses.

(c) *Selection against cells damaged by the exposure does not occur.* This assumption is obviously invalid for cytogenetic studies enumerating dicentrics because cells bearing these chromosomes are unstable through cell division. However, this assumption may be valid for stable aberrations (translocations), but estimates of translocation stability over long periods of time have been more qualitative than quantitative, or have been based on small sample sizes (e.g., 11-13). The development of painting probes for mice (14-17) has led to at least one

experiment designed to provide quantitative estimates of the persistence of translocations (18). This persistence appears to be radiation dose-dependent, although more work must be done.

(d) *Tumor cells are not present in the tissue being analyzed.* As a general rule, tumors have stable chromosome rearrangements (19), and care must be taken to avoid accidental inclusion of these cells in biodosimetry studies. This is not a major concern for most studies, and will be related to exposure only if a sufficient number of years have elapsed. This a problem that could safely be ignored with dicentric-based analyses, but may be encountered and must be borne in mind when evaluating translocation frequencies.

(e) *The influence of other confounding exposures, which may fluctuate with time, are negligible.* This assumption is routinely violated because of the dynamic nature human behavior. One example is cigarette smoking, which is well known to vary in intensity as smokers attempt to quit. The importance of this issue was addressed many years ago in studies utilizing sister chromatid exchanges (20).

(f) *Differences between individuals with respect to the above assumptions are negligible.* Any two people with the same exposure will be assumed to have the same amount of damage at all subsequent times. This may be generally true for special cases (e.g., radiation shortly after exposure), but in general individual differences are likely to be important. The existence of rare genetic disorders (e.g., ataxia telangiectasia) and our rapidly increasing knowledge of human genomics precludes categorical disregard for differences in individual susceptibility. Individual differences in metabolism are well known (21), and an improved

understanding of the involvement of specific genes will become increasingly important for individual risk estimation. The importance of metabolism upon the induction, persistence and accumulation of genetic damage should not be underestimated.

(g) *Changes in the frequency of genetic damage with age must be well characterized.* The effects of aging have been examined for many genetic endpoints and most show at least a small increase with age. Recently, the frequency of stable chromosome aberrations was shown to increase more than 10-fold with age, and age accounted for 70% of the statistical variation between donors (8-9). The magnitude of this effect illustrates the importance of understanding the cytogenetic changes that occur with age.

To the extent that the above assumptions are valid, dosimetry can be accurately performed long times after exposure. However, in some situations, such as the influences of aging, the incorporation of appropriate factors into statistical dosimetry models is required. For cytogenetics this has not usually been done because the most common aberrations analyzed were unstable dicentrics, which show only a modest age effect. With translocations, however, the age effect is large and must be considered.

Clearly it is important to determine the age and smoking history of subjects before determining whether or not their exposure to a suspected toxic agent has caused an increase in the number of stable chromosome aberrations. The effect of age increases dramatically beyond age 50 so it is especially important when designing studies to match exposed and control subjects by age. Matching for smoking history

is almost as important as matching for age since even a moderate amount of smoking, over many years, will substantially increase the expected number of stable aberrations.

The tables show that it is necessary to score thousands of cell equivalents to detect the possible effect of exposure to a suspected toxic agent in young persons. This is due to the low expected frequencies of aberrations in these people. It is easier to detect the effects of exposure in older persons, but there may have been exposures to other (unknown) agents which could lead to a false conclusions concerning exposure to the agent under study. Thus, it is especially important when designing a study of older persons to include a comprehensive survey, by questionnaire or personal interview, of exposures to other possibly confounding agents.

There is considerable person-to-person variation in the frequency of stable aberrations. This variability is reflected in our finding that the variance is 3.6 times as large as that expected based on Poisson "counting" statistics. There may be additional variation within each subject which could be measured by re-examination of subjects at different times. Our results assume that this latter source of variation is no larger than the person-to-person variation. If the within person variation were larger, then the sample sizes in the tables would have to increase.

In summary, we have presented statistical results that can be used to help determine whether a putative exposure in a single individual has produced a significant increase in chromosome aberrations. Although thousands of cell-equivalents must be scored, the inherent speed of molecular cytogenetic analyses is

such that the amount of effort required for an exposure assessment is not unreasonable.

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Table 1. Number of cell equivalents that need to be scored in nonsmokers to detect a doubling or tripling of stable aberrations. **

Background Multiple	2	2	3	3
Power	0.9	0.8	0.9	0.8
age	Number of cells to score	Number of cells to score	Number of cells to score	Number of cells to score
0	11,872	8,279	7,287	4,872
10	11,656	8,128	7,155	4,784
20	10,341	7,211	6,347	4,244
30	7,917	5,521	4,859	3,249
40	5,435	3,790	3,336	2,231
50	3,584	2,499	2,200	1,471
60	2,376	1,657	1,458	975
70	1,616	1,127	992	663

** The first three rows of data are the same as in Table 2 because the model assumes that smoking begins at age 20.

Table 2. Number of cell equivalents that need to be scored in two-pack-a-day smokers to detect a doubling or tripling of stable aberrations. **

Background Multiple	2	2	3	3
Power	0.9	0.8	0.9	0.8
age	Number of cells to score	Number of cells to score	Number of cells to score	Number of cells to score
0	11,872	8,279	7,287	4,872
10	11,656	8,128	7,155	4,784
20	10,341	7,211	6,347	4,244
30	6,104	4,256	3,747	2,505
40	3,861	2,692	2,370	1,584
50	2,554	1,781	1,567	1,048
60	1,751	1,221	1,075	719
70	1,240	865	761	509

** The first three rows of data are the same as in Table 1 because the model assumes that smoking begins at age 20.

Legend to figure

Figure 1. Stable aberrations for 91 subjects aged 0 to 79. Nonsmokers are indicated by open circles (N=51) and smokers by filled circles (N=40). There are results for 14 cord bloods plotted at age 0 (N=8 from nonsmoking mothers and N=6 from smoking mothers). The best fit line for nonsmokers is shown by a heavy solid line; that for a hypothetical smoker who starts smoking 2 packs a day at age 20 is shown as a slender solid line with + superimposed. The 95% upper bounds, based on scoring 1000 cell equivalents, are shown as dotted lines, with + superimposed for smokers.

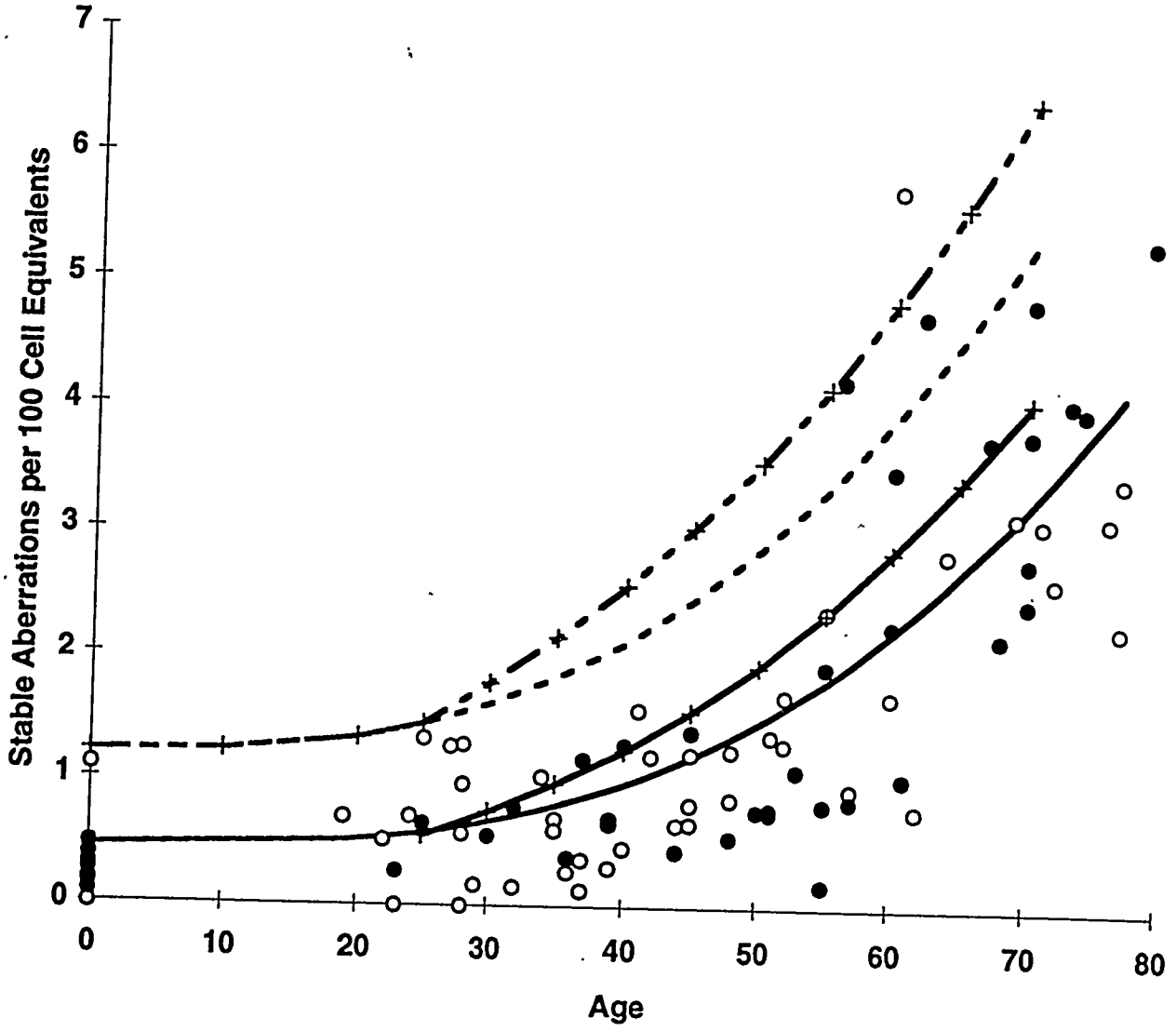

Figure 1

(Original)

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