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ıUIDELINES FOR THE STUDY OF NETIC EFFECTS IN HUMAN ULATIONS

ıed under the joint sponsorship of
ited Nations Environment Programme,
rnational Labour Organisation,
World Health Organization

World Health Organization
Geneva, 1985

The International Programme on Chemical Safety (IPCS) is a joint venture of the United Nations Environment Programme, the International Labour Organisation, and the World Health Organization. The main objective of the IPCS is to carry out and disseminate evaluations of the effects of chemicals on human health and the quality of the environment. Supporting activities include the development of epidemiological, experimental laboratory, and risk-assessment methods that could produce internationally comparable results, and the development of manpower in the field of toxicology. Other activities carried out by IPCS include the development of know-how for coping with chemical accidents, coordination of laboratory testing and epidemiological studies, and promotion of research on the mechanisms of the biological action of chemicals.

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CONTENTS

Page

GUIDELINES FOR THE STUDY OF GENETIC EFFECTS IN HUMAN POPULATIONS

CONTRIBUTORS 7

PREFACE 9

1. INTRODUCTION 11

2. METHODOLOGICAL AND EPIDEMIOLOGICAL ISSUES 13

2.1 General considerations 13
2.2 Methodological issues 13
2.3 Epidemiological considerations - common components 14
2.3.1 Study samples 14
2.3.2 Comparison samples 15
2.3.3 Confounding variables 15
2.3.4 Interaction 15
2.3.5 Methods for estimating exposure 16
2.3.6 End-points 16
2.3.7 Dose-response relationships 16
2.3.8 Time-response relationships 17
2.3.9 Problems in sample size and interpretation of findings 17
2.4 Long-term medical follow-up 18
2.4.1 Relevance and limitations 19
2.4.2 Personal identification 19
2.4.3 Starting-point records 21
2.4.4 End-point records 22
2.4.5 Methods required for integrating source files 22

3. MUTATIONS IN SOMATIC CELLS 25

3.1 General considerations 25
3.1.1 End-points 25
3.1.2 Study requirements 26
3.2 Chromosomal end-points 29
3.2.1 Structural aberrations 29
3.2.1.1 Methods of culture 29
3.2.1.2 Fixation and slide preparation . 32
3.2.1.3 Analysis of cells 32
3.2.1.4 Data analysis 41

Page

3.2.1.5 Radiation-induced aberrations and estimates of exposure . . . 41
3.2.1.6 Chemically-induced aberrations and estimates of exposure . . . 43
3.2.1.7 Conclusions 44
3.2.2 Sister-chromatid exchange (SCE) 45
3.2.2.1 Formation of sister-chromatid exchange 45
3.2.2.2 Relevance of sister-chromatid exchange 45
3.2.2.3 Factors potentially influencing SCE frequency 47
3.2.2.4 Methods for sister-chromatid exchange analysis 52
3.2.2.5 Data processing and presentation 53
3.2.2.6 Conclusions 54
3.3 Gene mutations 54
3.3.1 Principles and basis for the methods . . 56
3.3.1.1 Autoradiographic method 56
3.3.1.2 Cloning method 57
3.3.2 Relevance and limitations 57
3.3.2.1 Relevance 57
3.3.2.2 Limitations 58
3.3.3 Procedures for assay of TG^r T-lys arising *in vivo* in human beings 59
3.3.3.1 Autoradiographic method 59
3.3.3.2 Cloning method 60
3.3.4 Data presentation and analysis 62
3.3.4.1 Autoradiographic method 62
3.3.4.2 Cloning method 62
3.3.5 Conclusions 63

4. GERMINAL MUTATIONS 64

4.1 Introduction 64
4.1.1 Approaches for detecting germinal mutations 64
4.1.1.1 Detection of chromosomal mutations 64
4.1.1.2 The biochemical approach to detecting point mutations . . . 67
4.1.1.3 Indicator phenotypes 68
4.1.2 Methodological considerations and strategies 69
4.1.2.1 Sample acquisition and storage . 69
4.1.2.2 Timing of studies 69

Page

4.1.3 Summary 70

4.2 Germinal chromosomal abnormalities 70

4.2.1 Principles and basis of the method . . . 70

4.2.2 Relevance and limitations 72

4.2.2.1 Studies of induced abortions . . 72

4.2.2.2 Studies of fetal deaths 72

4.2.2.3 Studies of prenatal diagnosis specimens 73

4.2.2.4 Studies of live births 73

4.2.2.5 Studies of indicator phenotypes of chromosomal abnormalities . . 74

4.2.3 Procedures 75

4.2.3.1 Fetal specimens 75

4.2.3.2 Live births and other offspring 76

4.2.3.3 Detection of "indicator" phenotypes for germinal chromosomal mutations - trisomies 77

4.2.3.4 Data presentation 77

4.3 Biochemical approaches to detecting gene mutations in human populations 79

4.3.1 Biochemical methods for monitoring for gene mutations 79

4.3.1.1 One-dimensional electrophoresis 79

4.3.1.2 Two-dimensional electrophoresis 80

4.3.1.3 Enzyme activity 81

4.3.1.4 Other biochemical approaches . . 81

4.3.2 Analytical strategy and methodological considerations 82

4.3.2.1 One-dimensional electrophoresis 82

4.3.2.2 Two-dimensional electrophoresis 83

4.3.2.3 Enzyme activity 84

4.3.2.4 Sample acquisition and storage . 85

4.3.3 Data management 86

4.3.4 Considerations in screening for germinal mutations 86

4.3.4.1 Sample size 86

4.3.4.2 Distinction between "true" and "apparent" mutations 88

4.3.4.3 Implementation of gene mutation screening programmes 89

4.3.5 Summary 89

4.4 Sentinel phenotypes 90

4.4.1 Introduction 90

Page

4.4.2 Basis of method 90
4.4.3 Relevance and limitations 93
4.4.4 Procedures 94
4.4.4.1 Surveillance of sentinel anomalies 94
4.4.5 Data intrepretation 95
4.4.5.1 Surveillance of sentinel anomalies 95
4.4.6 Conclusions 96
4.5 Fetal death 97
4.5.1 Introduction 97
4.5.2 Procedures 98
4.5.3 Data processing and presentation 99
4.5.4 Conclusions 100

REFERENCES 101

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NOTE TO READERS OF THE CRITERIA DOCUMENTS

Every effort has been made to present information in the criteria documents as fully and as accurately as possible. In the interest of all users of the environmental health criteria documents, readers are kindly requested to communicate any errors found to the Manager of the International Programme on Chemical Safety, World Health Organization, Geneva, Switzerland, in order that they may be included in corrigenda, which will appear in subsequent volumes.

In addition, experts in any particular field dealt with in the criteria documents are kindly requested to make available to the WHO Secretariat any important published information that may have inadvertently been omitted, so that it may be considered in the event of updating and re-evaluation of the conclusions contained in the criteria documents.

PREFACE

Monitoring and assessment of effects on human health from exposure to environmental agents of all types, with monitoring to confirm that control measures are working effectively, are key aspects of the World Health Organization's Environmental Health Programme. Without objective data on effects, attempts to assign causes can only be speculative. Speculation does not provide a reliable basis for remedial action and the control of health hazards. Knowledge of the presence and identity of mutagenic agents in the environment and the extent of exposure to them is therefore extremely important in interpreting and using the results obtained from monitoring populations for genetic effects.

Within the World Health Organization, there are a number of programmes that are concerned with the monitoring and assessment of human exposures and effects on health.

Control of environmental health hazards is one such programme. This has the objective that, by 1989, the countries actively involved will have formulated and developed national policies and programmes for the protection of the health of their populations against environmental hazards. A number of projects on the monitoring of air and water quality, food contamination, and of selected pollutants in human tissues are carried out by the World Health Organization. These activities are complemented by a more recent component, the Human Exposure Assessment Location (HEAL) project, which is devoted directly to the monitoring of human exposure to certain pollutants in all environmental media. All of these monitoring projects are implemented in conjunction with the United Nations Environment Programme's Global Environmental Monitoring System (GEMS).

The International Programme on Chemical Safety is a cooperative activity of the World Health Organization, the International Labour Office, and the United Nations Environment Programme. It resulted from concern expressed on the hazards of exposure to chemicals and the pressing need to identify and assess them, so that control measures could be applied and safe use achieved. One of the means of achieving safe use is the preparation and dissemination of documents of practical use to those involved in implementing chemical safety. Publication in the International Programme on Chemical Safety environmental health criteria series emphasizes the importance of a systematic approach to monitoring human populations for genetic effects as an integral part of environmental health management.

These guidelines are intended as a source of practical information on the design and conduct of genetic studies on

human populations exposed, or suspected of being exposed, to mutagenic agents. As they are guidelines, they do no include comprehensive protocols for studies. However, attention is directed to important details that must be included, as well as pitfalls to be avoided. It is envisaged that this document will be of use both to those already involved in the assessment of human genetic hazards and to those who wish to become better informed on this subject. Although future requirements are not ignored, these guidelines principally encompass methods that are considered practicable at present.

The original concept for these guidelines arose from the recommendations of a WHO Consultation on Genetic Monitoring held in Ottawa, Canada, on October 17, 1980, organized by the WHO Collaborating Centre on Environmental Mutagenesis, Department of National Health and Welfare (DNHW) Canada, and held in conjunction with an International Conference on Chemical Mutagenesis, Population Monitoring, and Genetic Risk Assessment. The drafting of the monograph, implemented initially by the late DR K.C. BORA (DNHW), and subsequently directed by DR G.R. DOUGLAS (DNHW), who was also responsible for editing the final draft, began with a WHO/DNHW Consultation held in Ann Arbor, Michigan, USA, on July 27-28, 1981. This meeting produced a draft document containing material that formed the basis for this monograph. Subsequently, a joint WHO/DNHW Task Group Meeting, held in Ottawa, Canada, on September 18-21, 1984, completed the project.

* * *

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1. INTRODUCTION

The extent to which human somatic and germinal mutation frequencies may be increased by exposure to ionizing radiation and to the variety of chemicals that characterize modern societies has been a matter of concern in recent years. Somatic mutations, either genic or chromosomal, are not transmitted to the offspring of an exposed individual. However, increases in the frequency of these mutations may contribute to an increase in the frequency of acquired disorders, for example, cancer. Increases in the frequency of germinal mutations, genic or chromosomal, are likely to contribute to inherited defects in the offspring of individuals exposed to mutagenic agents. There is, therefore, a clear need to develop and apply methods to study exposed populations at risk of increased levels of somatic or germinal mutations.

Any effort to determine whether an increase in mutation rate has occurred in a given population must contend with formidable problems, one of the most significant of which is defining the genetic end-points suitable for study. Germinal mutations, either genic or chromosomal, can give rise to a plethora of phenotypes, only a few of which are useful and meet the criteria required for studies in mutation epidemiology. Likewise, using current techniques, only a subset of somatic-cell mutations are amenable to study. However, since it is much easier to obtain large samples of somatic rather than germinal events, it would be ideal if some measure of germinal mutations and the health effects of their consequent phenotypes could be conveniently extrapolated from studies of somatic events. Although such an extrapolation has been attempted for the effects of ionizing radiation (Brewen et al., 1975), it is not possible, at present, to attempt the same for chemical effects. Despite these difficulties, it is essential that efforts be made to initiate studies aimed at measuring somatic and germinal genetic changes and assessing the relationships between the two.

Although similar mutational changes will occur in somatic and germ cells,the methods for detecting them are quite different. An increase in the frequency of somatic mutations, genic or chromosomal, can be established from relatively few individuals, provided that a large number of cells is analysed from each sample (Bloom, 1981). In contrast, determining an increase in the frequency of mutation in germ cells by examining affected offspring involves large study populations; the smaller the increase to be detected (or excluded), the larger the sample needed (Neel, 1980; Vogel & Altland, 1982). International cooperation may be necessary to

obtain adequate samples. However, because of the costs involved in mounting large-scale epidemiological surveys, it is essential that they should be designed to make the most efficient use of resources and that the tests used should be designed to give meaningful results. The general principles are the same for all countries, and comparability can be achieved through standardization of many of the procedures. Since the practical details of the strategy and tactics in any one country will be influenced by local circumstances, the emphasis in these guidelines will be placed on general principles and examples of possible procedures.

2. METHODOLOGICAL AND EPIDEMIOLOGICAL ISSUES

2.1 General Considerations

Certain general principles influence the design of epidemiological studies for genetic effects and the nature of the procedures for data gathering and analysis. The information requirements are basically similar, whether changes in the genetic make-up of somatic cells or germ cells in the exposed individuals, or inherited changes affecting the offspring of such individuals are being investigated. The key elements for any such study should include:

(a) means of identifying the exposed (or affected) population to be studied;

(b) availability of a reasonably similar comparison group, to serve as the unexposed (or unaffected) controls;

(c) an idea of either the nature of the presumed mutagen, or, at least, of the general source of the anticipated harm;

(d) prior knowledge of the likely end-points that could serve as indicators of genetic damage;

(e) where possible, some separation into different levels of exposure with which to investigate a likely dose-response relationship; and

(f) a means of observing the time course of the response.

Not all of these will be equally available for any study. However, none of the elements that are available should be overlooked in the initial documentation or the subsequent analysis. In general, the need to guard against oversights in necessary information will tend to increase with the interval of time between the causal events and the resulting expressions of harm.

Wherever long-term follow-up is involved, particular attention should be paid to the personal identification of the individuals so that starting-point data and end-point data will be appropriately and unambiguously matched.

2.2 Methodological Issues

Not all studies will have a broad information base at the outset. However, useful work can still be done, even where

there is no prior indication of harmful exposures, no suggestion of which persons might be exposed, and no clear idea of the end-point effects to expect. In principle, watch can be kept over a wide range of potential indicators of genetic damage, among large populations, such as the new-born, and this should be continued over a long period of time. In this broad kind of continuing observation, it is the temporal changes in the frequency of end-points, above familiar background rates, that alert the investigator to a possible mutagenic effect. The term "surveillance" is used to describe this type of activity.

Methods for determining the frequency of a number of end-points are discussed in this monograph. Various techniques are used for determining germinal mutations. Indicators include potential germinal effects, adverse reproductive outcomes, or other health problems that may have a genetic basis.

Because of size considerations, it will often be necessary to combine data from many studies. This will be greatly facilitated if all investigators adhere to standardized laboratory procedures and record-keeping practices including identification of the individuals, with standard nomenclature for causes of death, congenital anomalies, and protein and chromosomal variants.

2.3 Epidemiological Considerations - Common Components

The following comments apply to all methods and end-points.

2.3.1 Study samples

The appropriate epidemiological study design for examining the possible hazards of agents must be determined. A cohort design will be used in most studies where there is known exposure to a suspected mutagenic agent. Alternatively, a case-control study design may be used when exposure is common (e.g., smoking, caffeine), or rare phenotypes are the end-points of interest (e.g., new mutant phenotypes). In a case-control study, groups of individuals are selected on the basis of whether they have the genetic end-point under study. Also, in a case-control study, a number of exposures can be evaluated in relation to a selected end-point, in contrast to a cohort study in which a number of diseases are evaluated in relation to one or more exposures.

In determining the population to be studied, several different approaches can be taken. For example, questionnaires can be used to determine if individuals in a particular setting have been exposed to a putative hazard, and to gather data on possible controls, or existing data sources

(e.g., vital records, census data, employment and work history records, special registries, populations of specific geographical locations) may be used. Regardless of the approach used, sufficient personal identifying information must be recorded regarding the individuals to allow for follow-up and for searching any large files.

2.3.2 Comparison samples

The identification and use of appropriate controls is essential. In both cohort and case-control studies, a basis for comparison is required, in order to evaluate whether the outcome observed differs from that expected, had there not been any increased risk from the agent under study. In certain cases a population may serve as its own control by sampling before, during, and after exposure (internal controls in a longitudinal study). Since the frequency of a given end-point may vary according to age, sex, socioeconomic status, and other variables, it is important that this is taken into consideration when making the comparisons.

2.3.3 Confounding variables

Confounding can be described as the mixing of effects caused by variables that are associated with both the exposures and end-points to be studied, or a distortion of the apparent effect of an exposure on risk brought about by the association with other factors that can influence the outcome. In this regard, the following should be considered:

(a) variables known to cause mutagenic effects should be identified as far as possible (e.g., cigarette smoking, individuals known to have undergone radiotherapy);

(b) cases and controls should be appropriately matched (e.g., for age and sex) in such a way that they have the same distribution with respect to known confounding variables; and

(c) appropriate statistical analysis to partition confounding variables should be used.

2.3.4 Interaction

If an exposure is associated with an increased risk of a particular end-point, it is important to determine whether the risk is additive to that of other known causes of disease, or synergistic in its effect.

2.3.5 Methods for estimating exposure

The following methods can be used to estimate either the levels of exposure, or values that are proportional to the levels of exposure:

(a) estimates of length of exposure as well as ambient levels of the agent may be the best or only estimate of dose in a working environment; it is recognized that such estimates are crude, but, in some situations, they may be all that is available;

(b) direct measurement of an agent in the environment (e.g., air or water sampling) may be used;

(c) direct measurement of an agent or its metabolites in body fluid and tissues to estimate body burden (e.g., blood, urine, hair, teeth);

(d) observation of other pathological evidence of organ or tissue damage (e.g., liver damage, chloracne) may be helpful in estimating doses; and

(e) the use of questionnaires related to work histories and lifestyles may assist in determining the exposure of concern or other exposures.

In some cases, there may be ample time to obtain exposure data, but, with emergency situations, the information may be only transiently available. Thus, it must be collected early and the people involved, recruited, or identified before they disperse.

2.3.6 End-points

Effects of somatic or germinal origin are considered in sections 3 and 4 of this document, respectively.

2.3.7 Dose-response relationships

The existence of a dose-response relationship - that is, an increase in disease incidence with increase in amount of exposure - supports the view that an association is a causal one. Thus, it is desirable to attempt to quantify exposure as far as possible. However, under certain circumstances, in order to make the best use of limited resources, initial studies could be restricted to those with high exposure doses. Individuals so exposed would be compared to an unexposed population. If no differences were found, the study

could be terminated, but it should be noted that, at high doses, excess cell lethality might mask genetic effects. If differences were observed, effects at intermediate dose levels should be studied, paying special attention to possible confounding synergistic effects.

Estimates of doses for populations that have been exposed accidently will most likely be less accurate than for those exposed occupationally and medically. In such cases, it may be more useful to estimate the upper boundary of the dose (i.e., maximum possible dose) rather than the mean, or to stratify the population into 2 or 3 groups whose bounds, though wide, probably do not overlap enough to wipe out the suspected differences between them.

2.3.8 Time-response relationships

Consideration of the optimal sampling time is critical for quantifying the somatic end-points in the exposed individual. Appropriate sampling times should be selected to maximize an effect, whenever this is possible. This is discussed further in section 3.

2.3.9 Problems in sample size and interpretation of findings

The ability to detect an effect of a given incidence will depend on the sample size that can be studied and the base-line frequency of a given end-point in the unexposed population. The more frequent the end-point, the smaller the sample size needed to detect a given effect. The required sample size will also depend on the chosen values of α, the probability of falsely rejecting the null hypothesis, and β, the probability of falsely accepting the null hypothesis. These are sometimes known as Type 1 and Type 2 errors. It is customary, in most experimental situations, to choose $\alpha = 0.05$ and $\beta = 0.20$ (2-tailed test). However, in order to be more certain that a real effect is not missed, then an $\alpha = 0.10$ (or 0.05 for a one-tailed test) and $\beta = 0.05$ could be chosen. However, this choice will increase the sample size required to rule out a given effect incidence.

It is possible, for given values of α and β, to calculate the sample size required to rule out a given effect incidence, for an end-point of known frequency in the exposed population. Conversely, the effect size detectable with a given sample size can be calculated. Table 1 shows the relationship between sample size and effect incidence for a given degree of assurance that the effect is real.

In any study, a negative result should be expressed in terms of the size of the effect that can be ruled out at a given power. For example, "this study rules out a 2-fold increase in the frequency of end-point X, with 80% confidence"

Table 1. Sample sizes needed to detect a given increase in frequency, with $\alpha = 0.05$[a]

Frequency of end-points	Sample size to detect a doubling		Sample size to detect a tripling	
	β = 80	β = 95	β = 80	β = 95
.000001	23 5551 082	39 001 131	7 850 356	13 000 369
.00001	2 355 076	3 900 058	785 021	1 300 011
.0001	235 475	389 951	78 487	129 976
.001	23 515	38 940	7834	12 972
.01	2319	3839	769	1272
.05	435	719	141	232
.10	200	329	62	102
.15	121	199	36	58

[a] Two-tailed probability without correction for continuity. For discussion of continuity correction, and computation formula, see Fleiss (1981).

or "this study has a 50% power to detect a doubling of the frequency of end-point X".

Further considerations of sample size will be discussed under the sections devoted to each specific test end-point.

2.4 Long-Term Medical Follow-up

People need to be identified uniformly on various files in order to monitor human populations for delayed effects caused by exposure to environmental agents. Epidemiological studies are greatly facilitated by the existence in many countries of centralized, computerized, accessible national registries relating to health outcomes.

The kinds of data required are concerned with establishing statistical associations, which may serve as pointers to possible "troublespots", or with investigating suspected exposed groups. An investigation involves defining the population cohort for study, the end-points of interest, and the appropriate study methods for carrying out individual long-term follow-up efficiently (WHO, 1983).

A number of existing population-based files, concerned with health events, are available as end-point records that can be used to measure various health effects among a cohort under study (Bloom, 1981). Normally, follow-up will consist simply of using a starting-point record, in its machine-readable form, to search by computer for an end-point record relating to the same individual or family. Procedures have been developed for doing this and for analysing the results, without loss of personal privacy (Smith & Newcombe, 1980).

2.4.1 Relevance and limitations

Lack of adequate recorded information and of organization and use of historical data in a consistent fashion are two major stumbling blocks in long-term epidemiological or genetic research. The term "record linkage" has been used to describe the process whereby two or more records relating to the same individual, family, or event are brought together. The success of this procedure depends on the quality, quantity, and discriminating power of the items of personal identification on the machine-readable records that are to be brought together.

Existing historical data should be more readily accessible for statistical analysis, but with built-in safeguards so that confidentiality of health records and personal privacy are not compromised. Examples of relevant data are: a) mortality data; b) birth defect monitoring programmes; c) health surveillance programmes; d) vital records, such as marriages and births; e) morbidity records; and f) cancer registries. In addition, where new data are being collected (e.g., on suspected high-risk groups, or on individuals who exhibit "early indicator" conditions), this should be done in such a fashion that it is possible to make international comparisons and to pool data.

2.4.2 Personal identification

Each record needs to contain enough information to indicate unambiguously the particular individual and/or family to whom it refers (Table 2). If a universal numbering system were available for each individual in the population, and were in general use on all medical and vital health records, the problem of matching would almost disappear. In the absence of such a universal number, the record should ideally contain full birth names, birth date, birthplace, sex, mother's maiden surname, and place of residence. Other useful items are full current surname and current address. Since records being linked will seldom have been generated at exactly the same time, and, since some of the items are subject to change with time, it is desirable that the date of the event and the type of event be known. For example, the date of event (e.g., hospital visit) may infer a "last-known alive date", which could substantially reduce the amount of scanning where a mortality search is being carried out.

To identify the family involved, the marital status of the individual, and, if applicable, the spouse's birth surname, forenames, and birthplace should be recorded. In the case of birth, marriage, death, cancer registries, and especially

Table 2. A list of items to be included on starting- and end-point records to facilitate follow-up studies[a]

*	1.	Surname
*	2.	Previous surname (if any)
*	3.	First given name
*	4.	Second and other given names
*	5.	Usual name (or nickname)
*	6.	Sex
*	7.	Birth date (year, month, day)
*	8.	Birth province or country
*	9.	Birth city or place
*	10.	Father's surname
*	11.	Father's first given name
*	12.	Father's second given name
+	13.	Father's birth province or country
	14.	Father's birth city or place
+	15.	Father's birth date (or age)
*	16.	Mother's maiden name
+	17.	Mother's first given name
+	18.	Mother's second given name
+	19.	Mother's birth province or country
	20.	Mother's birth city or place
+	21.	Mother's birth date (or age)
*	22.	Marital status
*	23.	Spouse's birth surname
*	24.	Spouse's first given name
	25.	Spouse's second given name
	26.	Spouse's birth province or country
	27.	Spouse's birth city or place
	28.	Social Security Number or equivalent
	29.	Health Insurance Number
*	30.	Place of residence - province or country
	31.	Place of residence - complete address including postal code
*	32.	Date of event
*	33a.	Last known alive date (e.g., date of last contact)
	33b.	Date hired by company
	33c.	Date left company
	34.	Principal lifetime occupation - type of work, type of business, length of time worked
	35.	Other items where applicable (e.g., birth order of child, status of birth, religion, race, etc.)
*	36.	Control code to indicate the kind of event
*	37.	A control code digit to indicate whether alternate entries for the same event are being recorded (e.g., where an individual may have alternate spellings for surname)
*	38.	A unique number (where no other suitable number is available)
	39.	Where applicable, an indicator to denote whether the individual is known to be dead,the date of death, and the province or country of death

[a] From: Smith (1977).

*,+ Top priority would be given to collecting the items identified with asterisks. For genetic studies, additional parental variables will be required, particularly those indicated with +.
Information relating to diagnostic procedures, work histories, exposure histories, etc., plus updates, are added to the basic record.
For marriages, the record should identify the groom, bride, groom's parents and bride's parents.

genetic registries, it is desirable to have parental birth names, birthplaces, and birth dates (or ages).

Records in files can contain erroneous information and omissions. Thus,in order to reduce the frequency of matching errors, it is necessary to introduce a measure of "redundancy" into the identifying information. Alternative entries can also be created, where the surname of an individual may have an alternative spelling (e.g., adoptions). It may also be useful to record nicknames, race, principal lifetime occupation, and birth order.

2.4.3 Starting-point records

Where information is sought concerning the likely health effects of possible harmful agents in the environment, and of various social and economic circumstances, the chief limiting factor is a shortage of starting-point records in a form suitable for searching death records, cancer registries, etc., for evidence of harm to the exposed individual or his/her offspring.

Collaboration and cooperation is required among a variety of organizations to help resolve this problem (e.g., Regulatory Agencies, Departments of Labour and Health, and research workers). Various laboratory tests and studies are being carried out to predict whether particular chemicals would be likely to cause mutations or cancers in human beings exposed over long periods to low or moderate doses. Human data relating to subpopulations can serve as a potential starting point for a variety of studies, but the records need to be uniformly stored and readily accessible. Studies involving large cohorts can be carried out at a national level. International cooperation is necessary in cases where the numbers are small, and pooling of data is required. An example would be to follow up people who show "early indicator" conditions. For instance, individuals who show chromosomal breakage could be identified, and followed up to see whether they subsequently developed cancer or had reproductive problems (e.g., stillbirths) or defective children. Similarly, children found by survey to have protein variants could be followed up to see whether they developed special health problems or died early.

Starting-point records can comprise a wide assortment of administrative and other kinds of microdata files. These may include *ad hoc* local files (e.g., nominal rolls created using personnel, pay, and pension records), specialized centralized registers (e.g., health surveillance registers), records of exposure to certain agents, records of employment, special survey records, vital registrations, or other sources.

2.4.4 End-point records

A number of health events are routinely documented in forms that are centralized to a certain extent and are in a machine-readable form that lends itself to studies in which a measure of the health impact on a particular cohort is required. Included among such sources are death registrations (Kinlen, 1980; Patterson, 1980; Smith & Newcombe 1980), and special registers of cancer, congenital anomalies, and other handicapping conditions. Secondary sources that might be helpful include marriage records, to give changes of name for follow-up and family composition, and birth records to indicate offspring at potential risk. Both birth and stillbirth records are of particular value, where fertility is of interest, for indicating a potential health effect. Hospital and medical insurance records, may be useful to help convert locally-based registers into population-based ones, and to give a measure of the social burden of disease.

2.4.5 Methods required for integrating source files

Computer methods have been developed to derive family statistics relating to hereditary and congenital disease and terms of other ill health (Newcombe, 1967, 1969, 1977; Smith, 1977, 1980). For example, combining records from the vital registration system, certain specialized disease registers, and records from a universal hospital insurance scheme made multi-generation and cousin studies possible (Table 3 and 4). However, individual follow-up is in much greater demand, primarily for detecting delayed cancers.

For linking an individual with health and other relevant records, a unique identity number assigned at birth would be extremely useful. These are not available in many countries. Where no unique number is available, the searching procedures to be used in bringing records together may involve probabilistic matching techniques. A generalized record linkage system and probabilistic matching procedures have been developed and used for such studies (Smith et al., 1980; Howe & Lindsay, 1981; Smith & Newcombe, 1982).

Linkage projects have been developed or are being planned in a number of international centres (Mi, 1967; Acheson, 1968; Skolnick, 1980; Beebee, 1981; Fox & Goldblatt, 1982; Smith, 1982), which facilitate the establishment of: (a) mortality data bases; (b) cancer incidence reporting systems; (c) death clearance of cancer registers; (d) procedures for supplementing cancer registers with hospital and medicare data; and (e) procedures for constructing better handicap registers.

Table 3. Identifying information available in the magnetic tape files of vital and health records example from British Columbia[a]

Event and its date and place			Father				Mother				Child							
Event	Date (or year*)	City & Province	Surname	Initials	Age	Province of birth	Maiden surname	Initials	Age	Province of birth	Surname	Given names	Sex	Birth order	Full birth date	City of birth	Province of birth	Other[b]
Marriage	X	X	X	X	X	X	X	X	X	X								(1)
Livebirth	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	(2)
Stillbirth	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	(2)
Death	X	X	X	X		X	X	X	X		X	X	X	X	X	X	X	(3)
Handicap	X*										X	X	X		X	X		(4)
Congenital malformation surveillance	X*										X	X	X		X	X		(4)
Hospital Separation	X	X									X	X	X		X			(5)

a From: Newcombe (1977).

b (1) Marital status of groom and bride.
(2) Legitimate or illegitimate status; singleton, twin, triplet, etc.
(3) If married women, own maiden name surname instead of mother's; marital status.
(4) Coded address representing city, school district and provincial health unit area; whether born in home province.
(5) Hospital code and admission number: maiden surname if different; marital status; address as city, street, and number; religion; three initials of head of family, or spouse, or guardian; plus codes for relationship and whether address is the same.

Table 4. Example of the file organization for a sibship history of disease (fictitious)[a,b]

Event (birth order in parentheses)	Year	Father				Mother				Child					
		Sur-name	Initials	Prov-ince of birth[c]	Age	Mai-den sur-name	Initials	Prov-ince of birth[c]	Age	Given names	Sex	Birth year	Birth month	Birth day	Disease
Marriage	1948	Cox	JW	07	25	Bell	MA	09	21						
Live birth (1)	1950	Cox	JW	07	27	Bell	MA	09	23	Annie May	F	50	01	09	
Live birth (2)	1952	Cox	JW	07	29	Bell	MA	09	25	Brian John	M	52	04	14	
Reg. handicap	1952	Cox	-	-	-	-	-	-	-	Brian John	M	52	04	14	Spina bifida
Hospitalization	1955	Cox	-	-	-	-	-	-	-	Brian John	M	52	04	14	Spina bifida
Death	1956	Cox	JW	07	-	Bell	MA	09	-	Brian John	M	52	04	14	Spina bifida
Stillbirth (3)	1954	Cox	JW	07	31	Bell	MA	09	27	Mary Jane	F	54	07	26	Anencephaly

a From: Newcombe (1977).

b Obtained by merging the individual histories of birth, ill health and death, with the sibship histories of parental marriages and births.

c Coded from a 2-digit geographical code for all Canadian provinces and all major countries; 07 = Saskatchewan 09 = British Columbia.

3. MUTATIONS IN SOMATIC CELLS

3.1 General Considerations

General reasons for studying somatic mutation in human populations include:

(a) to determine if an unsuspected introduction of new (or increase in already present) mutagens has occurred;

(b) to evaluate mutation frequencies in populations known (or suspected) to be exposed to mutagens;

(c) to monitor for changes in frequency in populations as a consequence of the removal of mutagens;

(d) to identify groups in the general population with high frequencies of somatic mutations, so that studies can be undertaken to attempt to detect the responsible factors; and

(e) to define the heterogeneity of susceptibility to genotoxic agents within the population.

3.1.1 End-points

Genetic damage can be assayed at the molecular, functional gene, and chromosomal levels. At the molecular level, DNA damage can be assessed in terms of adduct formation, strand breakage and repair, or base sequence alterations. At present, the measurement of adduct formation requires either prior knowledge of the nature of the adduct or limited population sizes, and is, therefore, not currently applicable as an assay for monitoring large populations. DNA strand breakage and repair are usually transient phenomena initiated at the time of insult and completed shortly afterwards, making them less than ideal for population monitoring purposes.

Recent research developments have made it feasible to use restriction enzyme mapping techniques to analyse alterations in DNA base sequence (Botstein et al., 1980; Skolnick & Francke, 1982; Southern, 1982). This approach is being used to define gene structure and identify polymorphisms for linkage analysis. The same approach could equally well be used to screen for mutational changes, but its application must await the availability of appropriate batteries of DNA probes. .

With current techniques, changes in gene function can be detected by the acquisition of resistance to selective agents

or by the alteration or loss of cellular constituents detected by immunological methods. Although these immunological approaches hold considerable promise, the available data are insufficient for application to routine monitoring. On the other hand, methods for the detection of thioguanine-resistant peripheral blood T-lymphocytes have been established and are being refined. A detailed description of the principles and methods of this approach is presented in section 3.3.

At the chromosomal level, genetic damage is observed as an alteration either in chromosome number or in chromosome structure. Although numerical changes represent a significant proportion of human heritable genetic diseases, their consequences in somatic cells are less well characterized. Moreover, estimates of numerical changes in somatic cells are heavily influenced by technical artifacts. Alterations in chromosome structure are more accurately assessed in somatic cells and are observed cytologically as chromosomal aberrations or sister chromatid exchanges (SCEs). These are currently the most readily-available and widely-used end-points for evaluating somatic mutations in human beings. There is extensive literature concerning these methods (Office of Technology Assessment, US Congress, 1983), .which are discussed in section 3.2. Micronuclei, which can result from structural or numerical chromosome aberrations, have been used as an indicator of such damage in human studies. Micronuclei have been enumerated in cultured lymphocytes to detect the effects of ionizing radiation (Krepinski & Heddle, 1983) and in the exfoliated epithelial cells of persons exposed to chemical carcinogens (Stich et al., 1982). However, this method, though showing potential for monitoring genetic damage in human beings, has not yet been accepted or used widely and so is not considered in detail.

3.1.2 Study requirements

Any study attempting to evaluate human populations for somatic cell mutations should satisfy the following requirements:

(a) the study should include exposed groups and appropriate concurrent controls;

(b) specimens from exposed and control individuals should be gathered, handled, transported, and evaluated at the same time and in the same way, using for example, the same culture medium, serum, and reagents;

(c) samples should be coded and evaluated in such a way that the the origin of the sample is not known, at the time of evaluation;

(d) attempts should be made to quantify levels of exposure and to determine dose-response relationships.

However, in some instances, concurrent controls may not be necessary, for example, in longitudinal studies to determine temporal changes in mutation frequencies. In these cases, any possible confounding variables, such as changes in batch or source of media, sera, or reagents used in the study should be minimized. Reagents should be stored and, when there is a batch change, comparative studies of selected individuals (or stored samples) with new and old reagents should be undertaken to determine if there are differences. It is recognized that it may be extremely difficult to find appropriate controls in all instances, because of various confounding factors that can influence somatic mutation frequencies (e.g., age, sex, exposure to ionizing radiation, medication, recent viral disease, smoking, etc). An appropriate control group is one that is matched as closely as possible with the exposed group for these confounding factors.

If it is impossible to find appropriate matched controls for everyone in an exposed group, but it is necessary to study all those exposed, the following alternatives are suggested:

(a) to obtain the best controls available and avoid the most significant confounding factors;

(b) to stratify the cases, prior to analysis, into those with well-matched controls and those with less well-matched controls;

(c) to give the greatest weight in the analysis and interpretation of results to the group with the well-matched controls.

In emergency situations, when appropriate matched controls are not available, it is important to obtain some control specimens that will be sampled, transported, and cultured at the same time as those from exposed individuals. Sources of such specimens are members of study teams, assuming that they have not been contaminated. Each batch of specimens, collected in emergency circumstances and transported to the laboratory, should include a specimen from at least one unexposed individual.

The questions arise as to when, and for how long after exposure to a mutagen, individuals can usefully be studied.

It may be important to follow populations with elevated mutant frequencies sequentially, to determine if such frequencies are changing. Because of the long life span of the circulating peripheral lymphocyte, some types of mutation can be detected many years after the exposure. However, the persistence of these events cannot be predicted with certainty and will vary with exposure duration, agent, and other factors. Thus, for chromosomal aberration and sister chromatid exchange measurements, samples should be taken as soon as possible after exposure. For example, persons receiving chemotherapy can have high frequencies of chromosomal aberration, sister chromatid exchange (SCE), and gene mutation in the lymphocytes. However, in the months following cessation of treatment, the chromosomal aberration frequency decreases, often to a level that may not be significantly different from that in the controls. The frequency of SCE also diminishes in time. For gene mutations, there may be a time interval between the mutational event and the expression of resulting mutants. This interval will vary from days to weeks and may vary with the markers. Thus, following exposure, somatic mutant frequencies may remain the same, decrease, or increase as a result of cell division, selection, or migration *in vivo*. Similarly, persons therapeutically exposed to ionizing radiation show elevated frequencies of chromosomal aberration and gene mutation, but not SCE. In these patients, the frequency of aberrations may remain elevated for many years after exposure. At present, there are few data relevant to the persistence of specific locus somatic mutants. It is recommended that more than one end-point for somatic mutational change be considered in any population study, because each end-point has limitations with regard to the conclusions that may be drawn. For example, negative findings for chromosomal aberrations do not preclude the possibility of increases in somatic mutations of other types. In a population presumably exposed to ionizing radiation, chromosomal aberration studies are most appropriate, but SCE could also be analysed as an indicator of exposure to confounding chemical agents. Conversely, in a population exposed to chemicals, SCE studies are more appropriate in terms of assay sensitivity, but chromosomal aberration studies provide additional important information.

Considerable effort can be expended in subject identification and sample collection; therefore, sufficient blood should be collected at one time to assay several somatic cell mutational end-points. Even though it may be necessary to evaluate a number of end-points in a particular study, it is worthwhile setting priorities for their analysis. For example, slides could be prepared for the determination of both chromosomal aberrations and SCE, but only one end-point

scored initially. This choice should be based on the type of end-point most likely to be elevated. The remaining slides can be scored later, according to the needs of the study.

In addition, blood collected from mutagen-exposed and control individuals should be sufficient to allow cryopreservation of cells and other components for later study. This will make more extensive evaluations possible as well as contribute to the creation of a repository of material from mutagen-exposed human beings for subsequent research.

3.2 Chromosomal End-Points

3.2.1 Structural aberrations

Chromosomal aberrations can be studied in any cycling cell population, or in any non-cycling cell population that can be stimulated by a mitogenic agent to enter the cell cycle. In animals, there are several cell types that fit these criteria, but for human studies, to all intents and purposes, there are only two cell types that are practically suitable. These are the bone marrow cells, which are a cycling population, and the peripheral blood lymphocytes, which are normally non-cycling, but can be stimulated to enter the cell cycle by *in vitro* culturing with a mitogen such as phytohaemagglutinin (PHA). However, because of the ease of obtaining blood samples, in contrast to bone marrow samples, lymphocyte assays have been used in the majority of studies on the induction of chromosomal aberrations in human beings.

Since the observation by Moorehead et al. (1960) that peripheral lymphocytes could be stimulated by PHA to enter the cell cycle and be observed at metaphase, an enormous amount of data has been obtained on the induction of chromosomal alterations by radiation and chemical agents using this lymphocyte assay system (Preston et al., 1981). It is often assumed that this is a simple and informative assay for human population monitoring, providing information on potential clastogenic (or mutagenic) exposures. However, it should be emphasized that such a view should be regarded with caution in the case of supposed chemical exposures, and the obvious distinction from its use for radiation exposures should be appreciated.

3.2.1.1 Methods of culture

There are numerous methods for culturing human lymphocytes. Many components of the methods are optional, and depend on individual preference. Those that are less variable are described here. More complete methods can be found in Evans & O'Riordan (1975), Bloom (1981), and Preston et al. (1981).

It appears to be advantageous to set up cultures from fresh blood samples. However, this is not always possible, usually because samples are taken at some distance from the laboratory and have to be transported. It is still possible to achieve good growth from samples taken several days before culturing. Although samples can be maintained at 4 °C during shipping and storage, it appears to be better to maintain them at room temperature whenever possible (Dzik & Neckers, 1983).

Cultures can be established from whole blood (approximately 0.5 ml per culture), buffy coats, or purified lymphocytes. However, if a large number of samples are to be handled at one time, it is clearly advantageous to use whole blood. For buffy-coat cultures, it is preferable to use the white-cell layer from about 3 ml of centrifuged whole blood, thus larger total blood samples are needed. One of the advantages of buffy-coat cultures is that the cell fixation procedure is not hampered by the large volume of red cells, present in whole-blood cultures, which are often difficult to disperse. A possible disadvantage of whole-blood cultures is that a small quantity of the agent under study, or another contaminating agent, could be carried over with the donor's serum. However, there seems little probability that this would influence the results, and thus, it would not negate the use of whole-blood cultures. It is also important to establish cultures in duplicate, as a precautionary measure against culture failure or sparseness of analysable cells.

A large number of different tissue culture media have been successfully used, and the choice is a matter of personal preference. This is also true for the serum type used, with the added proviso that it should be virus-free. Several different T-cell mitogens are also suitable, PHA being the most frequently used. One additional point concerns the use of tissue culture media containing a low concentration of folic acid. It has been noted that the growth of human lymphocytes in such media (notably TC 199) can result in the appearance of specific chromosome breaks at so-called "fragile sites" (Jacky et al., 1983), or in an increase in aberrations at possible fragile sites (Reidy et al., 1983). It is recommended that, for population-monitoring studies, low folate media should be avoided, though it is suggested that parallel cultures with low folate media could still be used to provide additional or different types of information.

While it is appropriate for each laboratory to use a medium and serum that provides good lymphocyte growth conditions, it is most important that each laboratory should determine the rate of progression of mitogenically-stimulated cells through their first and subsequent cell cycles with their specific culture conditions, using blood samples from several individuals. The rate of cell progression can be

variable, depending on the mitogen, culture medium, serum, and temperature (usually 37 °C in a 5% CO_2/95% air environment).

The progression of cells can be measured by culturing in the presence of bromodeoxyuridine (BrdU), sampling cells over a range of times (for example, every 2 h, from 42 h to 54 h after mitogenic stimulation), and staining the fixed preparations using a technique for obtaining differentially-stained chromosomes (Goto et al., 1978). In this way, the cells that have replicated their DNA once, in the presence of BrdU, will contain evenly- and darkly-stained chromatids. Those that have replicated twice, in the presence of BrdU, will contain differentially-stained chromatids, one light blue and one dark blue, when Giemsa stain is used. Cells that progress through 3 or more cell cycles, in the presence of BrdU, will contain some differentially-stained and some evenly but lightly-stained chromosomes. In studies on chromosome aberrations, it is important to analyse cells in their first metaphase after mitogenic stimulation; thus, a fixation time should be chosen when a high proportion of analysable cells are at the first division stage. Generally, it is not feasible to use a fixation time when all the cells are at this stage, because this requires very early fixation (approximately 42 h after stimulation), when the number of mitotic cells is too low. A compromise time (for example, 48 h) is selected, when, for the average individual, about 90% of the cells are at the first division stage. There is considerable variation from individual to individual in the percentage of cells at the first or subsequent division, even 48 h after culture initiation, and it is good practice to check the proportion of first mitoses in a series of cultures containing BrdU, separate from the series established for aberration analysis. In this way, the possible effects on aberration frequencies of analysing different proportions of first division cells from different samples can be ascertained. It is also possible to analyse chromosome aberrations from cultures grown with BrdU, where preparations have been differentially stained: cells showing no differentiation, and cells that are clearly first metaphase (M1) can then be analysed.

The decision on selecting or rejecting samples with a high proportion of 2nd divisions is optional, but should, at least, be consistent. If resampling is possible, then this should be done using an earlier fixation time than that for the first sample. There is also a possibility that, at the standard fixation time for any particular laboratory, there may be insufficient analysable mitotic cells, as a result of an unusually long cell cycle, either inherent or induced. The only solution to this problem is to resample using a later fixation time.

3.2.1.2 Fixation and slide preparation

Many different methods are available for obtaining conventional, banded or harlequin-stained metaphase preparations, and essentially any one that produces well-spread complete metaphases is acceptable (MacGregor & Varley, 1983). There is little likelihood of this step of the assay influencing the results.

3.2.1.3 Analysis of cells

There are many schemes available for the classification of chromosome aberration types, and comprehensive descriptions can be found in Savage (1975) and Bloom (1981). The following section includes descriptions of the abberations most commonly observed in control samples or samples from individuals exposed to radiation or chemical agents.

(A) Chromosome-type aberrations

(1) Terminal and interstitial deletions

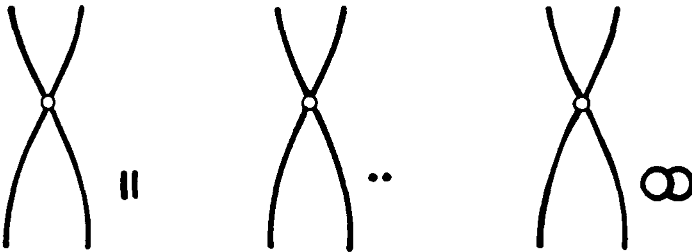


Fig. 1a. Chromosome-type deletions.

It is not possible to distinguish between chromosome-type terminal deletions and non-sister union isochromatid deletions (Fig. 2d), and so, in cases of radiation exposure, when induced aberrations are of the chromosome-type, it is appropriate to classify all paired acentric fragments as terminal deletions (Fig. 1a).

The small interstitial deletions appearing as paired dots are classified as "minutes" (Fig. 1a). The larger interstitial deletions in which there is a clear space in the centre of the ring are usually classified as acentric rings. The distinction is not particularly clear-cut, and, in general, is merely an indication of the different sizes of interstitial deletions. Acentric fragments associated with inter- or intra-changes are not classified as terminal deletions.

(2) Asymmetrical interchanges (usually dicentrics)

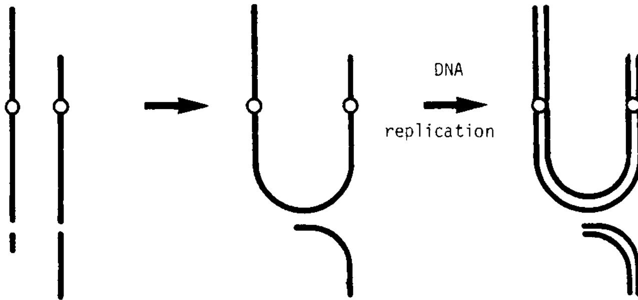
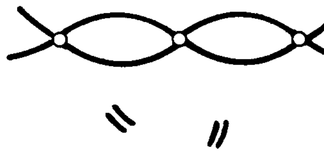


Fig. 1b. Dicentric and acentric fragments.



Tricentric + 2 acentric fragments

Fig. 1c. Tricentric and acentric fragments.

It is assumed that a dicentric, analysed at the first _in vitro_ metaphase will be accompanied by an acentric fragment (Fig. 1b) and a tricentric by 2 acentric fragments (Fig. 1c). A cell with a dicentric and two acentric fragments is, by convention, classified as a dicentric with its accompanying fragment and a terminal deletion. The two acentric fragments could be the result of incomplete rejoining during the formation of the dicentric. However, although this cannot be ascertained, it has been shown experimentally that its probability of occurrence is rather low (< 10%) (Schmid & Bauchinger, 1980).

Asymmetrical interchanges, i.e., dicentrics, can be analysed with greater efficiency than any other aberration type (> 95%), and it is their frequency that is generally used for estimations of radiation dose. For such determinations, a tricentric, for example, is assumed to be equivalent to 2 dicentrics.

(3) Asymmetrical intrachange (centric ring)

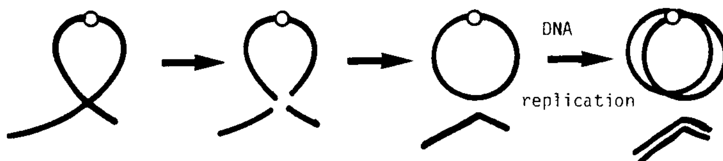


Fig. 1d. Centric ring.

In asymmetrical intrachanges as in interchanges, a centric ring is accompanied by an acentric fragment, and the same classification scheme applies to these as for dicentrics ((2), above) (Fig. 1d).

(4) Symmetrical interchanges (reciprocal translocations)

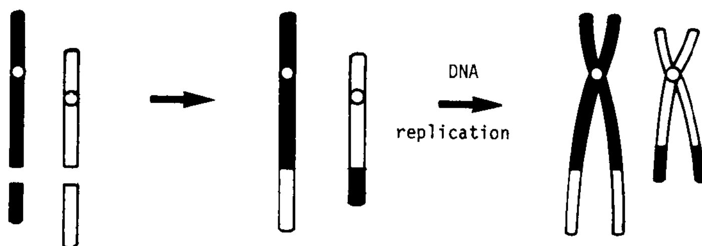


Fig. 1e. Reciprocal translocation.

Symmetrical interchanges (Fig. 1e) are particularly difficult to observe in conventionally-stained preparations, unless the exchanged pieces produce 2 chromosomes, very different from the normal karyotype. However, it is suggested that obvious symmetrical interchanges should be recorded, but giving less weight to their frequency than to that of most other aberration types.

It is not usually possible to observe these symmetrical intrachanges (Figs. 1f, 1g) in conventionally-stained preparations, unless a pericentric inversion produces a chromosome that is distinctly different from the normal karyotype. Any obvious symmetrical intrachanges should be recorded, but less weight given to their frequency than to that of most other aberration types.

(5) Symmetrical intrachanges (peri- and paracentric inversions)

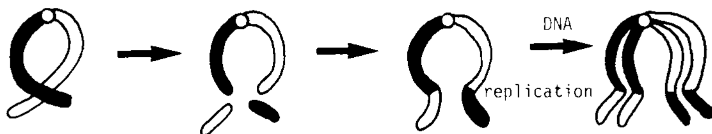


Fig. 1f. Pericentric inversion.

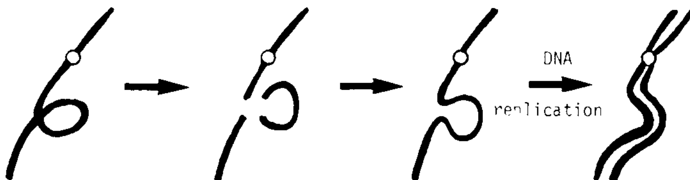


Fig. 1g. Paracentric inversion.

(B) Chromatid-type aberrations

Chromatid-type aberrations are generally classified in the same way as chromosome-type aberrations; the apparent unit of involvement in a chromatid-type aberration is, in most cases, the single chromatid, and not the whole chromosome, as seen for chromosome-type aberrations.

(1) Terminal deletions

A terminal deletion is a distinct displacement of the chromatid fragment distal to the lesion, or, if there is no displacement, the width of the non-staining region between the centric and acentric regions is greater than the width of a chromatid (Fig. 2a). The latter definition is used to distinguish between terminal deletions and achromatic lesions or "gaps" (section (2), page 36).

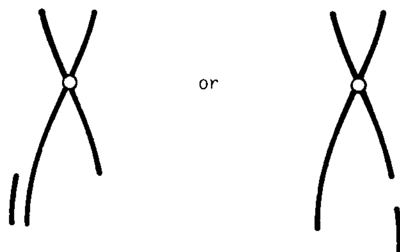


Fig. 2a. Terminal deletion.

(2) Interstitial deletions

Chromatid-type interstitial deletions (Fig. 2b) are not as readily observable as their chromosome-type counterpart, partly because the small deleted fragment is often separated from the deleted chromosome, and is not observed.

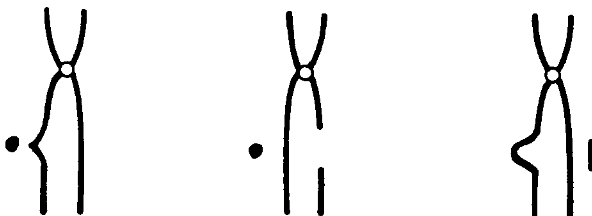


Fig. 2b. Interstitial deletion.

(3) Achromatic lesions ("gaps")

Achromatic lesions or gaps are non-staining or very lightly stained regions of chromosomes, present in one chromatid (single) or, in both sister chromatids, at apparently identical loci (double). If the non-staining region is of a width less than that of a chromatid, the event is recorded as an achromatic lesion (Fig. 2c). This is clearly only a working definition. It is generally suggested that achromatic lesions should be recorded, but always separately from chromatid deletions. Their frequency should not be included in the totals for aberrations per cell, since their significance and relationship to other "true" aberration types is not clear at present.

Fig. 2c. Achromatic lesions.

(4) Isochromatid deletions

Isochromatid deletions appear as exceptions to the class of chromatid-type aberrations, since they involve both chromatids, apparently with "breaks" at the same position on both. However, in suitable material they can be shown to be induced by radiation in the S and G_2 phases of the cell cycle, as is the case for other chromatid-type aberrations.

There are several possible types (Fig. 2d), depending on the nature of the sister unions. If sister union occurs, it is possible to distinguish isochromatid aberrations from chromosome-type terminal deletions.

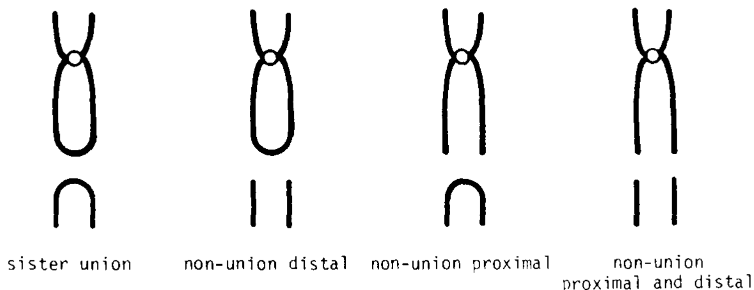


Fig. 2d. Isochromatid deletions.

In mammalian cells, however, sister union is a rare event, and most of the isochromatid deletions are of the non-union proximal and distal type. The acentric fragment is most often not associated with the deleted centric part of the chromosome. The convention for analysis (discussed for chromosome-type terminal deletions, above) is that, for radiation exposures, paired acentric fragments are recorded as chromosome-type terminal deletions, but, for chemical

exposures or control samples, they are most appropriately recorded as isochromatid deletions.

(5) Asymmetrical interchanges (interarm interchanges, asymmetrical chromatid exchanges)

Asymmetrical interchanges are the chromatid-type equivalent to chromosome-type dicentrics (Fig. 2e).

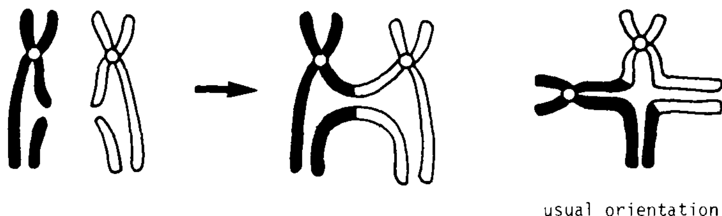


Fig. 2e. Asymmetrical chromatid interchange.

(6) Symmetrical interchanges (symmetrical chromatid exchanges)

Symmetrical interchanges are the chromatid-type equivalent of chromosome-type reciprocal translocations. In the case of chromatid-type symmetrical exchanges, somatic pairing maintains an association between the chromosomes involved in the exchange, and thus they can be readily observed in the absence of any chromosome-banding procedures (Fig. 2f).

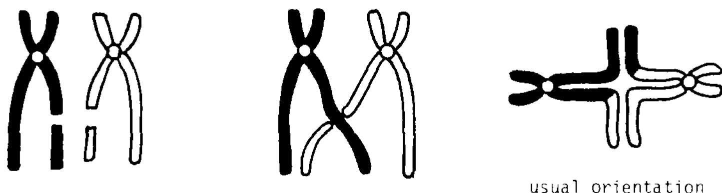


Fig. 2f. Symmetrical chromatid interchange.

(7) Asymmetrical and symmetrical intrachanges (interarm intrachanges)

There are 2 forms of symmetrical and asymmetrical interarm intfachanges but, when analysing metaphase cells, only one of each is distinguishable (Fig. 2g). The symmetrical intrachange can be observed in somatic pairing.

Fig. 2g. Chromatid intrachanges.

(8) Triradials

A triradial (3-armed configuration) can be described as the interaction between one chromosome having an isochromatid deletion and a second having a chromatid deletion. Many types of triradials can be formed but, in mammalian cells, where the frequency of sister union is low, the 2 illustrated are the most common (Fig. 2h).

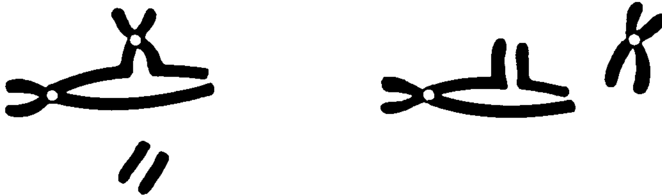


Fig. 2h. Triradials.

It is important to note that the different types of chromosome aberration should be recorded separately. The results for any sample should be presented as the aberration frequency per cell for each aberration type, or frequency of aberrant cells for each aberration type. It is not appropriate to express

the results as total aberrant cells or total breaks per cell, as this method of presentation can hide important facets of the data. For the statistical analysis of data, it is legitimate to combine aberration classes into the general categories of chromatid-type deletions, chromosome-type deletions, chromatid exchanges, and chromosome exchanges. Occasionally, cells are seen with multiple aberrations, and an attempt should be made to analyse such cells in detail, rather than to record them simply in a category "multiple aberration", since this classification will mean a loss of information. If it proves impossible to analyse a cell, it should be recorded in a category of "too many aberrations to analyse", and not included in the total of cells scored.

The selection of cells to be analysed is particularly important, to avoid observer bias. Such bias can result in either a higher or lower measure of the unbiased aberration frequency, depending on the nature of the selection. The generally accepted method of cell selection is to scan slides at low magnification; cells that appear to be suitable are then analysed at high magnification. Once a cell is observed at high magnification, it should only be rejected if: (a) the cell cannot be analysed because of the number or complexity of aberrations; in this case, it should be appropriately recorded, but not included in total cells analysed; (b) the cell is not sufficiently well spread when seen at high magnification, and the overlap of chromosomes prohibits an accurate analysis; (c) non-chromosomal material, such as dirt or stain crystals, not discernible at low magnification, prevent complete analysis; (d) the cell contains fewer centromeres than the observer's acceptable cut-off point; this may be the analysis of cells with 46 ± 2 centromeres or only cells with 46 centromeres, either cut-off point is acceptable, provided that the criterion is consistently applied.

It is particularly important in population-monitoring studies for the samples to be analysed under code, and for the slides from exposed and control individuals to be randomized in such a way that the observer is unaware of the identity of the sample. There are strong arguments in favour of more than one laboratory, with at least 2 individuals in each laboratory, being responsible for the analysis, in order to remove possible laboratory or individual scoring bias, which could influence the results. Microscope co-ordinates of every suspected aberrant cell should be recorded. In order to provide further consistency, the classification of any cell as aberrant by one individual should be confirmed by a second, and it is advantageous to keep a photographic record of all confirmed aberrant or otherwise suspect cells.

The number of cells to be analysed from any sample cannot be fixed with any certainty, because it can be argued that

this number depends on the aberration frequency, and thus is retrospective. A general suggestion would be to analyse 200 cells per sample.

The pros and cons of analysing banded preparations have been discussed frequently, and do not warrant repetition. The amount of time expended on the preparation and analysis of banded samples does not seem to be justified by the increase in sensitivity of analysis for aberration types detectable by banding. However, the choice of scoring conventially-stained or banded preparations should be left to the discretion of each laboratory.

3.2.1.4 Data analysis

Many statistical methods are available for the analysis of data obtained from human population-monitoring studies. The appropriateness of any particular method will depend, to some extent, on the design of the study and the nature of the data collected. Two examples of general approaches are given in Brewen & Gengozian (1971) and Evans & O'Riordan (1975).

3.2.1.5 Radiation-induced aberrations and estimates of exposure

For a variety of reasons, it is sensible to discuss the use of the lymphocyte assay for estimating exposure to ionizing radiation separately from cases of chemical exposure. In some ways, it was fortunate, but, in retrospect, perhaps also unfortunate, that all the original studies on the analysis of chromosomal aberrations in the lymphocytes of exposed or possibly-exposed persons applied to radiation exposure. The fact that, in these cases, estimates of exposure could be made with some accuracy has often led to the assumption that similar exposure-estimating procedures could be applied to persons, groups, or populations exposed environmentally or occupationally to chemical agents. A clear note of caution in accepting this assumption is made here, and will be discussed further.

Ionizing radiation and a small number of chemical agents (e.g., streptonigrin, bleomycin, neocarzinosatin, cytosine arabinoside, and 8-methoxycaffeine) are able to produce aberrations in all stages of the cell cycle including chromosome-type aberrations in G_1 and chromatid-type aberrations in S and G_2.

The peripheral lymphocytes, a subpopulation of which is mitogenically stimulated when blood samples are placed in culture, are essentially non-cycling, and are in the G_0 stage of the cell cycle, the term usually applied to non-cycling G_1 cells. Thus, following radiation exposure,

chromosome-type aberrations will be induced in these non-cycling G_1 lymphocytes, and can be observed at the first metaphase following mitogenic stimulation in culture. The fact that aberrations can be produced in G_1 cells means that their frequency can be directly related to the dose received (i.e., aberration frequency is proportional to dose). In addition, it has been shown for many species (Brewen & Gengozian, 1971; Preston et al., 1972; Clemenger & Scott, 1973), including man (Brewen et al., 1972), that the frequency of aberrations induced in vitro is the same as the frequency induced by the same dose delivered in vivo. This means that a standard dose-response curve, for any particular radiation type, can be obtained for in vitro exposures, and can then be used for estimating doses received by individuals as a result of radiation accidents during medical or other environmental exposures. The frequency of aberrations (usually dicentrics) is measured in cultured blood samples from exposed individuals, and then converted into a dose estimate from the standard dose-response curve. There is extensive literature on the use of the lymphocyte assay as a biological measurement of dose for many different types of radiation (Bender & Gooch, 1966; Lloyd et al., 1983). However, there are still some limiting factors in the use of the assay. These include the length of time of blood sampling after exposure, partial-body exposure, and non-homogeneous exposures.

It is also apparent that the lymphocyte assay can be used for estimating doses following long-term radiation exposure (Evans et al., 1979). Again, this is possible because the aberrations are induced in G_0 cells, and their frequency will be directly related to exposure. Cells containing radiation-induced aberrations as well as normal cells, will gradually be lost from the peripheral lymphocyte pool, as a result of lymphocyte turnover. However, the cells repopulating the peripheral pool will be derived mainly from normal precursor cells, and not from those containing aberrations. Many chromosome-type aberrations are lethal to the cell as the result of loss of acentric fragments at division, or because of the mechanical interference of the aberration with division. Thus, during long-term exposures (months or years), the aberration frequency will not be additive with time (or dose), but will reach an equilibrium where new aberrations are formed and existing ones are lost from the sampled population.

A similar argument holds for determining the length of time after short-term exposure when samples can provide a measure of the maximum aberration frequency or true induced frequency. The lymphocyte turnover will result in loss of cells from the peripheral pool, both normal and abnormal, and repopulation largely by normal precursor cells. The

aberration frequency appears to stay constant for about 6 weeks after exposure (Brewen et al., 1972).

Samples have been taken many years after exposure, for example, with radiation-treated ankylosing spondylitis patients (Buckton et al., 1978) and human beings exposed to the atomic bombs in Hiroshima and Nagasaki (Awa et al., 1978). The fact that some lymphocytes are very long-lived, in excess of 20 years (Awa et al., 1978; Buckton et al., 1978), means that radiation-induced aberrations can still be observed in cells that were present as peripheral lymphocytes at the time of exposure. If it is assumed that the aberration frequency declines exponentially with time, an approximate estimate of the dose received can be made; the word approximate is emphasized.

3.2.1.6 Chemically-induced aberrations and estimates of exposure

On the basis of the success of the lymphocyte assay for estimating radiation exposures, it seemed appropriate to analyse chromosome aberrations in blood samples from individuals, occupationally exposed to single chemicals or complex mixtures (Office of Technology Assessment, US Congress, 1983), to determine whether there had been exposure to a clastogen, and to relate aberration frequencies to dose level. However, the ability to estimate dose levels from aberrations in populations exposed to chemical mutagens is not reliable at present. More research is necessary to make the assay more reliable and applicable, and to make the results more readily interpretable.

The present low sensitivity of the assay for measuring the frequency of chromosome aberrations following chemical exposure is due to the mechanisms of induction of aberrations by chemical agents. These mechanisms may also result in induced aberration frequencies being only indirectly related to exposure dose, in contrast to radiation-induced aberrations.

Chromosome aberrations induced by chemical treatments are almost exclusively produced during the S-phase, irrespective of the cell-cycle stage treated. Thus, the majority of aberrations will be of the chromatid-type, although there are exceptions to this general hypothesis (Evans & Vijayalaxmi, 1980; Preston & Gooch, 1981). Therefore, it is more appropriate to consider that the probability of producing aberrations in G_1 or G_2 cells following chemical treatments is low, but is considerably increased when treated cells are in, or pass through, the S-phase.

Chemically-induced DNA damage in non-cycling lymphocytes will not generally be converted into aberrations until the cells are stimulated to re-enter the cell cycle *in vitro*, and

undergo DNA replication. Since repair of DNA damage can take place in G_0 cells as well as during the long first *in vitro* G_1 stage, the aberration frequency will not necessarily be proportional to the amount of induced DNA damage but rather to the amount of DNA damage remaining at the time of DNA replication. It is clear that the amount of DNA damage, present at the time of replication, which has the potential to be converted into aberrations, will depend on several factors including: (a) the dose received; (b) the induced amount of the particular types of DNA damage that can result in aberrations (a value that can vary with the agent); (c) the amount of DNA repair in G_0 cells before sampling (i.e., time between exposure and sampling); and (d) the amount of repair in G_1 cells from mitogenic stimulation to the first *in vitro* S-phase. Many of these will also be subject to individual variation. The outcome is that, for most chemical agents, only a proportion of induced DNA damage can be converted into aberrations, at the time of replication. These DNA repair factors all work to reduce the sensitivity of the lymphocyte assay for measuring chemical exposure, and will result in the aberration frequency being, at best, only indirectly proportional to exposure.

If an increase in chromosome aberrations is observed in a potentially exposed group when compared with a matched control group, it is reasonable to conclude that there has been exposure to a clastogenic agent, but no estimate of exposure level or of subsequent adverse health effects (genetic or somatic) can be made. On the other hand, if there is no difference in aberration frequency between the possibly-exposed group and a matched control group, it is not possible to rule out an exposure. However, in this case, it may be possible, on the basis of previous experience, to deduce a maximum exposure level that is not associated with a detectable difference in aberration frequency.

3.2.1.7 Conclusions

Future research should enhance the sensitivity of the assay system. In the meantime, it is suggested that care be taken in the choice of groups to be considered for monitoring studies, with particular regard to the usefulness of the information that might be obtained, and to the likelihood of being able to draw definitive conclusions. Cytogenetic monitoring can be an appropriate method for the detection of chemical exposure, but, clearly, it cannot, at present, be considered universally decisive. It should be used in conjunction with other end-points.

3.2.2 Sister-chromatid exchange (SCE)

3.2.2.1 Formation of sister-chromatid exchange

SCE results from the breakage and rejoining of DNA at apparently homologous sites on the 2 chromatids of a single chromosome. They were first visualized by Taylor et al. (1957) who used tritiated thymidine to differentially label the DNA of replicating cells and autoradiography to distinguish the silver grain pattern on the 2 sister chromatids. Advances in cytochemistry over the subsequent 15 years led to greatly simplified procedures for visualizing these exchanges. Latt (1973) demonstrated that bromodeoxy-uridine (BrdU), an analogue of thymidine, when incorporated into DNA, could quench the fluorescence of the fluorochrome Hoechst 33258. Perry & Wolff (1974) found that incorporated BrdU also diminished the uptake of Giemsa stain into the chromatin. In principle, SCEs can be observed in any cell that has completed two, or the first of two, replication cycles in the presence of BrdU. The most common procedure for human lymphocytes is to grow the cells in BrdU for two replication cycles.

SCEs are most efficiently induced by substances that form covalent adducts with the DNA, or interfere with DNA precursor metabolism or repair (Perry & Evans, 1975; Wolff, 1977; Perry, 1980; Carrano & Thompson, 1981; Latt, 1981). It is well documented that SCEs are produced during DNA replication (Wolff et al., 1974) and that the polarity of DNA is maintained in the process of exchange (Taylor, 1958; Wolff & Perry, 1975). The molecular mechanism by which the exchange is formed is not known but, since there is an absolute requirement for DNA synthesis, hypotheses have implicated the mechanics of this process in SCE formation (Bender et al., 1974; Painter, 1980; Cleaver, 1981, Shafer, 1982). Baseline SCE frequencies are increased in some human diseases such as Bloom's syndrome (Chaganti et al., 1974) and multiple sclerosis (Sutherland et al., 1980; Vijayalaxmi et al., 1983a). Cells from patients with the inherited disease Xeroderma pigmentosum are hypersensitive to the induction of SCEs by ultraviolet radiation and alkylating agents (Wolff et al., 1975, Wolff, 1977). There is no clear relation, however, between the repair capacity of these cells and their sensitivity to SCE induction (de Weerd-Kastelein et al., 1977).

3.2.2.2 Relevance of sister-chromatid exchange

An SCE represents the breakage of 4 strands of DNA (2 double helices), a switch of the strands from one to the other arm of the same chromosome, and the rejoining of the strands

in their new locations. The question is whether the breakage and rejoining events occur without producing any modification in the genetic code. There is evidence suggesting that this exchange process might not always be error-free. In particular, a considerable amount of information has been gathered concerning the relationship between induced SCEs and other genetic effects in vitro and in vivo.

Because the technique for enumerating SCEs is relatively simple, this assay has been used quite extensively in genetic toxicology studies. It has been amply demonstrated that the frequency of SCE is dramatically increased when cells, or animals, including human beings, are exposed to known mutagens and carcinogens (Perry & Evans, 1975; Latt et al., 1981; Lambert et al., 1982). Moreover, in early experiments using Chinese hamster cells in culture, it was found that the induction of SCEs was linearly related to the increase in single-gene mutations (HPRT locus), when the cells were exposed to several substances each of which differed in the type of lesion produced in DNA (Carrano et al., 1978; Carrano & Thompson, 1982). The ratio of induced SCE to induced mutation differed for each of the agents, suggesting that each type of lesion formed is processed differently by the cell favouring either the formation of SCEs or mutations. It is possible that the same lesion is capable of producing both an SCE and a mutation and/or that the lesions that form mutations are a subset of those that form SCEs. The induction of mutations has been shown recently to be correlated with the induction of SCEs in mice for one chemical, ethyl nitrosourea (Jones et al., in press). In this study, the induction of SCEs in mouse splenocytes, following intraperitoneal administration of the chemical, was linearly proportional to the increase in mutations at the HPRT locus in the same splenocyte population, suggesting that the mutation-SCE relation is not unique to in vitro studies.

Other investigations compared the induction of SCEs to either the induction of transformation in vitro or induction of tumours in vivo. In a series of experiments, the induction of SCEs was shown to be linearly and positively correlated with the induction of transformation of Syrian hamster embryo cells in vitro (Popescu et al., 1981). This relationship held for exposure to 5 chemicals, but not for X-irradiation. The ratio of induced SCEs to induced transformation frequency varied for each chemical. This is analogous with the results found for the SCE-to-mutation ratio described above. Cheng et al. (1981) examined the induction of SCEs in mouse bone marrow, regenerating liver, and alveolar macrophages following intraperitoneal injection of ethyl carbamate and related chemicals. The relative potencies in inducing SCE parallelled the reported activities for the induction of lung adenomas in

mice. For ethyl carbamate, the doses for the significant induction of SCEs in alveolar macrophages and lung adenomas were similar. Further, the slopes of the dose responses showed that the two end-points were of comparable sensitivity.

The results of early studies on rabbits demonstrated that it was possible to measure an increase in SCEs in peripheral blood lymphocytes for many months following repeated low-level exposure to mitomycin C in vivo (Stetka et al., 1978). Increased SCEs in human beings have been observed in cigarette smokers (Lambert et al., 1978; Carrano, 1982) and in individuals undergoing chemotherapy (Lambert et al., 1978; Musilova et al., 1979; Gillian Clare et al., 1982). The results of these studies suggest that a subpopulation of lymphocytes with high SCE frequencies may be present for long periods after termination of exposure. These cells may represent persistent damage in long-lived lymphocytes or a sensitive subpopulation (Carrano & Moore, 1982). If this is true, they may offer a more appropriate approach to the quantification of persistent damage in vivo.

3.2.2.3 Factors potentially influencing SCE frequency

The baseline SCE frequency in human peripheral lymphocytes averages about 7 - 10 per cell, but has been reported to range from about 2 to 45 per cell in unexposed individuals (Alhadeff & Cohen, 1976; Lambert et al., 1976; Crossen et al., 1977; Galloway, 1977; Carrano et al., 1980). Even within the same laboratory, the baseline frequency may vary by a factor of two or more. Several potential sources of variation have been identified and they generally fall into 2 categories: a) culture factors associated with the in vitro growth of the lymphocytes; and b) biological factors associated with the genotype, lifestyle, or general health of the individual. The biological factors usually cannot be mitigated and must be accounted for in the selection of appropriate control cohorts or ultimately in the statistical analysis.

(a) Culture-associated factors

A major source of variation can be attributed to the concentration of BrdU relative to the number of lymphocytes in the culture. The frequency of SCE has been shown to increase by as much as 50%, when the BrdU concentration is raised 10-fold (Carrano et al., 1980). The ability of BrdU to induce SCE has been well documented in human and other cell systems (Wolff & Perry, 1974; Lambert et al., 1976; Latt & Juergens, 1977; Mazrimas & Stetka, 1978) and it has been further shown that the SCE frequency can increase if the BrdU concentration

is held constant, but the cell number decreased (Stetka & Carrano, 1977). The number of lymphocytes that respond to mitogen and eventually incorporate the analogue is generally not controllable, and, therefore, the effect of BrdU can best be minimized by standardizing both the concentration of BrdU and white blood cells at culture initiation. Since the SCE increase above baseline following most long-term exposures may be small, careful consideration of the influence of BrdU is warranted.

Other culture-associated factors have been examined to determine whether they influence the SCE frequency. One concern has been the optimal time to harvest cells. In at least three studies, it has been concluded that the baseline SCE frequency does not depend on the time of harvest. Carrano et al. (1980) found that, for any individual, the SCE frequency could vary by as much as 30% as a function of culture time, but that the pattern was not consistent among donors. Similarly, Beek & Obe (1979), Becher et al. (1979), and Morimoto & Wolff (1980) did not observe any significant alteration in the baseline SCE frequency as a function of culture time. In a study by Snope & Rary (1979), for 4 individuals, the baseline SCE frequency observed in 58-h cultures was about 30% less than in 70-h cultures. The authors interpreted this to indicate that there were at least 2 subpopulations of lymphocytes that differed in SCE frequency. Studies by Lindblad & Lambert (1981) and Das & Sharma (1984) also revealed that the more slowly cycling lymphocytes had a higher SCE frequency. The possibility that this effect might be due to T- and B-lymphocyte subpopulations has not been completely resolved. Santesson et al. (1979) found that B-lymphocytes had a significantly lower SCE frequency than T-lymphocytes, but Lindblad & Lambert (1981) could not confirm this. In order to optimize an experimental design to account for such an effect, all samples should, where possible, be harvested at the same time.

Two other factors have been shown to influence the baseline SCE frequency, i.e., the serum used and the culture temperature. Using sera from 4 different sources, McFee & Sherrill (1981) showed that the SCE frequency could increase by 50% as the serum concentration increased. Kato & Sandberg (1977) also demonstrated an effect of sera on the production of SCEs. The frequency of SCE in amphibian cells increased almost 5-fold with increasing culture temperature (Speit, 1980). In Chinese hamster cells, both lower (33 °C) and higher (40 °C) temperatures increased the frequency of SCE by approximately 60%. Because the alteration in temperature was only effective in altering SCE frequencies while BrdU was being incorporated, the author concluded that SCEs might arise

via a temperature-dependent disturbance of DNA replication. In a study by Das & Sharma (1984), the frequency of SCEs in human lymphocytes increased as a function of culture temperature and reached a maximum at 40 °C. The authors concluded that temperature-dependent DNA replication enzymes might be responsible for this effect.

In many human population studies, the cohort to be sampled may be far away from the laboratory performing the cytogenetic analysis, and it will often be necessary to ship the samples. If blood is shipped commercially, recording thermometers in the shipping container will provide some quality control. The effect of blood storage on SCE frequency has been examined (Carrano et al., 1980). No consistent alteration in SCE frequency was observed for blood held at either 4° or 22 °C for up to 48 h. This was confirmed by Sharma & Das (1984) for blood stored at 5 °C for up to 7 days, at 22 °C for 3 days, and at 37 °C for 2 days. However, it should be noted that such storage can lead to an overall decrease in lymphocyte numbers and modification of lymphocyte subclasses (Dzik & Neckers, 1983). Thus, the samples should be placed in culture as quickly as possible.

Finally, Deknudt & Kamra (1983) evaluated 4 different mitogens for their effects on SCE frequency. They found that PHA, concanavalin A (Con A), Wistaria fioribunda (WFA), and lentil lectin (LcH-A) extracts did not result in different baseline SCE frequencies in the same donors. However, significant differences among mitogens were noted for lymphocytes that had been exposed to cyclophosphamide or mitomycin C. The use of a single T-cell mitogen, such as PHA, for all studies is therefore recommended. The experience accumulated from the use of lymphocyte cultures suggests that there are many culture-associated factors that could influence the SCE frequency. In order to minimize variation and maximize sensitivity, the use of standardized procedures is essential.

(b) Biological factors

Factors, unique to the individual being sampled, include such confounding variables as sex, age, diet, genotype, medication, and smoking. Each of these potentially plays a role in the induction or expression of SCEs. Although some information is available on the influence of these factors, the consequences are not always clear-cut. Moreover, it is likely that they represent only a small number of possible confounding factors.

Several investigators have compared the baseline frequency of SCEs in male versus female donors and have not observed significant differences (Galloway & Evans, 1975; Alhadeff &

Cohen, 1976; Crossen et al., 1977; Latt & Juergens, 1977; Morgan & Crossen, 1977; Cheng et al., 1979; de Arce, 1981; Waksvik et al., 1981; Carrano & Moore, 1982; Livingston et al., 1983). The absence of clear documentation of a sex difference for baseline SCE frequencies suggests that this factor may not have to be matched in a population study. However, that there is evidence for increased SCE frequencies in women taking oral contraceptives (see below). No studies relating SCEs to cyclic hormonal variation in the female have been reported.

Another variable of concern in population studies is the age of the donor, since it is not always possible to obtain accurate age matching. The independence of age and baseline SCE frequency in lymphocytes in adults has been observed (Galloway & Evans, 1975; Morgan & Crossen, 1977; Hollander et al., 1978; Cheng et al., 1979; Lambert & Lindblad, 1980; Carrano & Moore, 1982; Livingston et al., 1983). Lower SCE frequencies have been found in infants (Seshadri et al., 1982), in cord blood (Ardito et al., 1980), and in children with a mean age of 1.5 years (Husgafvel-Pursiainen et al., 1980). De Arce (1981) reported that people in the age range of 30 - 40 years had a higher number of SCEs per cell than those between the ages of 0 - 10 or 60 - 70 years. Schmidt & Sanger (1981) found a significant increase in SCE with age when comparing age groups of 1 - 18 months, 10 - 13 years, 26 - 32 years, and 63 - 85 years. However, this might be due to differential BrdU incorporation between cultures. With the exception of lower SCE frequencies in new-born infants, the weight of evidence suggests that baseline SCE frequencies do not change with age (at least up to 60 years).

In has been suggested that genetic factors play a role in the baseline SCE frequency. When Cohen et al. (1982) compared SCE frequencies, both within and among 12 pedigrees (2 generations), they found significant differences between, but not within, the families, suggesting a genetic contribution to baseline SCEs. Pedersen et al. (1979) examined the cultured lymphocytes from 11 monozygotic and 9 dizygotic twins of the same sex. They did not find any significant differences in the SCE frequency in monozygotic versus dizygotic twins and concluded that genetic factors do not play a role in baseline SCE frequency. Waksvik et al. (1981) also did not find any genetic contribution to SCE frequencies in their studies on twins. In order to determine whether baseline SCE frequencies were related to ethnic origin, SCEs were measured in adults of both sexes with no interracial family backgrounds from Caucasian, American black, oriental, and native American races (Butler, 1981). There was no significant difference in the average frequency of SCEs in the 4 races. Further research is needed to resolve the issue of genetic factors.

The potential influence of other factors on the frequencies of human SCEs are worth considering. Because a large number of women use oral contraceptives, care must be taken that this does not confound the consequences of exposure to other agents. A study of oral contraceptive users demonstrated a significant increase in lymphocyte SCE associated with the daily intake of d-norgestrel or ethenyl estradiol over a period of 6 - 24 months (Balakrishna Murthy & Prema, 1979). Additional studies are needed to confirm and extend these findings.

Despite knowledge of natural mutagens in food and the role of food processing in creating mutagens (Nagao & Sugimura, 1981; Ames, 1983), little attention has been given to the role of diet in causing baseline SCE variation. It has been reported that severe protein calorie malnutrition in children is associated with increased lymphocyte SCE frequencies (Balakrishna Murthy et al., 1980). Following nutritional rehabilitation, SCE frequencies were seen to decrease. Increased SCEs have also been reported in chronic alcoholics (Butler & Sanger, 1981). For most human studies, however, the nutrition factors are more likely to involve long-term, low-level intake of caffeine, saccharin, other additives, or well-cooked foods. Each of these factors has been shown to be genotoxic in microbial, mammalian _in vitro_, or animal assays, but there are no data on how these might influence SCE frequency in human beings. Dietary information should be collected in the initial questionnaire. Such information may be useful in interpreting a population difference or can be used in large composite data bases to search for diet-related factors. The use of prescription and non-prescription medication, or vitamins, should also be identified and appropriate information should be collected before blood sampling, so that matched controls can be employed.

Cigarette smoking is, perhaps, the most important confounding factor in the interpretation of human SCE frequencies. The preponderance of evidence indicates that individuals who smoke have elevated SCE frequencies (Lambert et al., 1978; Ardito et al., 1980; Hopkin & Evans, 1980; Lambert & Lindblad, 1980; Carrano, 1982; Husum et al., 1982; Livingston et al., 1983). However, this effect has not been observed in all studies (Hollander et al., 1978; Hedner et al., 1983). Since the elevation can be so marked, it is important to control for cigarette smoking in any population study.

Taken together, the culture and biological factors that potentially confound the baseline SCE frequency in human beings are numerous and contribute to individual variation.

3.2.2.4 Methods for sister-chromatid exchange analysis

The procedures for SCE analysis in human populations will depend, to some extent, on the specific circumstances of the study. The following generalized outline of a procedure may have to be modified to fit special situations.

In order to identify any of the factors that may confound the analysis of SCEs, it is important to obtain relevant information on the background of each individual in the study population. This information is best obtained through a questionnaire. The questionnaire should attempt to determine the obvious exposure and potential confounding factors. In addition to the questions related to personal and family cancer incidence, birth defects, known genetic disorders, and current health, there are specific questions related to the interpretation of the SCE results. These include: a) smoking history; b) medication and drug use; c) known or suspected exposures to physical or chemical agents occurring at work, at home, or during the pursuit of various hobbies or recreation; and d) nutrition, particularly the adherence to special diets or the use of artificial sweeteners or caffeine. Because a complete list of agents that may directly or indirectly affect SCE frequencies has not been established, it is better to collect too much information than too little.

When the blood is collected, it should be mixed with heparin, acid-citrate-dextrose, or another appropriate anticoagulant. As already stated, the cultures should be established as soon as possible after the blood is withdrawn, preferably within 24 h. Just prior to establishing the culture, a total white blood count and a slide for a differential should be made from the blood sample. These parameters can serve as indicators of abnormally high or low lymphocyte counts, factors that can have a bearing on the observed frequency of SCEs.

It is important that a standard procedure be employed and, although the exact procedure may differ among laboratories, there are some common requirements. Because BrdU can induce SCEs in human lymphocytes, both the BrdU concentration and the number of lymphocytes potentially incorporating the drug, should be stabilized. In order to ensure this, it is important that each laboratory uses a fixed BrdU concentration and a consistent number of lymphocytes per culture. Selection of culture medium, serum type and concentration, whole blood versus purified lymphocyte cultures, and duration of cultures is somewhat arbitrary and should be based on experience. The serum lot should be pre-tested for its potential to support lymphocyte growth and the reproducibility of SCE frequencies in duplicate samples from the same donor. It is recommended that the same serum lot be used for each population study.

The duration of culture should be such that it yields a high proportion of second division cells so that the SCE frequency is representative of a major fraction of the lymphocytes. Exposure of the cultures to other than yellow or red light should be avoided. Replicate cultures should be established for each individual, and, when a large number of individuals is processed, the inclusion of blood cultures from historical controls is useful.

At the appropriate culture time, the cells can be arrested in mitosis with Colcemid®, colchicine, or other suitable agents. Several slides should be prepared from each culture and the remaining cell pellet can be stored in absolute methanol:glacial acetic acid (3:1) at a temperature of 4 °C or lower, for future recall. The slides can be stained for analysis by fluorescence alone (Latt, 1973), fluorescence plus Giemsa (Perry & Wolff, 1974), or a suitable alternative. Once a staining procedure has been standardized and adopted, it should be used throughout the whole course of the study.

Slides should be coded and scored blind. The coded slides can be scanned under low magnification (100 - 200X) and selected for scoring on the basis of good staining and chromosome number. Only well-differentiated metaphases should be accepted for scoring. It is not essential for all the chromosomes in a cell to be counted. Overlapping chromosomes can be disregarded and the remaining chromosomes scored. At least 43 chromosomes should be analysed per cell. The SCEs are counted for each chromosome in the cell and expressed as SCE per chromosome or converted to the number of SCEs per diploid cell. Although the precise number of cells to be scored per individual will ultimately depend on the desired statistical sensitivity, a minimum of 50 cells should be scored, with 25 cells from each replicate culture.

3.2.2.5 Data processing and presentation

The standard statistical descriptors such as mean SCE per cell or per chromosome, standard deviation or standard error, and number of cells scored should be determined for each individual. For heteroploid cells, the SCE frequency should be normalized to a diploid cell. Population means and their errors should be presented similarly and information on the number of cells scored per sample as well as the number of people per group should be given.

Student's t-test is commonly used for the statistical analysis of population data. When comparing population groups, however, the sample size is the number of people in each group, not the number of cells scored for each group. Caution is advised with the use of t-tests. Individual and/or population means may not be normally distributed and hence the

t-test may be inappropriate. Analysis of variance or appropriate non-parametric statistics may be more rigorous for the particular population under study (Carrano & Moore, 1982). Data transformation can be useful in the statistical analysis, if the mean and the variance are independent (DuFrain & Garrand, 1981). No single statistical procedure can be recommended for universal application. The investigators must carefully examine the data and apply the most appropriate test for the questions being addressed.

3.2.2.6 Conclusions

There are many examples of populations exposed to mutagens in which large increases in SCE have been demonstrated. These studies show that the end-point can be a very sensitive indicator of chemical exposure. In contrast, SCEs are not a useful indicator of human radiation exposure. From the many reported studies, it is evident that further quantitative information on variations in SCE level between people, and the factors that influence these frequencies, are required. For this reason, marginal increases above a baseline SCE frequency must be interpreted with caution. Significantly increased levels are an indication that the population has been exposed to a genotoxic agent; on the other hand, failure to observe increased levels does not necessarily indicate the absence of exposure. Any increase in SCE frequency for an individual or group cannot be interpreted as an indication that the individual or group is likely to suffer adverse health consequences.

3.3 Gene Mutations

New procedures are being developed to detect mutations that lead to the production of abnormal haemoglobins (Bigbee et al., 1981), the loss of cell surface proteins (Bigbee et al., 1983), or the loss or modification of enzymes used in the synthesis of DNA in human somatic cells. The last of these procedures has been used for human population studies and is considered in detail.

Resistance to 6-thioguanine is a marker that has been used widely to detect somatic mutations occurring _in vitro_ in cultured mammalian cells (Hsie et al., 1979). Since similiar somatic mutations occur _in vivo_, quantification of 6-thioguanine resistant (TG^r) cells in peripheral blood provides a measure of specific-locus somatic mutations occurring _in vivo_.

In human beings, a naturally-occurring germinal mutation of the X-chromosomal gene for hypoxanthine-guanine phosphoribosyltransferase (HPRT) (EC 2.4.2.8) provides a

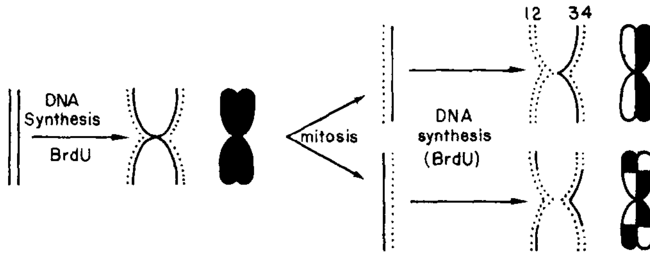


Fig. 3. The formation of sister chromatid exchanges when BrdU is present for 2 cycles of DNA replication.

The solid lines represent a DNA double helix normally-substituted with thymidine in an unreplicated chromosome.

During DNA replication, BrdU (broken line) is substituted in the newly forming daughter strands of each double helix. Since each chromatid contains one DNA strand with thymidine and one with BrdU (singly- substituted), they do not stain differently at mitosis. The metaphase chromosome would appear uniformly dark (shown to the right).

After mitosis, each chromatid enters a different daughter cell and it is singly-substituted with BrdU.

During the subsequent DNA synthesis in the presence of BrdUrd, the newly forming DNA is substituted with BrdU. This results in one chromatid being doubly-substituted with BrdU and the other chromatid, singly-substituted. The singly-substituted chromatid stains dark. The appearance of a second division metaphase chromosome without any SCEs is shown at the top. If SCEs occur, DNA polarity must be maintained. Strand 1 must interchange with 3 and strand 2 with 4. In the lower portion of the figure, 2 SCEs have occurred. The appearance of the metaphase chromosome with these exchanges is shown to the right.

prototype for 6-thioguanine resistance at the somatic level (DeMars, 1971). HPRT normally converts hypoxanthine and guanine to inosine monophosphate, and it phosphorylates some purine analogues (e.g., 6-thioguanine) to produce cytotoxic products (Caskey & Kruh, 1979). Mutant cells, deficient in this enzyme, are unable to use exogenous hypoxanthine as a purine source and are resistant to killing by these purine analogues.

TG^r somatic cells arising in vivo can be shown to be mutants by the stability of their phenotype and deficiency of HPRT activity (Albertini, 1982; Albertini et al., 1982a; Morley et al., 1983b). Also, molecular studies have demonstrated changes in the HPRT locus at the DNA level (Albertini et al., in press).

3.3.1 Principles and basis for the methods

TG^r T-lymphocytes (T-lys), present in human blood, can be measured either by autoradiography to determine TG^r T-ly variant frequencies (Vfs), or by cloning to determine TG^r T-ly mutant frequencies (Mfs). Autoradiography is the earlier method (Strauss & Albertini, 1977, 1979) and has been used for some human population studies (Strauss & Albertini, 1979; Strauss et al., 1979; Cole et al., 1982; Lange & Pranter, 1982; Morley et al., 1982; Vijayalaxmi et al., 1983b; Albertini, 1983; Albertini et al., 1983a); it is simple, inexpensive, and can be automated (Anmeus et al., 1982; Zetterberg et al., 1982). Cloning is a more recent development by which it is possible to recover the TG^r cells for characterization of the nature of the mutations (Albertini, 1982; Albertini et al., 1982; Strauss, 1982; Morley et al., 1983a; Vijayalaxmi & Evans, 1984). This characterization may be necessary in order to quantify the different types of somatic mutations responsible for a collection of mutants.

3.3.1.1 Autoradiographic method

The autoradiographic detection of rare TG^r T-lys is based on the ability of these cells to incorporate tritiated thymidine (HTdR) in vitro following short-term lectin stimulation in the presence of cytotoxic concentrations of 6-thioguanine. The vast majority of human peripheral blood T-lys are in the G_0 stage of the cell cycle in vivo. When stimulated in vitro with lectins, such as phytohaemagglutinin (PHA), T-lys are activated to early G_1, which is characterized by the acquisition of T-cell growth factor (TCGF) receptors on their surfaces (Maizel et al., 1981). These receptors are also called interleukin-2 (IL-2) receptors or Tac antigens (Deeper et al., 1983). A series of cellular events results in the production of interleukin-1, (IL-1) and IL-2, and other factors in short-term peripheral blood mononuclear cell cultures, with IL-2 driving the activated T-lys through G_1 to DNA synthesis and subsequent events in the cell cycle.

The initial round of lectin-stimulated T-ly DNA synthesis in vitro is inhibited by 6-thioguanine. Inhibition probably is at the activation step in 6-thioguanine sensitive cells and occurs without incorporation of the analogue into DNA. The interval of lectin stimulation before the addition of HTdR in the autoradiographic assay is short, so that cell division in vitro does not occur (Strauss & Albertini, 1979). Under

proper conditions, the T-lys that incorporate this label in the presence of 6-thioguanine are TG^r variants. These cells can be enumerated by autoradiography and the TG^r variant frequency (Vf) calculated.

3.3.1.2 Cloning method

Human T-lys are easily propagated for extended periods in vitro by supplying TCGF to cells that have been activated with lectin or antigen (Paul et al., 1981). Thus, mass populations derived from peripheral blood can be developed and maintained. Moreover, freshly-obtained human T-lys may be cloned directly in vitro, in the presence or absence of 6-thioguanine. In the first case, large cell innocula must be used, to ensure the presence of rare TG^r cells (Albertini, 1982; Albertini et al., 1982a; Strauss, 1982; Morley et al., 1983a,b; Vijayalaxmi & Evans, 1984). Activating intervals are short so that cell division does not occur in vitro prior to exposure to cytotoxic concentrations of 6-thioguanine, thus ensuring that the TG^r cells recovered in vitro actually arose in vivo. The number of TG^r T-lys determined by cloning is used to derive in vivo TG^r T-ly mutant frequencies (Mfs).

3.3.2 Relevance and limitations

3.3.2.1 Relevance

The ability to measure TG^r T-lys arising in vivo is important, because it provides a genetic end-point for assessing human genotoxicity that may be qualitatively different from somatic cell chromosomal damage. Thus, the ability to measure gene mutation provides a broader basis for human monitoring. Furthermore, gene mutations, as assessed by TG^r T-lys, are end-points that are qualitatively different from other measures of in vivo DNA damage. Measuring human somatic cell gene mutations occurring in vivo has the following advantages:

(a) The effects of metabolic and kinetic factors are included in measurements of somatic cell mutation occurring in vivo.

(b) Mutagenicity assays for in vivo events assess the genetic effects of given environments, rather than the genotoxic potential of targeted chemicals or other agents. Thus, these assays can be used to measure the effects of environmental mixtures in

which the components and/or their synergistic actions may not be known.

(c) Mutagenicity assays, *in vivo*, may define human population heterogeneity in susceptibility to various mutagens/carcinogens (Vijayalaxmi et al., 1983b). Such individual susceptibilities may be important in assessing human genotoxic risks.

(d) Mutagenicity assays, *in vivo*, have the potential for being developed into a means of making quantitative human genetic health risk assessments, provided that appropriate correlations can be made between individual assay results and individual health outcomes (Fowle et al., 1984).

3.3.2.2 Limitations

Although progress has been made in demonstrating that TC^r T-lys are mutant somatic cells, at least 3 major problems remain for human *in vivo* somatic cell mutation assays. Two are concerned with quantification and apply primarily to gene mutation assays. The third applies to all human somatic cell mutation assays and the relevance of the results for making estimates of human health risks.

(a) The *in vivo* sensitivities of the human TG^r T-ly assays in terms of dose-response characteristics are not known at present. However, because mutant cells are also detectable in animals (Garcia & Couch, 1982; Gocke et al., 1983; Recio et al., 1983; Jones et al., in press), these studies may provide useful information.

(b) Although somatic cell mutations are the events of relevance for human population monitoring, TG^r T-ly assays measure the frequency of somatic cell mutants, not the number of mutations. In order to derive mutation frequencies from mutant frequencies, it is necessary to have information including cell pool sizes and distributions *in vivo*, representativeness of test samples, *in vivo* cell kinetics, and *in vivo* positive or negative selection of mutants. One method for circumventing this problem is to determine whether the mutants scored in a mutagenicity assay are qualitatively heterogeneous, which will yield a minimum number of independent mutational events giving rise to the observed collection of mutants.

The ability to recover TG^r T-lys from clonal assays makes these determinations possible.

(c) Assays of genetic damage occurring in vivo in human somatic cells have not been validated in that it has not been possible to use the results as predictors of human genotoxic health risks. Before such results can be used as surrogate markers for human health outcomes, high-risk populations will have to be monitored and correlations made between the results from mutagenicity assays and epidemiological outcomes. The importance of other factors (e.g., immunotoxicity) in influencing human genotoxic disease risks remains to be assessed.

3.3.3 Procedures for the assay of TG^r T-lys arising in vivo in human beings

3.3.3.1 Autoradiographic method

TG^r T-ly Vfs for normal, control adults, as determined autoradiographically by the method described here, rarely exceed 10×10^{-6}. By contrast, chemotherapy-treated cancer patients frequently have values several times higher (Strauss & Albertini, 1977, 1979; Lange & Pranter, 1982; Albertini, 1983). Other laboratories report somewhat higher normal values, as well as a positive correlation with age (Morley et al., 1982), indicating that local ranges of normal values must be developed. In one laboratory, approximately 75% of placental cord blood values fell within the normal range for adults, while the remainder were elevated (Albertini et al., 1983). Whether this reflects maternal exposure to genotoxic agents, or an altered ratio between mutants and mutations in the fetus remains to be determined.

The basic autoradiographic method for assessing TG^r T-lys has been described in detail by Albertini et al. (1982b) and Albertini & Sylwester (1984). Peripheral blood mononuclear cells (MNCs), which include the T-lys, are separated from whole blood by the Ficoll-Hypaque method (Boyum, 1968). After washing, the MNCs are suspended in a dimethyl sulfoxide-containing medium and aliquoted into ampules for controlled freezing and storage. Freezing is important for holding and shipping samples and serves also as one method for eliminating the phenocopy effect of cycling cells as described below. For assaying, MNCs are thawed, washed, and suspended in appropriately supplemented medium for short-term tissue culture. A minimum of 5×10^6 MNCs are cultured without 6-thioguanine (control culture), while several times this number of cells are cultured with 2 x

10^{-4} M 6-thioguanine (test cultures). All cultures contain phytohaemagglutinin (PHA), for activation of T-lys, and are incubated under standard conditions. After approximately 24 h (Strauss & Albertini, 1979), ^{3}HTdR is added to all cultures, and incubation continued for an additional 18 h. Cultures are terminated by adding cold 0.1 M citric acid, to prepare suspensions of free nuclei. The nuclei are then washed and suspended in methanol-acetic acid fixative, counted, diluted if necessary, and added to coverslips affixed to microscope slides. Slides are dried, stained with aceto-orcein and subsequently autoradiographed by standard methods. Slides are scored and Vfs are calculated as described in section 3.3.4.1.

A subclass of 6-thioguanine-sensitive T-lys in human peripheral blood may become labelled and scored as TG^r (i.e., as variants), when using the autoradiographic method, unless certain precautions are taken (Albertini et al., 1982a,b; Albertini, 1982). These phenocopies occur because, at any given time, a small minority of human T-lys are in an activated state, *in vivo*. These cells have the Tac antigen, when initially put into culture, and do not require the activation step before proceeding to *in vitro* DNA synthesis. Although ultimately 6-thioguanine sensitive, these cells probably are not effectively blocked from accomplishing at least one round of DNA synthesis *in vitro*, even in the presence of 6-thioguanine. Thus, activated T-lys in the peripheral blood constitute a potential source of phenocopies, when TG^r T-ly Vfs are determined by autoradiography. Cryopreservation appears to remove this phenocopy effect by forcing the *in vivo* activated T-lys to proceed to DNA synthesis *in vitro* at a time when a label is not present (Albertini et al., 1982a,b). These cells are not scored as variants. Cryopreservation is a critical step, although other methods, such as prolonged incubation in 6-thioguanine, or immunological elimination of Tac positive T-lys at the initiation of culture, may accomplish the same purpose.

3.3.3.2 Cloning method

From the limited data available, normal control adults have shown peripheral blood TG^r T-ly Mfs of $< 20 \times 10^{-6}$ when based on clonal assays in which single-cell cloning efficiencies achieved values ≥ 0.10. Similar findings from a number of laboratories have been reported (Strauss & Albertini, 1979; Morley et al., 1983a; Vijayalaxmi & Evans, 1984). Results for placental blood and for mutagen-exposed adults remain to be determined. TG^r T-ly Mfs have been slightly higher than TG^r T-ly Vfs, concurrently determined (Albertini et al., 1984).

The cloning method for assessing TG^r T-lys has been described by Albertini et al. (1982a). Peripheral blood MNCs are obtained from whole blood as for the autoradiographic method, but freezing is not required. The MNCs are incubated with PHA in order to activate the T-lys. The duration of activation is sufficiently short to ensure that cell division does not occur. Activated cells are then innoculated in limiting dilutions into the wells of microtitre plates, in the presence or absence of 6-thioguanine (approximately 10^{-5} M). In addition to the appropriate medium, wells contain an optimum concentration of crude TCGF and feeder cells. X-irradiated B-lymphoblastoid cells are well suited for use as feeders. A TG^r lymphoblast line is used to avoid interference with subsequent HPRT enzyme assays of the recovered TG^r T-lys. An average of 1 activated cell per well is innoculated into the non-selection wells (containing no 6-thioguanine); 10^5 activated cells per well are innoculated into selection wells (containing 10^{-5} M 6-thioguanine). Wells are scored after approximately 2 weeks of culture with one change of medium, by microscopy, by scintillation spectrometry of HTdR incorporation (added during the last day of culture), or by cell transfer and clonal expansion. *In vitro* cloning efficiencies (CEs) of T-lys are determined from wells receiving, on average, 1 cell per well. By assuming a Poisson distribution of cells in wells, the average number of clonable cells per well is derived from the P_0 class of that distribution, i.e., the observed fraction of wells without growing cells. Similarly, the incidence of TG^r cells in wells receiving 10^5 activated T-lys in 6-thioguanine is determined from the P_0 class of wells in the plates containing 6-thioguanine. The TG^r T-ly Mf is the incidence, divided by 10^5, corrected for the CE.

TG^r T-lys obtained by cell transfer and clonal expansion may be characterized for T-ly subset markers, stability of the TG^r phenotype, HGPRT enzyme activity, and, by using suitable cDNA probes, for the nature of the molecular lesion at the DNA level (Albertini et al., 1982a, in press).

For reasons not known at present, human T-lys, activated *in vitro* as described, may fail to clone as efficiently when present as single cells than when present in large numbers (i.e., 10^5 cells/well), even when the latter are selected in 6-thioguanine (Albertini et al., 1984). Clonal assays with a single cell CE of < 0.10 have the potential for yielding falsely elevated Mf values, because of an underestimate of clonable cells in selection wells. Current research is directed at increasing and/or standardizing single-cell CEs. At present, however, only Mfs determined in clonal assays achieving single cell CEs of 0.10 or more should be considered valid.

3.3.4 Data presentation and analysis

3.3.4.1 Autoradiographic method

Slide scoring is done by one of 2 methods that give almost identical results. One method involves determining labelling indices (LIs) for both control (no 6-thioguanine) and test (6-thioguanine containing) cultures for each individual. The LI of test cultures (LI_t) is determined by counting all labelled nuclei on all slides made from test cultures, and dividing this number by the total number of nuclei (determined in suspension) added to all slides.

$$LI_t = \frac{\text{Number of labelled nuclei on all test slides}}{\text{Number of nuclei on all test slides}}$$

The LI of control cultures (LI_c) is determined from a differential count of 2500 nuclei on slides from control cultures.

$$LI_c = \frac{\text{Number of labelled nuclei per 2500 nuclei}}{2500}$$

The TG^r T-ly Vf for each individual is calculated from the LI of test cultures (LI_t) divided by the LI of the control culture (LI_c).

$$Vf = \frac{LI_t}{LI_c}$$

An alternative method of scoring autoradiographic assays has recently been described for mouse TG^r T-lys (Gocke et al., 1983), and is equally applicable to human assays. Statistical methods for deriving confidence intervals for variant frequencies are described by Sylwester & Albertini (1984).

3.3.4.2 Cloning method

Cloning efficiencies (CEs) are calculated from the P_0 class of the Poisson distribution:

$P_0 = e^{-x}$, or

$x = -\ln P_0$ (control)

where x is the average number of clonable cells per well. When CE is determined by single cell innocula, x = CE.

The incidence of TG^r T-lys in selection wells, receiving on average 10^5 cells per well, is also determined from the P_0 class of the Poisson distribution:

$$P_0 = e^{-y}, \text{ or}$$

$$y = -\ln P_0 \text{ (test)}$$

where y is the average number of clonable TG^r T-lys per well.

The TG^r T-ly Mf is determined from the incidence of TG^r T-lys per well divided by the average cell innocula, corrected by the CE. When 10 cells are innoculated into selection wells:

$$Mf = \frac{-\ln P_0 \text{ (test)}}{CE \times 10^5}$$

Appropriate statistical procedures for handling Mf data, differences between Mfs, etc. are being developed by methods analogous to those referred to in section 3.3.4.1.

3.3.5 Conclusions

TG^r T-lys arise *in vivo*, are present in human peripheral blood, and their frequencies are measurable. Known mutagen exposure increases the frequencies of these cells and their characterization *in vitro* has shown them to be somatic mutants. Thus, methods are available for measuring specific locus somatic mutants occurring *in vivo* in human beings, for purposes of human genetic monitoring. The 2 assays described here continue to be developed. There are potential technical sources of error. Among the limitations in the interpretation of the results of these assays is the fact that the numbers of somatic mutants, and not the numbers of somatic mutations, are being measured. The latter may be of most interest for human population monitoring. However, characterization of the recovered mutant cells, which is possible in the cloning assay, may make it possible to estimate the minimum number of mutations responsible for a given number of mutants.

Methods described here can be applied to the detection of mutations, occurring *in vivo* at other genetic loci in human T-lys, in order to broaden the base for human monitoring.

4. GERMINAL MUTATIONS

Methods and technical aspects of approaches available for detecting germinal mutations in human populations are reviewed in this section. It should serve as a guide, when such studies are initiated, and as a reference for defining specific methods for estimating human mutation rates.

4.1 Introduction

Germinal mutations include a spectrum of alterations in either the structure or quantity of DNA in germinal cells. The study of germinal mutations is the quantification of transmitted genetic damage. Within the context of monitoring for induced genetic damage, this is an important consideration, because it is the offspring of exposed individuals, rather than the exposed individuals themselves, that are the focus of concern. It is generally assumed that a significant proportion of all mutations have deleterious effects on both the health and the genetic constitution of future generations. Thus, the increased genetic risk and associated health effects are of ultimate concern, following exposure to a putative mutagen, when possible increases in germinal mutation rates are being ascertained.

Germinal mutations are usually classified in 2 categories. Chromosomal mutations are operationally defined as changes in either chromosome number or structure observable with standard karyotypic techniques. More minute changes in DNA structure are classified as gene mutations, often referred to as "point" mutations.

4.1.1 Approaches for detecting germinal mutations

The 3 approaches to monitoring for germ cell mutations are generally divided as follows: (a) chromosomal, (b) biochemical, and (c) the indicator phenotype; each detects a different set of end-points.

4.1.1.1 Detection of chromosomal mutations

Germinal chromosomal mutations occur in about 5% of recognized conceptions (Hook, 1981a,b), making them more amenable than the rarer specific locus mutations for study in small exposed populations. Unbalanced chromosomal complements are almost always associated with deleterious phenotypic effects, leading to fetal death, livebirths with anomalies and mental retardation, and/or sterility. Balanced chromosome complements may occur as mutations without phenotypic effect.

However, their deleterious effects will be seen primarily in the next generation in individuals with unbalanced chromosomal complements.

Chromosome abnormalities can originate in four different ways: a) they may be inherited from a parent; b) they may result from errors during gametogenesis (e.g., meiotic non-disjunction); c) they may result from events during conception (e.g., dispermy resulting in triploidy); d) they may result from events after conception (e.g., mitotic non-disjunction). Only the second category listed above is regarded as a germ-cell mutation in the strict sense, but to avoid unnecessary complexity in the discussion that follows, triploidy and tetraploidy will be considered in the review of germinal events.

Chromosomal mutations can be subdivided into numerical and structural anomalies.

(a) Numerical aberrations

Numerical chromosomal abnormalities include trisomy, monosomy, triploidy, and tetraploidy. Most instances result from events that occur during gametogenesis in a parent, or at the time of fertilization, although strict proof of the time of origin is often lacking.

Apart from triploidy and tetraploidy, numerical chromosome abnormalities involving sex chromosomes without a mosaic 45, X cell line, or involving autosomes are usually presumed to have resulted from a germinal mutation. The only exceptions are situations in which numerical abnormalities occur in only a single tissue, as for example in some malignancies. It is often difficult, however, to exclude formally the possibilities that they: a) have been inherited from a parent who is a cryptic mosaic for the abnomal line; or b) have resulted from a somatic event, mitotic non-disjunction, early in the development of the organism.

The strongest risk factor known for numerical abnormalities is older maternal age, which is highly associated with the frequency of trisomy (Hassold et al., 1980, 1984). Paternal age seems to have little, if any, effect (Hook, in press). In studies using chromosomal markers, it has been demonstrated that at least 60% of trisomy 21 appears to be the result of maternal 1st division non-disjunction (Juberg & Mowrey, 1983).

Numerical abnormalities almost always occur in offspring of parents who have normal chromosomal complements, and thus, such abnormalities are presumably the result of germinal mutations. Although there is not a great deal of experimental or epidemiological evidence to link numerical chromosome abnormalities with environmental agents, increases in such

anomalies must be considered as possible outcomes of exposure to possible mutagens. An unkown proportion of trisomy or monosomy may result from post-zygotic non-disjunction, or, in the case of trisomy, may be inherited from parents who are cryptic carriers, and thus may not be the result of germinal mutations in a strict sense. For trisomy, the results of studies of the parental origin of the extra chromosome suggest that a maximum of 25% of trisomies are due to post-zygotic non-disjunction, and it may well be considerably less (Juberg & Mowrey, 1983).

Evidence suggests that mosaic trisomies (47/46) do not originate from post-zygotic events any more often than non-mosaic trisomies (Hassold, 1982).

Ninety percent of numerical anomalies in recognized conceptuses terminate as fetal deaths; thus, a study restricted to live births will miss a major proportion of detectable abnormalities.

Only a few kinds of numerical anomalies commonly survive to birth. The most common is Down's syndrome, trisomy 21, occurring in about 1 in 1000 live births. Other numerical anomalies such as trisomy 18 and trisomy 13 sometimes survive to occur as live births, but these are much less common.

(b) Structural aberrations

The proportion of individuals with detected structural cytogenetic abnormalities is likely to vary with technical factors. Recent advances in the resolution of chromosome substructure (high resolution banding) have reduced significantly the size of detectable lesions and such advances appear likely to continue. Thus, it is difficult to specify precisely the proportion of recognized conceptuses with a structural abnormality. With currently available techniques, these are much less frequent than those with numerical abnormalities. Among live births, the ratio of detected structural to numerical abnormalities is about 1:4 to 1:5 (Hook & Hamerton, 1977). Among fetal deaths, the ratio is much lower, about 1:30 (Warburton et al., 1980). Unlike numerical abnormalities, a significant fraction of detected structural abnormalities are known to be inherited, so that inferences concerning mutation are not possible unless both parents are studied and found not be be carriers of the aberration present in the offspring. The possibility of false assignment of paternity must also be considered. Unlike trisomies, structural chromosome abnormalities, in general, show little if any association with parental age, although markers and X-isochromosomes may represent exceptions. In instances in which the parental origin of a structural mutation has been investigated, many have been found to be

predominantly paternal in origin, unlike trisomies (Magenis & Chamberlin, 1981).

In general, mosaicism, involving a normal cell line and a cell line with a structurally abnormal chromosome, is not attributable to germinal cell mutation, but may be safely inferred to be of post-zygotic origin (supernumerary markers are, however, exceptions).

In terms of sensitivity to environmental factors, mutant germinal structural chromosome aberrations are likely to be more similar to germinal specific locus mutations than numerical abnormalities, as both involve alterations in the structure of the genetic material. Germinal chromosomal rearrangements, unlike specific locus mutations, show little association with advanced paternal age in human beings (Hook, in press) indicating that there are etiological differences between these effects.

4.1.1.2 The biochemical approach to detecting point mutations

Many biochemical approaches for the study of germinal mutations have been proposed (Bloom, 1981), but most are not feasible at present. Two general approaches are currently used to study the proteins in offspring of selected individuals. Electrophoresis is used to detect a significant proportion of amino acid substitutions while enzyme activity measurements are used to detect major losses in protein function or protein quantity.

Studies involving genetic typing of large numbers of individuals, either to analyse population structure or to develop mutation monitoring programmes, have demonstrated the feasibility of large-scale screening using one-dimenional electrophoretic techniques (Harris et al., 1974; Neel et al., 1980a; Altland et al., 1982). Through the introduction of high-resolution two-dimensional electrophoresis (Klose, 1975; O'Farrell, 1975), the number of proteins that can be studied in a single sample has been increased to several hundred. The search for mutational events resulting in loss of a functional gene product, using quantitative techniques, is complementary to the electrophoretic assay, in that this assay detects genetic events not normally detectable by standard electrophoretic assays. Both the electrophoretic and enzyme activity approaches have been used in studies to determine the induced mutation rate in exposed mice (Johnson & Lewis 1981; Johnson et al., 1981; Bishop & Feuers, 1982) and *Drosophila* (Racine et al., 1980). Thus, it should be possible to acquire data on the mutation rate in human populations and also to examine directly some of the problems associated with extrapolation from experimental animals.

One other biochemical approach has been proposed for detecting mutational events in human populations. It is possible to study mutations at the DNA level by employing the restriction enzyme mapping approach. One potential advantage of the restriction enzyme mapping approach (or other techniques that directly monitor specific alterations in DNA structure) is the ability to examine larger portions of the genome and thereby acquire increasing amounts of data from each individual. Since this approach is currently in the developmental stage, appropriate samples should be retained, as far as possible, to take advantage of this or other techniques that may be developed in the near future.

The major constraints in relation to the biochemical approach include: (a) the large number of determinations and, to date, the large populations necessary to detect statistically-significant increases in the mutation rate; (b) the distinction between "apparent" mutations and nonparentage; and (c) the problem of confirming that a suspected variant is the result of an alteration in DNA structure, i.e., is a transmissible trait.

4.1.1.3 Indicator phenotypes

Three types of indicator phenotypes are considered in this section: Down's syndrome, fetal death, and sentinel phenotypes. Down's syndrome will be discussed in section 4.3, which deals with chromosomal mutations. The potential use of fetal death as an expression of genetic damage arising from genic mutations will be discussed in section 4.5. Although the term "sentinel phenotype" is relatively new (Sutton, 1971), the notion of studying mutations in human beings by counting the frequency of dominant traits is credited to Danforth (1921). Mulvihill & Czeizel (1983) have reviewed the current status of the concept.

As indicators of germinal genic mutations, sentinel phenotypes are a significant health problem and are recorded in a variety of health facilities. The sentinel phenotype is a clinical disorder that: (a) occurs sporadically as a consequence of a single, highly penetrant mutant gene, (b) is a dominant or X-linked trait of considerable frequency and low fitness, and (c) is uniformly expressed and accurately diagnosable with minimal effort, at or near birth. Individuals with such traits are important for the surveillance and monitoring of germinal genic mutations because affected persons of unaffected parents arise from a new mutation. Their use has severe disadvantages connected with the difficulty of accurate nosological diagnosis arising from their genetic heterogeneity and a general lack of clinical expertise. The sentinel phenotype approach, like

other strategies in mutation epidemiology, is burdened with problems created by the inability to link separate data files, the necessity to maintain confidentiality, and the difficulty in collecting sufficiently large study populations or study samples. The best course of action for the present is to obtain field experiences and to sustain critical discussion of the approach.

4.1.2 Methodological considerations and strategies

4.1.2.1 Sample acquisition and storage

Future access to study subjects may be limited; therefore, the maximum quantity of sample, usually blood, should be obtained on the initial contact. When more than one test is to be performed, attempts should be made to coordinate the appropriate collection procedures and minimize the number of different acquisitions. In situations where fetal tissue is being collected for chromosomal studies, it is suggested that, where possible, a sample of kidney tissue should be collected and stored for possible biochemical analysis.

Adequate samples should be stored for confirmation of any observation and also for future analysis using new and/or refined techniques. Lymphocytes should be stored in a manner that allows for the retrieval of viable cells and the subsequent expansion of the number of cells, thus providing material for future studies.

4.1.2.2 Timing of studies

With specific regard to mutation studies, following suspected exposure to mutagens, women of child-bearing age who have been exposed to a mutagen and wives of exposed men should be identified and surveyed as soon as possible. Questionnaires or interviews should be undertaken and used to obtain data on basic biological and demographic variables (and information on other possible mutagenic factors). The factors include, but are not necessarily limited to, age, race, previous pregnancy history, smoking, and drug use. Women should be encouraged to participate in a continuing evaluation of future pregnancy outcomes. If a woman thinks she is pregnant but is uncertain, pregnancy testing should be carried out as soon as possible. A control population of similar ethnic and socioeconomic background should be identified. This could be done at the time of identification of women at risk of pregnancy, or could be done after pregnancy is confirmed, using as a control group, women pregnant at the same gestational age.

Each of these approaches has difficulties. To wait until the exposed woman is pregnant before selecting a matched

(pregnant) control may result in the pregnancy of the exposed woman being terminated by the time the control is chosen. It may be extremely difficult to avoid biasing the results towards a more favourable outcome in the controls. If controls are selected prior to the pregnancy of the exposed woman, there is no certainty that the exposed and control women will become pregnant at the same time, if in fact they conceive at all. One possible approach is to select several possible controls for each exposed woman, before pregnancy, and follow the reproductive history of all of them. Ideally, these controls should be matched as far as possible in age, race, socioeconomic status, and previous reproductive history.

Infants, children, and young unmarried males, who have been exposed should also be identified early and followed for possible inclusion should a subsequent long-term study be undertaken (see section on record linkage).

Women recruited into the study should be interviewed by investigators, at least once a month, to ascertain if they are pregnant or think they may be pregnant. If there is any question, pregnancy testing should be carried out.

4.1.3 Summary

Given the current state of knowledge in the area of human mutations, all 3 approaches, namely indicator phenotype, chromosomal, and biochemical, to estimating germinal mutational damage in human populations should be considered complementary. They detect different types of genetic "damage" or "end-points", have different degrees of relevance for estimating potential health effects and probably different sensitivities. Therefore, a collaborative monitoring exercise should employ all 3 in order to obtain the maximum amount of data possible.

4.2 Germinal Chromosomal Abnormalities

4.2.1 Principles and basis of the method

Chromosomal aberrations associated with germinal cell mutation have already been discussed in section 4.1.1.1.

Depending on their origin and consequences, chromosomal mutations may be detected in gametes, the embryo or fetus, in live births, or at later stages in life.

If a population is exposed to a mutagen, the most direct method of detection of germinal chromosomal mutants would be examination of the gametes themselves. At present, there are no methods for evaluating human ova. Preparations of human sperm chromosomes can be made, but these methods are difficult and time-consuming, and few laboratories have yet been

successful with this technique (Martin et al., 1983). It is possible that improved techniques for studying sperm chromosome constitution will be available in the future, but, at present, this method cannot be regarded as practical for most situations involving mutagenic exposures. Also, at the present time, alterations in sperm morphology or function cannot be regarded as a reliable index of germinal cell mutation, nor can changes in the proportion of sperm showing double "Y" bodies. Thus, the following discussion focuses on detection of chromosomal mutations in the offspring of those exposed to known or suspected mutagens.

A clinically-recognized pregnancy is generally diagnosed after the first missed menses, at about 4 weeks of gestation. The frequency of chromosome abnormalities at this point in gestation has been estimated to be about 5% (Hook, 1981a). About 15% of recognized pregnancies terminate in fetal death, approximately one-third having a chromosome aberration (Harlap et al., 1980; Hook, 1981a). The rate of loss between conception and recognized pregnancy is not known, but it is generally agreed to be very high (Kline et al., 1980). The proportion of these early losses that is associated with chromosomal aberration is unknown. The bulk of the evidence from the use of sensitive human chorionic gonadotropin serum assays, capable of diagnosing pregnancy immediately after implantation (usually 7 days after conception), suggests that loss between implantation and the first missed menses may be at least as common as loss afterwards (Miller et al., 1980; Edmonds et al., 1982), but not all results are in agreement (Whittaker et al., 1983). Preimplantation losses are still unmeasurable. Only clinically-recognized pregnancy in the usual sense will be dealt with here, since the other types of study are unlikely to be feasible on the necessary scale, and chromosome studies cannot be performed on these early losses.

The proportion of chromosome abnormalities in fetal deaths varies with gestational age, being highest (about 50%) in the 8 - 12 weeks range, and then decreasing with gestational age (to about 7%) at more than 22 weeks (Warburton et al., 1980). The kinds of chromosome abnormalities found in fetal deaths include many rarely, or never, seen in live births, e.g., triploidy, tetraploidy, and trisomy for most whole chromosomes. Monosomy X, though occurring in only about 1 in 20 000 live births, occurs in about 7% of early fetal deaths; triploidy occurs with about equal frequency, as does trisomy 16, an anomaly never seen at term and compatible only with very rudimentary embryonic development (Warburton et al., 1980).

Chromosome abnormalities are found in about 7% of late fetal deaths (including still births) (Sutherland et al., 1978). At birth, the proportion has been found to be 0.6% (Hook & Hamerton, 1977). About 90% of all recognized

conceptuses with chromosome abnormalities terminate as fetal deaths.

Most recognized abnormalities involve numerical aberrations that are presumptive mutants. Structural chromosome abnormalities make up only about 5% of the chromosome abnormalities seen in fetal deaths (Warburton et al., 1980). About 40% of structural abnormalities found in fetal deaths or at amniocentesis are de novo mutational events: the rest are inherited from a carrier parent (Jacobs, 1981; Hook et al., 1984).

4.2.2 Relevance and limitations

4.2.2.1 Studies of induced abortions

Chromosome studies of induced abortions are a useful source of data. Such investigations are, of course, only possible where induced abortion is a legal option. Advantages are that the frequency of chromosomal abnormalities is still high at the point in gestation when most induced abortions are performed, that the specimens are likely to be viable in culture, and that the procedure can be scheduled for collection purposes. A disadvantage is that it is likely to be more difficult to obtain specimens from the controls than from the exposed population. If, for example, exposed women were more likely than controls to provide specimens of early abortions, the unadjusted rate of chromosome abnormalities would appear, incorrectly, to be higher.

4.2.2.2 Studies of fetal deaths

Studies in which the products of conception from fetal deaths are examined cytogenetically are likely to be the most productive in terms of the proportion of chromosome abnormalities that are detected. However, such studies are difficult because of problems in specimen retrieval, culture failure, and the high cost of the tissue culture procedures required. Usable specimens will be obtainable from only a portion of cases, even with the best retrieval systems, since viable fetal tissues are sometimes not present at the time of expulsion. Organization of retrieval systems can be a challenge, even in such ideal situations as a teaching hospital, and are extremely difficult outside a medical setting. Through special education, attempts can be made to collect specimens from women having early abortions, where the tissue is passed at home, and medical attention may not be sought. In New York City, an interview study suggested that 40% of women having a first trimester fetal death did not seek medical attention (Kline et al., 1981). Factors affecting the

probability of specimen retrieval must always be examined to rule out biases that would affect the outcome of the study.

When cytogenetic analysis is performed, further case loss occurs because not all will be successfully karyotyped. This might produce differences in studies done in different laboratories or at different times. Thus, cases and controls should be studied at the same time, in the same laboratory. Ideally, the laboratory carrying out the study should not know if a specimen is from a control or a case.

One further problem in interpreting studies of fetal death is the possibility that an environmental agent might influence not only the rate of occurrence of a genetic abnormality, but also the probability that a conceptus with an abnormality can survive to a particular point in gestation. If an exposure reduces the viability of a conceptus with a chromosome abnormality, such as monosomy X, so that it is lost even earlier in gestation, before recognizable pregnancy, a reduction in that anomaly will be seen in all recognized conceptuses. An exposure that postpones the fetal death of a conceptus with abnormality from before the usual recognition of pregnancy to later in gestation would result in an increased proportion of detected abnormalities.

4.2.2.3 Studies of prenatal diagnosis specimens

In some locations, amniocentesis for prenatal diagnosis is widely available. This procedure is usually done at 16 - 20 weeks of gestation. In such areas, many women exposed to putative mutagens might seek such procedures, though a much smaller proportion of controls might do so. While data available from such an outcome should be used in any analysis, this cannot be regarded as a plausible sole source of pertinent data on chromosome abnormalities. It should be noted that, if all women in a population undergo amniocentesis, the expected proportion of chromosomally abnormal fetuses is 1%.

4.2.2.4 Studies of live births

Studies of live-born infants and older children are easiest to undertake, and cheapest to perform. However, because only a small proportion would be expected to be affected (0.6%), a relatively large-scale study would have to be undertaken to detect a statistically-significant increase in the proportion affected. A sample of 25 000 births is needed to detect a doubling of the trisomy rate at birth ($P \leq 0.05$)[a].

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[a] For the purposes of discussion, cases with an extra sex chromosome have been included.

Some inferences are possible in relation to large exposed populations, based, in part, on phenotypic evaluation. Cytogenetic study for mutation investigation might, for example, be carried out only on children with major malformations at birth, i.e., about 2%. In any event, such studies would be indicated on this group of children for clinical reasons. This approach would reveal primarily only unbalanced autosomal abnormalities, about 1/3 - 1/2 of all chromosomal abnormalities in live births (both structural and numerical abnormalities would be detected). Such a restriction in a cytogenetic study would result in perhaps 10 - 20% of those evaluated being found to have an abnormality. This reduces 50-fold the total number of cytogenetic studies required in the population, but does not avoid the need for a large original exposed population from which the selected group with malformations would be drawn.

4.2.2.5 Studies of indicator phenotypes of chromosomal abnormalities

Inferences in large populations might be possible on the basis of simple enumeration of Down's syndrome individuals, 98% of whom are likely to be trisomic or carry a de novo chromosome rearrangement. The expected frequency is about 1 in 1000 births. Similarly, cases of Patau syndrome and Edwards syndrome (resulting from 47, +13 and 47, +18, respectively) might also be enumerated. The expected frequencies, however, are much smaller (each about 1 in 10 000 live births (Hook & Hamerton, 1977), and the phenotypes are not as useful an indication of karyotypic abnormality.

Some conclusions on the probability of karyotypic abnormality can be reached through morphological examination and classification of specimens from fetal death. A useful classification scheme, suggested by Byrne (1983), provides categories reflecting the degree of organization of development, as well as developmental age. These categories range from an "empty sac" with no visible embryo, to an apparently normal fetus greater than 30 mm. Studies associating specimen morphology with karyotype have shown that, while about 1/2 of the most poorly-developed specimens have a chromosome abnormality, less than 2% of fetuses greater than 30 mm in length and with no visible malformations externally, will have a chromosome abnormality (Byrne, 1983). Thus, if resources are limited, such specimens might be left unstudied without much loss of information.

True hydatidiform moles represent a category of conception where the chromosome constitution is usually 46,XX, reflecting 2 identical male haploid complements in an egg with no maternal chromosomes. Although other rare karyotypes occur,

single morphological examination is sufficient to indicate the chromosome abnormality with over 98% accuracy.

4.2.3 Procedures

4.2.3.1 Fetal specimens

In a prospective study, women can be provided with sterile containers containing a balanced salt solution for collection of specimens from early fetal deaths. Later fetal deaths are likely to reach medical attention, and collection systems must be organized at likely treatment areas. Specimen containers should also be available at such possible collection points as clinics, emergency rooms, and wards.

(a) Morphological examination

Specimens should be examined externally and described in a classification scheme that reflects the degree of development as well as developmental age. Fetal autopsies can be performed on specimens of 30 mm or more. A careful description of the classification scheme must be provided, with photographs, whenever possible, of abnormal specimens.

(b) Karyotyping

Successful cultures can be obtained, up to 5 days after expulsion, though such a delay is not recommended. Thus, transport of samples over some distance is possible. As the products of conception are almost never obtained in a sterile state, the use of culture medium for storage is not recommended, since it will encourage the growth of contaminants. Specimens should be kept refrigerated (but not frozen), during storage or transport over long distances.

Specimens from induced abortions should be obtained in a sterile container from the operating room or clinic. Care must be taken to prevent the usual routine fixation of such specimens.

A most important aspect of the procedure is the careful examination of the specimen to ensure that only fetal tissues are taken for culture. Some specimens will contain only decidua, and cannot be used. Any fetal tissue can be used for karyotyping; if available, fresh tissues from the embryo or fetus are most desirable; however, the actual embryo may be very small for the length of gestation, very macerated, or absent altogether. In this case, fetal membranes (amnion and/or chorion), or placental villi, carefully dissected away from maternal tissues, may be used. Maternal cell contamination is an inevitable possibility in such studies,

but evidence suggests that it is infrequent in experienced hands (Warburton et al., 1980; Hassold, 1982).

Fetal tissues may take from a few days to several weeks to reach the stage where they can be karyotyped. Cover slip preparations with in situ chromosome preparations allow faster karyotyping, but may not yield as large a number of analysable cells as cultures in flasks, which must be trypsinized before harvesting (Byrne, 1983).

Banded chromosome analysis should be carried out on all specimens, and on at least 10 cells from each culture analysed. Mosaicism is not uncommon among cultures from spontaneous abortions. More cells must be counted if a non-modal cell, not accounted for by random loss, is found among the first 10 cells, e.g., if a normal cell is found in an otherwise trisomic culture, or a trisomic cell or a 45,X cell is found in an otherwise normal culture. In general, the proportion of mosaics among all abnormalities detected, is expected to be small (Warburton, 1980). For the purposes of initial analyses, it is suggested that they be classified with non-mosaic aberrations with the same abnormal line.

Almost all instances of numerical chromosome abnormalities are presumably the result of a mutation in the most recent generation. Thus, if monosomy, trisomy, triploidy, or tetraploidy is discovered, then study of the parents to confirm a mutation is not necessary, as the likelihood of mutation is perhaps 0.99. However, this is not the case for structural cytogenetic abnormalities. At about 18 weeks of gestation, for instance, only about 40% of structural abnormalities are the result of a recent mutation, 60% being inherited from carrier parents. Thus, if a structural rearrangement is observed, study of the parents should be undertaken to determine if this is the result of a mutation first manifest in the offspring.

4.2.3.2 Live births and other offspring

Chromosome studies will normally be performed on PHA-stimulated lymphocyte cultures from specimens of peripheral blood. Specimens should be collected as soon as possible after birth in order to minimize losses of neonatal deaths. Cord blood can be used, and is easy to collect routinely.

Preservation of whole blood samples by deep-freezing (-70 °C.) in DMSO will make possible a good retrieval of cells for chromosome analysis (or other kinds of studies) for up to a year (Nakagone et al., 1982). Furthermore, specimens can be collected and stored before laboratory facilities have been organized for the study, and, if necessary, can be transported to a laboratory some distance away.

If a structural rearrangement is found, parental studies are necessary.

4.2.3.3 Detection of "indicator" phenotypes for germinal chromosomal mutations - trisomies

Cases of Down's syndrome can be identified through medical records, vital statistics, registries, or education settings. Identification is likely to be incomplete through any of these routes, and care must be taken that both exposed and unexposed women are equally likely to be discovered.

4.2.3.4 Data presentation

Results in those exposed should initially be presented according to broad categories of events (Table 5) and also within subcategories, when sample size allows, because different chromosomal abnormalities may reflect different etiological factors. Trisomy 21 and trisomy 16 may well result from different mechanisms, for example, while increased triploidy might indicate increased dispermy.

Data should be presented on "conceptions" followed from detection of pregnancy, and results compared by category between exposed and control groups. Results should be expressed as the proportion of either the exposed or control group with a specific abnormality. Data should be stratified by maternal age, or other adjustment made for this variable, because of the very strong association of maternal age with trisomies (Hassold et al., 1980, 1984). In addition, exploratory analysis should be undertaken of other possible confounding variables such as ethnicity, socioeconomic status, cigarette smoking, and alcohol consumption. Any differences between exposed and control groups in these variables should be adjusted for in the analysis by sample stratification, or multivariate analyses. Results should also be analysed according to whether the father, the mother, or both parents have been exposed. If sufficient data are available by exposure category, a dose-effect relationship should be sought. With regard to structural rearrangement, cases in which one or both parents cannot be studied should be scored separately, and regarded as possible mutants. The mutation rate for structural rearrangements should be reported as a range. The minimum boundary excludes such cases as being of unknown status. The maximum boundary includes such cases.

It should also be recognized that lack of an observed difference during the first few years after exposure does not preclude the possibility of late manifestations of effects. In one study, it has been claimed that a radiation effect on

Table 5. Types of germinal chromosomal abnormalities

Numerical Aberrations: all presumed mutants

Monosomy (almost entirely X monosomy)

Trisomy	16
	21
	others

Triploidy[a]

Tetraploidy[a]

Structural aberrations[b]

Unbalanced: Robertsonian

deletions and rings
markers and fragments
other

Balanced:

Robertsonian
inversions
reciprocal

[a] Most triploidy results from dispermy, and tetraploidy from mitotic non-disjunction. Thus, they might not be regarded strictly as germinal cell mutations.

[b] Both parents must be shown to have normal chromosomes, before a case can be regarded as new mutant.

trisomy 21 frequency first manifested itself 10 years after exposure (Alberman et al., 1972).

A problem that may arise in the evaluation of results is that of missing data. Pregnant exposed and control individuals may die, or withdraw before the outcome of the pregnancy is established. Moreover, if a fetal death occurs, tissue may not be collected and, if collected, the specimen may not include fetal tissue. Even if fetal tissue is set up in culture, the karyotyping procedures may not be successful. In experienced laboratories, the proportion of successful cultures has varied from 65% to 90% (Warburton, 1980). Moreover, culture failures are likely to be preferentially higher for fetuses with chromosome abnormalities because the greatest proportion of culture failures occurs in specimens with poorly "organized" morphogenesis, which have been dead for some time *in utero*. Among specimens cultured successfully, the proportion of cytogenetic abnormalities in specimens with poorly organized morphologies is at least 50%, at least twice as high as that of specimens whose

morphogenesis is more normal (Byrne et al., in press). Assuming that this occurs also among unsuccessful cultures, then those of unknown outcome will contain a higher proportion of chromosomally abnormal specimens than successful cultures.

Another problem arises if the effect of exposure to a suspect substance is not to increase the proportion of conceptuses with abnormalities but only to alter the proportion of conceptuses whose karyotypes can be determined. If, for example, such a detection effect occurs selectively more often on fetuses of abnormal karyotype, then there will be a bias towards an apparent mutagenic effect of the substance.

For these reasons, inferences about differences between exposed and control populations (on the basis of comparison of the observed proportion of the abnormal among those studies) require explicit assumptions about the conceptuses of unknown karyotype. The characteristics of this group, for example, the proportion of them among the total number of conceptuses in the sub-populations, their fetal pathology, and their maternal age association, should be investigated closely. If there are striking differences in the nature of the conceptuses of unknown karyotype between exposed and unexposed populations, great caution is necessary in drawing conclusions from comparisons involving conceptuses of known karyotype.

4.3 Biochemical Approaches to Detecting Gene Mutations in Human Populations

4.3.1 Biochemical methods for monitoring for gene mutations

Two approaches have been used to study the proteins in offspring of selected individuals. One is based on the detection of differences in charge, shape, or size of proteins by one- or two-dimensional electrophoretic techniques. In the other, quantitative enzyme activity measurements are used to detect enzyme variants associated with either the loss of enzyme function or the absence of the enzyme protein.

4.3.1.1 One-dimensional electrophoresis

The introduction of new electrophoretic technology has increased the proportion of variants detectable at the protein level and the number of gene products that can be studied. Using these techniques, it has been shown that, in contrast to previous assumptions that only one-third of the amino acid substitutions could be detected, many neutral change substitutions can also be detected. This reflects the alterations in higher-order protein structure associated with these later substitutions, which change the charge distribution on the molecule. Experimental data suggest that

80 - 90% of all amino acid substitutions may be detectable with refined techniques (Johnson, 1976, 1977; Ramshaw et al., 1979; Fuerst & Ferrell, 1980). The use of narrow-range pH gradients for isoelectric focusing has also increased the detection and resolution of the variants at many loci (Altland et al., 1979; Chramback et al., 1980).

The number of gene products that can be studied by electrophoretic analysis depends on the availability of techniques to demonstrate selectively the position of the allele products following electrophoretic separation. In addition to previously developed protein specific stains (Harris & Hopkinson, 1976), immunological techniques (e.g., immunoblotting and immunofixation) (Chapuis-Cellier et al., 1980; Tsang et al., 1983) have become more widely used, especially with the recent modifications. One additional approach has involved several sequential steps of electrophoresis to separate the protein of interest from the remaining proteins, so that standard protein-staining techniques can be used for visualization (Altland & Hackler, 1984).

The results of many studies involving genetic typings of large numbers of individuals, either for population structure studies or in the context of the development of mutation monitoring programmes, have demonstrated the feasibility of sizeable screening efforts using blood samples as the source of material (Harris et al., 1974; Neel et al., 1980a,b). The products of at least 40 - 50 loci can be routinely screened by electrophoresis with either starch and/or polyacrylamide as the support medium. For many proteins, either support can be used, with polyacrylamide having the advantage of requiring smaller sample aliquots and potentially increased resolution. Isoelectric-focusing is feasible for many of these proteins, especially when the potential for increased resolution of some proteins is considered. Electrophoretic techniques have been used in several human mutation screening programmes (Neel et al., 1980a,b; Altland et al., 1982a,b). They have also been used for estimating the mutation rate in mice (Pretsch & Narayanan, 1979; Johnson & Lewis, 1983; Neel, 1983) and *Drosophila* (Mukai & Cockerham, 1977; Voelker et al., 1980). Electrophoretic mobility variants have been identified and the genetic transmission of the trait to subsequent generations has been confirmed.

4.3.1.2 Two-dimensional electrophoresis

The high resolution two-dimensional (2-D) electrophoretic techniques described by O'Farrell (1975) with many subsequent modifications (Dunn & Burghes, 1983a,b) has been used to separate the large array of proteins found in cells and tissues. The positions of the several hundred proteins in

many types of samples can be visualized by protein staining, especially when the highly sensitive silver stains (Merril et al., 1981; Sammons et al., 1981) or isotopic labelling of proteins in nucleated cells followed by autoradiography or fluorography (McConkey et al., 1979; Thomas et al., 1984) are used.

The ability of the 2-D system to resolve electrophoretic mobility variants has been shown by Warner et al. (1982). In other studies, the level of heterozygosity for polypeptides has been examined in several types of human cells and tissues (McConkey et al., 1979; Walton et al., 1979; Comings, 1982; Hamaguchi et al., 1982). More recently, extensive studies have been reported on the level of genetic variation in plasma proteins and other blood proteins (Rosenblum et al., 1983, 1984). One advantage of the 2-D approach over the single-dimension electrophoresis techniques is the ability to study the gene products of at least several hundred loci in each sample (individual). The 2-D electrophoresis technique has been used to estimate the induced mutation rate in mice (Klose, 1979; Marshall et al., 1984).

4.3.1.3 Enzyme activity

Much of the background to the search for enzyme deficiency variants is derived from the study of metabolic diseases. The extensive lists of inborn errors of metabolism indicate not only the prevalence of genetic events associated with loss of enzyme function but also the usefulness of quantitative enzyme assays as a tool for studying this class of genetic events (Beutler, 1979; Kahn et al., 1979; Miwa, 1979).

The search for mutational events resulting in loss of function, owing to either loss or nonfunctionality of the gene product, is complementary to the electrophoretic assays, in that it detects genetic events, not normally detectable by standard electrophoretic assays. Current data suggest that inherited rare enzyme deficiency variants occur more frequently in human populations than rare electrophoretic variants (Mohrenweiser, 1981; Mohrenweiser & Neel, 1981; Satoh et al., 1983). In addition, mutations resulting in loss of enzyme function occur at least as frequently as electrophoretically identifiable mutations in mice and *Drosophila*, following exposure to mutagenic agents, either radiation or chemical (Racine et al., 1980; Charles & Pretsch, 1981; Johnson & Lewis, 1981).

4.3.1.4 Other biochemical approaches

Alterations in base sequence can be analysed using the restriction enzyme mapping techniques (Botstein et al., 1980; Skolnick & Francke, 1982; Southern, 1982). Most of the current

effort, in addition to defining gene structure, has been directed towards identification of polymorphisms at restriction enzyme sites and subsequent linkage analysis, but similar techniques could be employed for screening for mutational events (Beaudet, 1983; Cooper & Schmidtke, 1984 for recent listings of cloned human DNAs). Although it is clear that it is now technically possible to detect base sequence changes, incorporating this approach into a monitoring protocol must await further developments that will facilitate the generation of the quantity of data necessary for a mutation screening programme.

In addition to the sensitivity of the method, another potential advantage of an approach that studies mutations by examining the DNA directly, is the ability to obtain very large quantities of data from each individual. Thus, this method can be employed for the study of small populations.

4.3.2 Analytical strategy and methodological considerations

Biochemical analyses of the samples collected from the F population (offspring of exposed individuals) will be carried out usually in a number of laboratories while population and health data will be derived from various sources. It is unlikely that all laboratories will be able to complete the entire battery of assays or that the techniques will be identical in each laboratory or for each study, thus each laboratory should receive samples from both exposed and control groups. The key for the successful completion of the analytical aspects of any study will be for each laboratory to establish and maintain high standards of technical competence and performance. Provision should be made for the exchange of samples, as this will increase the amount of data obtained from each individual and also serve to confirm the existence of interesting observations. The basic technical procedures for electrophoresis and enzyme activity assays, are available. These techniques form the basis for establishing a new laboratory effort. The methods described in each section are current routine laboratory procedures; thus, the biochemical techniques for monitoring germinal mutations in a human population are available.

4.3.2.1 One-dimensional electrophoresis

Approximately 40 - 50 blood proteins have been examined for electrophoretic variation in many laboratories including the laboratories at the University of Michigan, USA (Neel et al., 1980a) and in Japan at the Radiation Effects Research Foundation (RERF) (Neel et al., 1980b). Approximately half of the proteins studied at the University of Michigan use

polyacrylamide as the support medium while the laboratory at RERF relies more on starch as the support medium. Many of these proteins are also being studied by isoelectric focusing techniques as a component of mutation screening programmes in other laboratories (Altland et al., 1982a,b).

Techniques are available for the electrophoretic analysis of approximately 20 proteins, when the sample is obtained from dried blood (Altland et al., 1979, 1982b; Metropolitan Police Forensic Science Laboratory, 1980). The general principals of electrophoretic separation and isozyme (protein) identification using samples collected as dried stains are as described for other blood samples.

High-speed one-dimensional electrophoretic screening procedures have been developed for vertical polyacrylamide gel electrophoresis, flat bed isoelectric focusing, and the sequential combinations of these procedures. With these techniques, using multiple sample handling procedures, 96 samples are analysed simultaneously (Altland et al., 1982a). This is particularly useful when large numbers of samples are being analysed.

An electrophoretic mobility variant is identified by an alteration in the standard profile that is consistent with the appearance of a new allele product. This decision process must include previous knowledge of the protein subunit structure, chromosomal location (e.g., hemizygous males) and factors such as age, sex, etc., which are important in interpreting the data. The new protein should have characteristics that indicate that it differs from the original protein by an amino acid change. It is important to confirm, using as many additional techniques as possible, that any new variant is not the result of an artifactual or secondary alteration in protein structure. Ultimately, such confirmation would require amino acid or DNA sequencing studies. This is important because of the low probability of obtaining data to confirm genetic transmission of a new mutation in human populations. The proteins that are being studied in the above-mentioned studies have been selected because of the low frequency of artifactual findings. But, as with any technique, it is important for each laboratory to undertake appropriate control experiments.

4.3.2.2 Two-dimensional electrophoresis

The 2-D electrophoresis techniques, used for human mutation monitoring studies, have been described by Neel et al. (1983, 1984) and modifications of the technique of O'Farrell (1975), by Anderson & Anderson (1977) and Anderson et al. (1980). The blood sample is fractionated into various components, e.g., plasma, erythrocyte membranes, haemolysate,

platelet, etc., the proteins of which are then analysed. The proteins in the cell fraction to be studied are solubilized in 4 - 8 M urea, which dissociates the multimeric proteins into component subunits. The component polypeptides are separated on the basis of charge by isoelectric-focusing in the first dimension. Electrophoresis in the second dimension is in the presence of sodium dodecylsulfate, so that the proteins are separated in this dimension on the basis of apparent molecular size. The sensitive silver-based staining techniques are most often used for identifying the positions of the separated polypeptides (Merril et al., 1981; Sammons et al., 1981). Analysis of the results of 2-D electrophoresis can be scored by visual inspection (Hanash et al., 1982; Rosenblum et al., 1983, 1984), though recently, significant progress has been achieved in automating and computerizing the analysis of these gels (Skolnick, 1982; Skolnick et al., 1982; Skolnick & Neel, in press). Computerized analysis can increase the amount of data obtained from each gel (the number of locus tests completed for each sample) and also reduce the workload at this step.

4.3.2.3 Enzyme activity

The methods for detecting enzyme deficiency variants, defined as a level of enzyme activity that is less than 65% of the mean for the population and more than 3 standard deviation units below the mean, have been described by Fielek & Mohrenweiser (1979), Mohrenweiser & Fielek (1982), and Mohrenweiser (1983a). Additional or alternative methods have been described by Bulfield & Moore (1974), Krietsch et al. (1977), Eber et al. (1979), and Satoh et al. (1983). The general analytical strategy for analysing large groups of samples has been outlined by Mohrenweiser (1983b). Enzymes that are: (a) present with reasonable levels of activity; (b) primarily the gene product of a single locus; (c) not influenced by environmental factors (e.g., nutritional status); (d) easy to assay; and (e) exhibit little total variation among individuals, are good candidates for inclusion in this approach (Mohrenweiser, 1982; 1983a,b). The gene products of at least 12 - 14 loci can be routinely monitored for the presence of null variants in erythrocytes.

Each of these biochemical approaches either has been, or is being, used to obtain data for the estimation of radiation-induced mutation rates in human populations. Detailed techniques are available for each component in this section. The most significant problem is obtaining the commitments (funding, study and control populations, etc.) necessary to initiate a major mutagenic study. Obtaining agreements on general approaches, necessary standardization of

technical aspects, and scoring of significant events is a lesser problem.

4.3.2.4 Sample acquisition and storage

There are two general strategies for sample acquisition. The first involves obtaining a large volume (up to 20 ml, although 5 ml is an adequate sample) of whole blood. All members of a nuclear family are sampled to the maximum extent possible, so that the material for family studies to determine heritability of the characteristic is routinely available. Experience has shown that when the gene products of 40 - 50 loci are studied, family studies are necessary in at least 10% of the families, even when only a single child per family is studied. With the number of loci studied with the 2-D electrophoresis technique, obviously the percentage of family studies increases to include almost every family. In either case, when the number of locus tests per sample or family unit increases to this level, the work involved in recontacting families for additional samples becomes a considerable task.

The blood sample is fractionated into at least plasma, buffy coat, and erythrocyte fractions. Each part is subdivided into a fraction for routine analysis as well as a fraction for long-term storage and potential additional analysis. An aliquot of red cells is stored in glycerol-sorbitol solution and retained in liquid nitrogen for blood group analysis in the event of a potential mutation being observed. The "white cell" component could be fractionated into several components, e.g., platelets, polymorphonuclear leukocytes, etc., which could be used as samples for 2-D electrophoretic studies. Alternatively, either all or fractions of the lymphocyte samples could be retained as a source of DNA for future restriction enzyme mapping studies. Another alternative to the above protocol would be to store the white cell fraction under such conditions that intact, viable cells could be recovered and, with appropriate cell culturing techniques, the number of cells expanded for use in future studies. In certain situations, where a specific population is of special interest and the potential for follow-up studies is limited, it may be advisable to establish and store transformed lymphoblast cell lines in order to maintain the genetic information contained in this population for future studies. The second acquisition strategy involves obtaining a smaller volume of sample, often in the form of a dried blood stain, which is usually obtained in conjunction with other studies, usually with new-born metabolic screening programmes. Procedures have been established for screening for gene mutations at 20 loci using a blood sample obtained on a Guthrie Test card. With these techniques, 300 samples or 6000

locus tests per day can be completed (Altland et al., 1982a,b; Vogel & Altland, 1982). As the new-born metabolic screening programme in many countries encompasses the total new-born population, this strategy appears useful for establishing a surveillance progamme for determining the human germinal mutation rate. The number of locus tests from each individual is somewhat less than with the larger samples, thus the frequency of family studies is less, though obviously as the number of tests per sample increases, it may be important, whenever possible, to obtain concurrent parental samples.

It has been suggested that children with congenital birth defects could be of special interest for monitoring studies because of a high gene mutation rate in such children (Dubinin & Altukhov, 1979). However, study has not been able to confirm a similar increased or high mutation rate among a group of children with apparently similar congenital birth defects (Neel & Mohrenweiser, 1984). The usefulness of the approach of targeting specific F individuals within a population for detailed study, rather than all F individuals, needs further study.

4.3.3 Data management

Neel et al. (1979) have developed a computer-based data management system for handling the types and volume of data generated in a population monitoring programme. This includes a sample identification system with a letter designation for the geographical site of sample acquisition, a number code for family identification, and a single digit indicating family position. This identification, which is the first entry into a computerized data management system, is used for identification of all data generated, thus it is used for linkage to the electrophoretic and enzyme activity files, which are additional components of the computer-based data management system. It is linked to family identification (e.g., name) only when follow-up is necessary, in order to maintain confidentiality of records. A similar data management system is currently in operation at RERF. It is important that the data-management system should be complete and compatible with all aspects of the study (section 2).

4.3.4 Considerations in screening for germinal mutations

4.3.4.1 Sample size

The logistic problems associated with a germinal mutation screening programme are significant because of the scale of the effort. The magnitude of the effort reflects both the rarity of mutational events (background mutation rates of

10^5 - 10^6) and the need for statistical methods to ascertain generally small (10 - 100% increase) differences between background and induced mutation rates. The sample sizes necessary for any of these approaches to monitoring depend on the baseline rate of the effect under consideration and the magnitude of the increase in mutation rates that can be left undetected.

Vogel (1970), Neel (1980), and Vogel & Altland (1982) have calculated the sample sizes necessary for a research design involving the contrasting of 2 samples. The samples can be either collected consecutively, as when studying the possibility of a changing mutation rate in a defined population, or simultaneously, when contrasting a control group of children with a group at high risk of mutation because of the occupational or other exposure of their parents. The minimum sizes of the 2 samples are determined by the magnitude of the errors, type I (α) and type II (φ) that are considered acceptable. The minimum increase in mutation rate that is to be detected (or the maximum to be deemed insignificant) also influences the sample size necessary to detect an increased mutation rate. Most geneticists would agree that any monitoring programme should detect an increase of 50% above background. Using type I and type II error limits of 0.05 and 0.20 and a mutation rate of 1×10^{-5}, it would require some 7.5 million observations (Neel, 1980). There is considerable disagreement on how much effort should be devoted to detecting smaller increases in mutation rate. Given the exponential nature of the relationship, detecting an increase of 20% involves 2 samples 5 times as large as those required to detect a 50% increase. It is obvious that, although the set of assumptions and limits may change, generating a data base necessary for estimating changes in human mutation rates (or finding that at some limit of power, the rate has not changed) requires many observations and considerable analytical effort.

With the biochemical approach, in which many proteins are examined in any given child, and with 2 loci at risk of mutation for each polypeptide included in the study, the number of children will be only a small fraction of the number of determinations. For example, in a pilot study (Neel et al., 1980a), 50 proteins were examined for mutations influencing electrophoretic behaviour. Thus, the demonstration of a difference of 50% between the 2 groups could be accomplished with 2 samples of approximately 90 000 children (it is assumed that each protein of a child is an independent test of mutation and that each child constitutes 2 tests of each protein, i.e., both parents exposed). With the two-dimensional gel electrophoresis method, which could permit simultaneous monitoring of 500 (or more) different proteins of

the blood serum, erythrocytes, and/or leukocytes, with the same assumptions as above, the size of the 2 samples is reduced to approximately 7500 individuals.

4.3.4.2 Distinction between "true" and "apparent" mutations

Whenever a genetic trait is encountered in a child, neither of whose parents is affected, the possibility that it is due to a discrepancy between legal and biological parentage rather than the result of mutation must be considered. This is less likely when the mutation involves a major chromosomal abnormality rather than a biochemical marker, because individuals carrying the former are often sterile or have a severly reduced life expectancy. With the sentinel phenotypes or biochemical variants, when an apparent mutant is encountered it is always necessary to carry out extensive genetic typings on both parents, and the child. At present, with the battery of test traits usually available, including enzymes, blood groups, and HLA, a child whose legal father is not the biological parent can be detected with an assurance approaching 98%. Thus, in a situation characteristic of many countries, such as the USA, where 2 - 3% of the children born in wedlock have fathers other than the legal one and where variants of the type under consideration have a frequency of 2 - 4/1000 examinations, "false" mutations (i.e., undetected parentage exclusions) will have a frequency not very different from the present expectation for mutation.

It should be pointed out that recent developments, which could be used in a study such as this mutation protocol, are also useful for paternity studies. Many gene products studied using two-dimensional electrophoretic techniques are characterized by genetic polymorphisms useful in parentage studies, resulting in an improvement in the ability to detect discrepancies between legal and biological parentage. The study of DNA restriction site polymorphisms will also decrease the frequency of undetected non-parentage, thus the probability of an apparent mutation being due to non-parentage should become very small with the increased number of loci that can now be studied.

In the final analysis, there may always be some uncertainty as to whether a particular apparent mutant is due to an undetected discrepancy between legal and biological parentage. If the control population has been properly chosen, however, the amount of non-parentage could be demonstrated to be the same in the two populations, so that this factor would be a constant diluent in the 2 samples. Furthermore, with a statistical approach, recently developed by Rothman et al. (1981), under suitable conditions,

probabilities can be developed for parentage, and apparent mutations ranked according to these probabilities.

4.3.4.3 Implementation of gene mutation screening programmes

Final decisions regarding analytical strategy and technical approaches, beyond the points described above, must await further information regarding the population of interest. The magnitude of the study helps determine the number of laboratory centres necessary to complete the study within a reasonable time. Similarly, the size of the study population (and estimated mutation rate) will dictate the amount of data that must be obtained from each individual studied for the generation of a statistically significant data base. The size of the study population (as well as the age of the individuals from whom blood is obtained) will also need to be considered when blood fractionation and allocation procedures are finalized. Furthermore, techniques are continually being refined and new approaches being developed, and it is unwise to dictate the specific technical details until such time as the commitment to proceed with a study has been made. At this point, laboratories should be able, with relative ease, to define the technical details for the laboratory and analytical protocols.

4.3.5 Summary

In many respects, with the limitations of the current state of knowledge regarding the detection and subsequent consequences of gene mutations, all practical approaches to estimating the germinal mutation rate in human populations, following exposure to a putative mutagen, should be considered to be complementary. This is important as each technique differs in sensitivity, detects different types of genetic damage or end-points, and has different degrees of relevance for estimating potential health effects. Therefore, it is logical in a collaborative monitoring exercise to use all of the approaches in order to obtain the maximum possible amount of data.

The technical aspects of a collaborative effort to use biochemical methods to monitor selected human populations for germinal mutation frequencies are available. Such programmes have been conducted in the Federal Republic of Germany (Altland et al., 1982b), Japan (Neel et al., 1980b; Satoh et al., 1983), and the USA (Neel et al., 1980a; Mohrenweiser, 1981). Thus, feasibility has been tested at a significant level of effort. There is no technical reason for not expanding such an effort to other populations of interest.

4.4 Sentinel Phenotypes

4.4.1 Introduction

A sentinel phenotype is a clinical disorder that occurs sporadically as a consequence of a single, highly penetrant mutant gene, which is a dominant or X-linked trait of considerable frequency and low fitness, and is uniformly expressed and accurately diagnosable with a minimal clinical effort, but a relatively high probability of ascertainment (Mulvihill & Czeizel, 1983).

A new dominant mutation can manifest itself at any time in the human life-span. In practice, it is worth distinguishing three separate groups of sentinel phenotypes: sentinel anomalies, easily observed at birth and generally recorded in birth defects registries (Table 6); sentinel childhood tumours, which occur in a well defined age-group and are recorded in childhood tumour registries; and genetic disorders with delayed onset, which are readily diagnosed and recorded in various types of genetic disease registries (Table 7). For practical reasons, sentinel anomalies are the easiest to handle.

4.4.2 Basis of method

Certain essential requirements for the surveillance or monitoring of sentinel anomalies include:

(a) Sensitivity and specificity

Theoretically, all dominant phenotypes might be suitable for these purposes. McKusick (1983) catalogues 1828 autosomal dominant traits in man. These probably occur at a total birth prevalence of 1% (Matsunaga, 1982). Nearly half of autosomal dominant disorders may be caused by new mutations (Holmes et al., 1981). Nevertheless, at present, no single dominant phenotype suits all, or nearly all, the criteria of the above definition. However, the 22 anomalies listed in Table 6 comply satisfactorily. The main problems are the validity of a diagnosis and the relatively uncommon occurrence of all these candidate anomalies. If the baseline prevalences are too low, both the specificity and sensitivity of population screening are decreased (Hook, 1981b).

(b) Reliable and accurate diagnosis

A sentinel phenotype must be clearly distinguishable from related disorders. However, each of the anomalies in Table 6 has features that overlap with so many other anomalies that

Table 6. Candidate sentinel anomalies and the figures of the Hungarian Programme per 10 000 births

Congenital anomaly (s = syndrome)	McKusick number (1983)	Expected rate
Achondroplasia	10 080	1.08
Acrocephalosyndactyly type I (Apert s.)	10 120	0.07
Acrocephalosyndactyly type V (Pfeiffer s.)	10 160	0.02
Aniridia, isolated	10 620	0.11
Van der Woude s.	11 930	0.02
Cleidocranial dysplasia	11 960	0.04
Contractural arachnodactyly	12 105	0.04
Crouzon s.	12 350	0.11
EEC s.	12 990	0.04
Holt-Oram s.	14 290	0.07
Treacher-Collins s.	15 450	0.04
Moebius s.	15 790	0.04
Nail-patella s.	16 120	0.04
Oculo-dento-digital (ODD s.)	16 420	0.02
Osteogenesis imperfecta, type I	16 620	0.22
Polysyndactyly preaxial IV.	17 420	0.22
Split hand and/or foot, typical	18 360	0.15
Spondyloepiphyseal dysplasia cong.	18 390	0.07
Thanatophoric dwarfism	18 760	0.04
Whistling face (Freeman-Sheldon s.)	19 370	0.02
Incontinentia pigmenti (Bloch-Sulzberger s.)	30 830	0.11
Oral-facial-digital s. (Gorlin-Psaume s. or OFD I.)	31 120	0.02
Total		2.59
Cataract[a]	11 620	0.71
Ptosis[a]	17 830	0.66
Grand total		3.96

[a] Excluded from the final list because ptosis was rarely ascertained and the nosological diagnosis of cataract was impossible.

highly-trained clinical experts must be involved in the diagnosis. In general, these well-known genetic anomalies have a considerable genetic heterogeneity. In addition, genocopies and sometimes phenocopies exist for all sentinel anomaly candidates. The task is to make a precise nosological diagnosis, i.e., to identify the effect of the specific locus or sometimes of the given allele of the specific locus.

Table 7. Candidate sentinel phenotypes without reliable nenonatal manifestations

Disorder	McKuskick number (1983)	Inheritance
Amelogenesis	10 450	AD[a]
Exostoses, multiple	13 370	AD
Fibrodysplasia ossificans progressiva	13 510	AD
Marfan's syndrome	15 470	AD
Myotonic dystrophy (Steinert disease)	16 090	AD
Neurofibromatosis (Recklinghausen disease)	16 220	AD
Polycystic kidneys	17 390	AD
Polyposis coli, I (familial)	17 510	AD
Polyposis intestinal, II (Peutz-Jeghers syndrome)	17 520	AD
Polyposis intestinal, III (Gardner syndrome)	17 530	AD
Retinoblastoma (hereditary)	18 020	AD
Tuberous sclerosis	19 110	AD
von Hippel-Lindau syndrome	19 330	AD
Waardenburg syndrome	19 350	AD
Wilms' tumour (hereditary)	19 407	AD
Haemophilia A (Classical)	30 670	XR[b]
Haemophilia B (Christmas disease)	30 690	XR
Muscular dystrophy (Duchenne type)	331 020	XR
Martin-Bell	30 955	XR

[a] AD = autosomal dominant.
[b] XR = X-linked.

(c) Complete ascertainment

In countries where most births occur in hospitals and there are neonatal check-ups, the easily-recognized congenital anomalies of autosomal dominant origin can be used as sentinel anomalies, particularly if a population-based registry or surveillance of congenital anomalies is functioning.

(d) The knowledge of pedigree data

Since only a new occurrence of sentinel phenotype in a family is a mutation, obtaining data from and/or clinical examination of both parents is necessary to verify that neither is affected. In practice, it is necessary to obtain clinical data on the grandparents in order to exclude the possibility of non-penetrance in the parents.

(e) Possibility of further action

After a significant increase in the rate of occurrence of one or more mutations, it is essential to initiate an

analytical epidemiological study to identify the responsible mutagens and to achieve the ultimate goal, i.e., prevention of further genetic damage.

4.4.3 Relevance and limitations

The theoretical advantage of using sentinel phenotypes is considerable. When a sentinel anomaly or sentinel childhood tumour is recognized in an infant or child, in the absence of an affected parent, there is little question that a germinal mutation has occurred.

From the practical point of view, there are three advantages:

(a) Since sentinel phenotypes are diseases rather than innocuous physical or biochemical traits or variants, the affected person in a developed country will enter the health-care system. Thus, affected persons will almost certainly be registered in a recording system for reasons other than their potential use in surveillance. Thus, the so-called opportunistic approach is feasible (this term is used to describe studies that are built on data, already collected for other reasons).

(b) In the case of sentinel anomalies and sentinel childhood tumours, parents with an affected infant or young child are likely to be willing to cooperate in additional investigations, with regard, for example, to family or environmental histories.

(c) In sentinel phenotypes in which there is a low fitness, there is a low probability of false paternity.

The main theoretical disadvantage is that the phenotype is not an immediate gene product, but a distant manifestation of altered DNA. Hence, intervening developmental events could obscure the relationship between a mutagenic event and its expression as a sentinel phenotype. In addition, dominant traits tend to have greater variation in clinical manifestations than most recessive and chromosomal syndromes. The main practical difficulty is that most clinicians lack experience in the accurate diagnosis of these rare disorders.

Since the expected incidence of mutant candidate sentinel anomalies is 2 - 3 per 10 000 live births, a very large study population is required in order to be able to detect any effects in exposed groups of individuals.

4.4.4 Procedures

4.4.4.1 Surveillance of sentinel anomalies

A programme may be based on existing congenital anomaly registries and surveillance systems. The target population comprises all the new-born infants and, when possible, those up to the age of one year. Several sources of ascertainment are desirable. Three criteria of congenital anomaly surveillance should be continuously checked:

(a) Completeness of ascertainment

In Hungary, the total birth prevalence of congenital anomalies included in Chapter XIV "Congenital Anomaly" of the International Classification of Diseases was estimated to be about 60 out of every 1000 new-born infants at, or after, birth (Czeizel & Sankaranarayanan, 1984). Individual types of congenital anomalies show a wide range of completeness of ascertainment.

(b) Validity of diagnosis

The validity of diagnosis may be known as a result of *ad hoc* epidemiological studies. However, there may be a wide range of misdiagnosis for different types of congenital anomalies.

(c) Base-line frequency

The baselines of all pregnancy outcomes (e.g., spontaneous and induced abortions, still births, low-birthweight infants, etc.) and other potentially confounding demographic variables (e.g., paternal and maternal age) are also taken into consideration in evaluating sentinel anomalies.

The surveillance of sentinel anomalies should consist of three steps:

(1) Indexed patients with sentinel anomalies, notified to the congenital anomalies registries and surveillance systems, depending on their type of sentinel anomalies, should be referred, with the help of their parents, to selected participating paediatric, orthopaedic, or ophthalmological institutions.

(2) Specialists in these institutions should examine these indexed patients to confirm (or exclude) the nosological diagnosis, to clarify family history (whether a sporadic or familial case), to obtain data on environmental history (with

the help of a specifically-designed questionnaire), and to give genetic counselling (if parents plan to have babies in the future).

(3) The data obtained by experts at medical institutions should be sent back to the programme director to be evaluated.

4.4.5 Data interpretation

4.4.5.1 Surveillance of sentinel anomalies

Preliminary Hungarian experiences, based on data obtained during 1980-82, are inconclusive. The registered prevalence at birth of 24 sentinel anomalies was 2.6/10 000 births. The rate of participation was only 41% (the main causes of case loss were the death or severe condition of certain indexed patients) and the diagnosis was confirmed in only 60% of the cases examined. Nevertheless, as an average of 7 possible new mutations per year was found over the 3-year period, during which nearly 430 000 births occurred, it is hoped that improving the completeness of notification, participation rate, and diagnostic skills will increase the number of new mutations identified in the future.

The possible causes of new mutations can be studied by obtaining the environmental histories of the parents. The environmental histories of the parents of familial cases can provide adequate control data.

The main problem is that the basic data concerning even common candidate sentinel anomalies are not known completely. Knowledge of their frequency in different populations, the rate of prenatal loss, definite figures on their penetrance,and a time-table of the expression of their manifestations would improve the efficient use of these sentinel anomalies.

The total prevalence at birth of the 24 sentinel anomalies studied is not low. Assuming a Poisson distribution of events, a background rate of 4.0 sentinel anomaly cases per 10 000 births, and a 50% proportion of sporadicity, a sample size of 38 000 would be needed to detect a doubling of mutation rate with probabilities of type I and II errors at the 0.05 level. The null hypothesis would be rejected, if 22 or more new mutations were seen.

The surveillance of sentinel anomalies has some other practical benefits. Experts at referral centres have the opportunity to improve their diagnostic skills, and to suggest prognosis and treatment. From this aspect, verifying a genocopy or phenocopy is important. Furthermore, surveillance may promote the detection of teratogens. One of the main practical advantages of surveillance is economy. In the

Hungarian programme, an opportunistic and cost-effective approach is used (Mulvihill & Czeizel 1983).

4.4.6 Conclusions

At present, most sentinel anomalies are not suitable for surveillance, because of the inability to validate the diagnosis and the incomplete ascertainment of some types. However, the reliability of the diagnosis of sentinel anomalies will improve as the number and skill of clinical geneticists increases. Thus, the material in national or regional surveillance systems with a large and unselected data base will become usable for studying sentinel anomalies. The main argument for surveillance is that data are already available in the registries and surveillance systems used for other important medical purposes. In addition, the existence of such programmes will improve existing medical care.

The monitoring of sentinel phenotypes has a specific purpose. Defined populations, such as the offspring of self-poisoned persons, epileptics, cancer patients (e.g., patients with acute lymphoid leukaemia), or workers exposed to chemical compounds, would be worth studying. However, the sentinel phenotype approach is not useful for the monitoring of a small circumscribed newborn population (< 50 000), exposed to known or suspected mutagens, because such a small sample cannot yield statistically significant results. Nevertheless, the importance of a opportunistic approach should be stressed, because some types of industrial and disease registries may provide data for this purpose.

There are a number of possibilities for improving the feasibility of the surveillance and monitoring of sentinel phenotypes. Because the birth prevalence of individual sentinel anomalies is relatively low, international collaboration should be encouraged. For example, the International Clearinghouse for Birth Defects Monitoring Systems (Flynt & Hay, 1979) represents a huge surveillance programme involving more than 24 countries and the records of more than 2 million births per year. The majority of the national and regional systems are able to evaluate the occurrence of sentinel anomalies. However, the fact that most sentinel anomalies do not have separate codes in the International Classification of Disease is a serious technical limitation. Unique codes need to be given to them so that international comparisons of rates of sentinel anomalies can be made.

An important approach involves the use of sentinel childhood tumours, mainly hereditary retinoblastomas and Wilms' tumours. Patients with these disorders are diagnosed and, in general, hospitalized in central institutions; thus, their records can also be used for monitoring purposes.

Although retinoblastoma and Wilms' tumour are not usually detectable at birth, they have a specific feature beyond those mentioned above. The vast majority of cases of either type occur sporadically, and all sporadic bilateral cases have an autosomal dominant origin, while most sporadic unilateral cases are non-hereditary, persumably arising from somatic mutation. Hence, these two types of tumour can be used for surveillance of both germinal and somatic mutations.

Registries of individual genetic disorders (e.g., Huntington, neurofibromatosis, polyposis coli) (Table 7), in several countries, also offer a possibility for the surveillance of sentinel phenotypes.

Improvement in diagnostic laboratory procedures has resulted in progress in the surveillance of sentinel phenotypes (Vogel & Altland, 1982). Recently, screening for the lipoproteinaemias has become widespread and affords an opportunity for detecting common familial hypercholesterolaemias of autosomal dominant origin. Finally, some X-linked recessive traits and disorders, such as Duchenne muscular dystrophy, Martin-Bell syndrome, or haemophilia A, would be useful sentinel phenotypes, particularly if: (a) a considerable fraction of cases were new mutations, and (b) heterozygosity in the mothers could be detected reliably and inexpensively by laboratory techniques.

Although, the monitoring of sentinel phenotypes cannot compete with the efficacy of studying protein variants and the methods of the new molecular genetics, simultaneous approaches can provide possibilities for comparison and, in addition, the sentinel phenotype approach may be more feasible in certain countries.

4.5 Fetal Death

4.5.1 Introduction

The proportion of human fetal deaths normally attributable to germinal genic mutations is not known. If rare recessive lethals are a common cause, studies on inbreeding have failed to show the increase in fetal deaths expected (MacCluer, 1980). Dominant lethal mutations acting in fetal life can occur, but their contribution to fetal death is unknown and difficult to study with present techniques. If this contribution were large, an increase in fetal death with increasing paternal age might be expected. Such an effect was seen in one study (Selvin & Garfinkel, 1976), but was not seen in another study of late fetal deaths (Hatch, 1984).

The occurrence of a fetal death, particularly an early fetal death, without any investigation of the products of conception, can be considered as an indicator of germinal

genic mutation. However, any increase in FD could be due also to exposure to a teratogen during pregnancy, or to interference with normal maternal hormonal or immunological functions during pregnancy.

Teratogenic effects could be separated from genetic effects, if the exposure could be allocated according to the sex of the exposed parent, and whether it was pre-conceptional or post-conceptional. Although the possibility exists that maternal exposure can occur indirectly through paternal contact, most paternal exposures are likely to affect fetal health via genetic changes transmitted in the sperm. Pre-conceptional exposures only, in females, are also most likely to induce genetic damage. However, it is not possible to distinguish between germinal chromosome aberrations and genic mutation using exposure data.

If morphological examination of the products of conception can be carried out (section 4.2), further information can be gained. Genetic effects, either chromosomal or genic mutations, are likely to result in developmentally abnormal specimens, whereas factors acting via interference with the maternal physiological response in pregnancy are likely to result in developmentally normal specimens. A change in the usual distribution of morphological types among specimens of FD can be used to document an effect of an exposure: a doubling of the number of "intact empty sacs" compared with controls would confirm the validity of an observed rise in over-all rate of FD.

If the products of conception can be examined cytogenetically (section 4.2), it may be possible to distinguish a class of FD most probably due to genic mutation. A rise in developmentally abnormal, but chromosomally normal specimens, after maternal pre-conception, or paternal, exposures, might possible be attributed to an increase in germinal genic mutations, lethal for fetal life.

4.5.2 Procedures

If exposed and unexposed populations are to be compared for overall frequency of fetal death, the population(s) to be studied must be clearly defined, and data collected on the total number of births over 28 weeks of gestation, i.e., late fetal death (still birth) and the total number of FDs. For purposes of comparison between an exposed and unexposed population, bias should not be introduced, if the ascertainment of either of these variables is not complete, as long as the exposure status does not influence the ascertainment. However, the higher the observed rate of early and intermediate fetal death (spontaneous abortion) the greater the power of the study, and the less likely it is to

miss effects limited to only particular subsets of the total (as is likely for genetic effects). As most genetic effects will probably be observed among the early spontaneous abortions, it is particularly important to ascertain these as completely as possible. This is not usually possible with most existing vital statistics sources of fetal deaths. In a retrospective study, it will probably be necessary to use either medical records or interviews to ascertain reproductive history.

In a case-control study, efforts would be made to ascertain spontaneous abortions through a medical treatment centre, and to compare exposure histories between women who have spontaneous abortions and women having live births. If the sample were relatively small, cases and controls would probably have to be matched for age, in order to control for this source of bias. Finding controls at a point in gestation that matches the cases of spontaneous abortion will also help to control for differences in time of ascertainment, and differences in recall of events occurring at the beginning of the pregnancy.

Data must be collected as carefully as possible on factors known to influence the frequency of fetal death (e.g., maternal age, socio-economic status, previous reproductive history, and gestational age at abortion). Gestational age is usually estimated from menstrual history. Even if data on the developmental stage of the fetus is available, it should not be used to estimate gestational age, since development often ceases some time before abortion. Smoking and alcohol consumption histories would also be useful, since these common exposures are associated with increased abortion risk and might differ in exposed and unexposed populations.

4.5.3 Data processing and presentation

Frequency is usually expressed as the number of fetal deaths divided by the number of births over 28 weeks plus the number of fetal deaths. Maternal age is the most important variable to be controlled in any analysis, since it will influence the overall frequency of abortion, and also the distribution of the kinds of chromosome anomalies expected. Trisomies in spontaneous abortions increase with maternal age in the same way as trisomies in live births. On the other hand, monosomy X shows a decrease in frequency with maternal age in spontaneous abortions. The risk of abortion increases with the number of previous abortions in a woman's history, and this variable should also be examined for possible confounding effects.

4.5.4 Conclusions

(a) Fetal death can be used as a possible indicator of germinal genetic mutation. Because of the high background frequency, and concentration of anomalies, an increase can be detected in a small sample size, e.g., a sample from only 200 women will detect a doubling with 95% power.

(b) Without cytogenetic studies, it is not possible to define the kind of genetic damage that has occurred (i.e., chromosomal or genic mutation) or to separate mutagenic from teratogenic effects. However, defining the exposure of the parent (pre-conceptional versus conceptional) may make more inferences possible about the cause of a rise in the frequency of fetal deaths.

(c) Estimates of the rate of fetal death may be imprecise because of difficulties in identifying all causes, and in determining the relevant population denominator of term births. Data on factors known to influence rates of fetal death, such as maternal age, socio-encomic status, and previous reproductive history, need to be collected, so that possible biases can be explored in the analysis.

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