Standardization of the Sister Chromatid Exchange Technique in Human Lymphocytes for Mutagen and Carcinogen Testing

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STANDARDIZATION OF THE SISTER CHROMATID EXCHANGE TECHNIQUE IN HUMAN LUMPHOCYTES FOR MUTAGEN AND CARCINOGEN TESTING

by

Kamlesh Sharma

A Dissertation
Submitted to the
Faculty of the Graduate College
in partial fulfillment of the
requirements for the
Degree of Doctor of Philosophy
Science Education

Western Michigan University
Kalamazoo, Michigan
December 1981

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STANDARDIZATION OF THE SISTER CHROMATID EXCHANGE TECHNIQUE IN HUMAN LYMPHOCYTES FOR MUTAGEN AND CARCINOGEN TESTING

Kamlesh Sharma, Ph.D.
Western Michigan University, 1981

The differential staining of chromosomes with the use of 5-bromo-2'-deoxyuridine (BUDR) was discovered in 1972. Since then, the BUDR technique has been modified and improved. It is used to study cellular kinetics; yet its most extensive use today is focused on studying sister chromatid exchange (SCE). Studies show that the frequency of SCEs increases significantly in the presence of mutagenic and carcinogenic agents. Hence, the BUDR technique (commonly referred to as the SCE technique) has been useful as a diagnostic tool to test for mutagenic and carcinogenic agents.

Investigators have used differential staining to obtain a baseline incidence of SCEs in human lymphocytes; but their results show a wide variation. This makes the testing of mutagenic and carcinogenic agents problematic. A more basic problem encountered in using the SCE technique is that the yield of differentially stained chromosomes is too low for analytical purposes.

The present study is designed to standardize the differential staining technique so that the problem of low yield can be overcome. This should help tackle the problem of establishing the baseline incidence of SCEs. The three specific objectives of this research, therefore, are to: (a) establish culture conditions that yield a high frequency of metaphase spreads; (b) determine the time of initiation of
DNA synthesis; and (c) establish staining conditions that yield an adequate number of differentially stained chromatids for SCE analysis.

These objectives were accomplished as follows. To obtain optimal cell proliferation, Ham's F-10 medium was selected out of five commonly used media on the basis of its high mitotic index (M1) result. The initial lymphocyte concentration was kept at $3.5 \times 10^5$ per ml. Cell proliferation was measured by determining the amount of tritiated thymidine--$^{3}$H-TdR--incorporated into the DNA. Optimal phytohemagglutinin (PHA) concentration was found to range between .36 and .84%. (It was discovered that the optimal range of PHA concentration varied from one lot to another.) PHA concentration of .48% was used in all further experiments. Optimal FCS concentration was found to be 13%.

Using these optimal conditions, the time of initiation of DNA synthesis in human lymphocytes was determined by $^{3}${H}-TdR incorporation as well as on a flow cytometer. The first method indicated a range of 30-40 h, the second indicated a range of 40-50 h. Thus it was found that BUdR should not be added later than 30 h after culture initiation to ensure its incorporation into the DNA.

Using 10 $\mu$g/ml of BUdR, clear differentiation of the maximum number of chromosomes was obtained under the following regimen:

- **Hoechst 33258**: 50 $\mu$g/ml
- **Soaking time in Hoechst 33258**: 10 min
- **Slide warmer (@ 55° C)**: 10 min
- **Black light exposure**: 50 min
- **Giemsa (3%) staining**: 15 min.

A procedure for standardizing the differential staining technique

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in human lymphocytes has been established in this research. It should prove useful in obtaining uniform in vitro culture conditions generally. Moreover, it should help to make SCE analysis a more reliable diagnostic tool for mutagen and carcinogen testing.
ACKNOWLEDGEMENTS

During the course of this research, I have accumulated a debt to several persons. I wish to acknowledge the guidance of my Advisory Committee, Drs. Gyula Ficsor (Chairman), Ronald J. Flaspohler, Leonard C. Ginsberg, Paul Lang, and George G. Mallinson; and Dr. Robert Poel, Coordinator of Graduate Science Education, Western Michigan University.

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To my family, I owe more than I can say. Without the patience and understanding of Namita, Radhika, Sujit, and Rahul, this work would not have been completed. I have saved for last my husband, Visho, who has been a constant source of inspiration, encouragement, and invaluable help.

Kamlesh Sharma

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Western Michigan University Ph.D. 1981

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CHAPTER I

INTRODUCTION

In our present day society, humans are constantly exposed to thousands of genotoxic agents, both natural and synthetic. For most of these agents, adverse long-term chronic health effects, such as cancer and heritable mutations, have not been thoroughly evaluated. Most short-term tests use microbial, plant, and animal cells for detecting carcinogenic and mutagenic agents (Hollstein et al., 1979). In order to assess the genetic and potentially carcinogenic damage in human tissue directly, we need easily available human cells in simple and sensitive tests. The sister chromatid exchange (SCE) analysis in human lymphocytes meets this criterion.

SCE analysis has been used by many investigators to test for mutagenic and carcinogenic agents in human lymphocytes. In order to observe SCEs, chromosomes have to be stained differentially, i.e., one chromatid stained dark and the other one light. The technique for differential staining that is now widely used was pioneered by Zakharov and Egolina (1972). Many modifications of the technique were introduced to improve differential staining (see CHAPTER II). However, using cultured human lymphocytes, the present investigator had difficulty in obtaining a sufficient number of differentially stained chromosomes with the methods reported by other investigators. Therefore, the development of a better technique for the differential staining of chromosomes was undertaken to:
1. Identify components in the procedure that are responsible for poor cultures and, insofar as possible, overcome these conditions.

2. Achieve staining conditions that would yield optimal number of differentially stained chromosomes.
CHAPTER II

LITERATURE REVIEW

Sister chromatid exchange (SCE) is any reciprocal exchange between two sister chromatids. In order to observe this exchange, the chromatids need to be stained differentially, i.e., one light and the other dark. The most commonly used technique today was developed only nine years ago (Zakharov and Egolina, 1972). Since then a number of studies have been done to determine the incidence of SCEs in chromosomes of several species, including human cells. The most easily available human tissue is peripheral blood (Moorhead et al., 1960). Hence, peripheral blood was used in this study to standardize the differential staining technique.

Components of an In Vitro Culture of Human Blood

Until 1960, human chromosome spreads were prepared from bone marrow cells or skin fibroblasts. Nowell (1960) showed that when human peripheral blood is cultured with phytohemagglutinin (PHA), it initiated mitotic activity in leukocytes. Moorhead et al. (1960) introduced the air-drying technique that facilitated the preparation of well spread chromosomes on glass slides from cultured human leukocytes. Today, in vitro culturing of human peripheral cells is a well established laboratory procedure.

The essential components of an in vitro human lymphocyte culture system are as follows.
The medium. It is made up of nutrients for cell growth, i.e., nucleic acids, amino acids, and vitamins in a phosphate buffered saline solution. There are various ready-made media available from commercial firms.

PHA. It is an extract of kidney beans (Phaseolus vulgaris). Its initial use was to separate red cells from whole blood because it agglutinated the red cells. Nowell (1960) was the first one to show that PHA can stimulate leukocytes into mitotic activity. Hesketh (1978) described the events that lead to and follow mitogenic activation.

Serum. It is the defibrinated and cell-free part of whole blood. Either homologous or heterologous serum can be used in a cell culture. Various types of sera are available from commercial firms. Serum is an important component because it promotes viability and duration of lymphocyte survival (Heilman & McFarland, 1966).

Antibiotics. Penicillin and streptomycin are added to safeguard against bacterial growth.

Blood inoculum. To a mixture of the above components, human blood is added either in the form of whole blood, leukocyte-rich plasma, or purified lymphocytes. The culture is incubated at 37° C, usually for 72 h, by which time maximal cell proliferation has taken place (Tice et al., 1979).

Composition of Human Peripheral Blood

Blood is a complex mixture of formed elements and a fluid substance, the plasma. The formed elements are erythrocytes or red blood cells
(RBCs), leukocytes or white blood cells (WBCs), and platelets in the ratio of 500:1:30, respectively. The formed elements are suspended in plasma. The major component of plasma is water. Plasma contains dissolved solutes such as salts, vitamins, end-products of metabolism, and hormones. It also carries, in suspension, colloids, such as proteins, blood coagulation factors, enzymes, and antibodies. An average adult has five liters of blood, that consists of 60% plasma and 40% formed elements.

Normal human adult blood has the following cellular composition (Lamberg and Rothstein, 1978):

- red blood cells $4-6 \times 10^9$/ml
- White blood cells $5-10 \times 10^6$/ml.

The six different types of leukocytes or WBCs are divided into two categories shown in Table 1. SCEs are observed in chromosomes predominantly from lymphocytes.

### Table 1

Classification of Leukocytes or WBCs

<table>
<thead>
<tr>
<th>Granulocytes or polymorphonuclear leukocytes</th>
<th>Agranulocytes or mononuclear leukocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophilic segmented cells</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td>Band cells (immature neutrophils)</td>
<td>Monocytes</td>
</tr>
<tr>
<td>Eosinophils</td>
<td></td>
</tr>
<tr>
<td>Basophils</td>
<td></td>
</tr>
</tbody>
</table>

The average percentage of the different types of leukocytes in a normal individual's blood appear in Table 2.

Table 2

<table>
<thead>
<tr>
<th>Type of Leukocyte</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophilic segmented cells</td>
<td>55-75</td>
</tr>
<tr>
<td>Band cells</td>
<td>2-6</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>20-35</td>
</tr>
<tr>
<td>Monocytes</td>
<td>2-6</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>1-3</td>
</tr>
<tr>
<td>Basophils</td>
<td>0-1</td>
</tr>
</tbody>
</table>


In preliminary experiments, this investigator observed that the number of WBCs and the percentage of lymphocytes vary from person to person, and indeed in the same person on different days (Table 3).

Human lymphocytes are of two types, B and T, in a proportion of 40:60 ±20%, respectively (Dwyer, 1976; Steel, 1974). However, Mangi et al. (1974) have reported a suppression of T cells in peripheral blood even with a mild respiratory tract infection. This may explain the day to day variation in the number of WBCs and lymphocytes in an individual. Studies show that PHA stimulates mitotic activity mainly among T lymphocytes (Oppenheim & Rosenstreic, 1976).
Table 3
Variation in the WBC Count and the Percentage Lymphocyte Counts in the Whole Blood of Various Individuals

<table>
<thead>
<tr>
<th>Individual</th>
<th>Date</th>
<th>WBC x 10⁶/ml</th>
<th>% Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6/1/80</td>
<td>6.3</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>1/20/81</td>
<td>5.8</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>4/21/81</td>
<td>12.6</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>6/16/81</td>
<td>6.4</td>
<td>46</td>
</tr>
<tr>
<td>D</td>
<td>9/1/80</td>
<td>4.9</td>
<td>39</td>
</tr>
<tr>
<td>E</td>
<td>9/1/80</td>
<td>12.5</td>
<td>28</td>
</tr>
<tr>
<td>F</td>
<td>9/1/80</td>
<td>6.9</td>
<td>21</td>
</tr>
</tbody>
</table>

Methods of Measuring Lymphocyte Proliferation in In Vitro Cultures

The proliferative response of lymphocytes can be measured in the following ways.

Mitotic index. The number of chromosome spreads per 100 nuclei on a slide give the percentage mitotic index (Mi). Usually 1000 nuclei are counted.

Autoradiography. ³(H)-TdR is incorporated in cell cultures. Slides are prepared and covered with photographic emulsion and they are left in the dark for a few days. Nuclei with grains in excess of background grains are counted manually under a microscope. The percentage of nuclei that have incorporated ³(H)-TdR (thus becoming heavily grained) is used as a measure of cell proliferation.

³(H)-TdR incorporation. Cell cultures are incubated in the
presence of $^3$(H)-TdR for a certain period of time. The amount of
labelling incorporated into the DNA is measured with a scintillation
counter as counts per minute. Thus, the amount of $^3$(H)-TdR incorpor­
ated into the DNA is used as a measure of cell proliferation.

**Flow cytometry.** Cells to be studied are stained in suspension
with a DNA-binding fluorochrome. Stained cells are passed in a single
stream perpendicular to a beam of laser light. Each cell generates a
fluorescent pulse, depending on its DNA content. This is displayed
electronically in the form of a dot-pattern distribution on an oscil­
loscope. The main advantages of this method are direct-staining of
the DNA, rapid counting of large numbers of cells (approximately
10,000/15 sec), and the assessment of percentages and absolute num­
bers of responding cells.

**Development of the Differential Staining Method**

SCE was first observed by Taylor et al. (1957) in mitotic chro­
omosomes of higher organisms. They used autoradiographic techniques
to differentiate between chromatids. Chromosomes were allowed to
duplicate once in the presence of $^3$(H)-TdR; then the investigators
followed the distribution of labelled DNA in subsequent division in
the absence of the labelled precursor (Figure 1).

There were two problems with the autoradiographic method. First,
the resolution was poor and, secondly, $^3$(H)-TdR itself caused SCEs

Zakharov and Egolina (1972) did the pioneer work in developing
a new method for differential staining. They used a halogenated
The Technique for Observing Sister Chromatid Exchange for Tritiated Thymidine

analogue of thymidine, 5-bromo-2'-deoxyuridine (B UdR), instead of $^3$(H)-T dR to label chromatids. They showed that when Chinese hamster cells were grown in the presence of BUdR for two rounds of replication, the two chromatids could be distinguished from each other by their unequal spiralization patterns. They also showed that after staining with Giemsa, occasionally one of the chromatids appeared weakly stained. Ikushima and Wolff (1974) allowed chromosomes to replicate for two cell cycles in the presence of BUdR. They showed that chromatids that had incorporated BUdR in both DNA strands stained lighter than chromatids
that had incorporated BUdR in only one strand. Although this method allowed SCEs to be observed more accurately than when $^3$H-TdR was used, the resolution obtained was not always satisfactory.

Latt (1974a and 1974b) developed a new method that allowed a clear demarcation between sister chromatids. He stained BUdR-substituted human chromosomes with the fluorochrome, Hoechst 33258. He observed that, under a fluorescence microscope, the chromatids in which both the DNA strands had incorporated BUdR fluoresced lighter than the chromatids in which only one DNA strand had incorporated BUdR. Now it was possible to observe SCE with great clarity. The only disadvantage was that the image of the differentially stained chromatids faded rapidly. Hence, the slide preparations were temporary. To overcome this problem, other researchers used Acridine Orange instead of the fluorochrome, Hoechst 33258 (Dutrillaux et al., 1974; Franceschini, 1974; Kato, 1974a). Although this allowed the use of stained preparations for several weeks, these preparations were still temporary.

Perry and Wolff (1974) developed a technique in which they combined the use of Hoechst 33258 with Giemsa staining and called it the fluorescence-plus-Giemsa (FPG) technique. This technique gave permanent slides with differentially stained chromatids. They allowed chromatids to replicate in the dark for two cell cycles in the presence of BUdR. Slide preparations were stained with Hoechst 33258, exposed to daylight for 24 h and then incubated in distilled water or 2 x SSC (.3 M sodium chloride, .03 trisodium citrate) for 2 h at 60° C. The slides were then stained in 3% Giemsa for 30 min.
The technique provided great clarity of image. The FPG technique, or modifications thereof, has since been used by many investigators. Kim (1974), independently of Perry and Wolff, reported a technique in which the incubation step used in the FPG technique is omitted. Korenberg and Freedlender (1974) reported that differential staining can be achieved without the use of Hoechst 33258. However, they preheated the slides for 10 min in 1 M NaH₂PO₄ at 89°C and then stained them with Giemsa. Goto et al. (1975) showed that metachromatic dyes such as thionine and toluidine blue could be used instead of Hoechst 33258. Banding techniques, such as the use of trypsin and urea treatment, have also been shown to result in differential Giemsa staining (Pathak et al., 1975).

All these staining methods are effective only under the condition that cells go through two replications in the presence of BUdR or iodo-deoxyuridine (lUdR). It is also possible to obtain differential staining by allowing chromosomes to replicate for one cycle in the presence, and for the second cycle in the absence, of BUdR in the culture medium in which they have been growing (Kato, 1974a; Kihlman & Kronberg, 1975) (Figure 2).

When cells are exposed to short-wavelength light in the FPG technique, the stain in the more heavily substituted chromatid fades faster than in its sister chromatid. Gratzner et al. (1976) used antibodies directed against BUdR to show the differentiation between two chromatids.
Figure 2
Sister Chromatid Exchange with the
Incorporation of BUdR/IUdR

Reasons for Differential Staining

The reason for differential staining of chromatids after BUdR incorporation in the DNA is not understood. Latt (1973) believes that the bifilarly BUdR-substituted chromatin binds less tightly to the dye, thus staining lighter than the unifilarly-substituted chromatin.
However, there are other investigators who believe that the lighter staining of the bifiliarily BUdR-substituted chromatid is due to an effect on the structural proteins that hold the helical DNA structure together. Zakharov and Egolina (1972) noticed that the pale chromatid (in which both strands were substituted with BUdR) was usually longer than the dark chromatid. They postulated that protein synthesis was delayed, which affected the condensation and spiralization of bifiliarily BUdR-substituted chromatid. Ikushima and Wolff (1974) attributed the differential staining to a differential binding of protein to the DNA of chromatin.

Korenberg and Ris (1975) studied Chinese hamster ovary chromosomes with the electron microscope. They showed that the bifiliarily-substituted chromatid is more open with looser gyres than is the unifiliarily-substituted chromatid. They attribute this effect to nonhistone protein affecting condensation of chromosomes. Wolff and Bodycote (1974) showed that differential staining also results without the dye. They showed that exposure of slides to high-pressure mercury burners or some chemicals in the dark, e.g., dithiothreitol or β-mercaptoethanol, and then staining in Giemsa produces differential staining. Both these agents break disulphide bonds in protein--thus again implicating non-histone structural proteins in inducing differential compaction and thus differential staining of chromatids.

Uses of the Differential Staining Technique

Differential staining using the BUdR technique has helped us to understand the basic structure of eukaryotic chromosomes. Much of the
recent interest in differential staining and SCE phenomena has been focused on studying (a) cellular kinetics, and (b) detection of mutagenic and carcinogenic agents.

**Cellular kinetics.** In circulating human peripheral blood, only .1 to .7% of the mononuclear cells are undergoing DNA synthesis (Bond et al., 1958). This percentage increases to approximately 45% after 72 h of *in vitro* activation with PHA (MacKinney et al., 1962). The time of initiation of DNA synthesis and the length of the cell cycles have been studied by following the autoradiographic patterns as a function of time. This method has several disadvantages, e.g., it requires a lot of time and effort, $^3$(H)-TdR itself causes cell cycle delay, and the resolution is poor.

Many investigators have used the BUdR technique to study various aspects of cellular proliferation in *in vitro* lymphocyte cultures (Kim, 1974; Craig-Holmes & Shaw, 1976; Dutrillaux & Fosse, 1976; Tice et al., 1976; Crossen & Morgan, 1977; Tice et al., 1977). Tice et al. (1976) showed that BUdR concentrations of 7 μM/ml to 35 μM/ml did not delay the cell cycle. All the investigators who have used the BUdR technique to study cellular kinetics, have used BUdR concentrations below this range. Tice et al. (1976) named the BUdR incorporation system for analyzing cellular kinetics BISACK. The analysis is simple and can be scored from differentially stained slides. Cells that have undergone one replication cycle in the presence of BUdR have these two features: their metaphase chromosomes contain unifiiliarly-substituted DNA in both chromatids; their chromosomes are uniformly stained. In second metaphase chromosomes, one chromatid is unifiiliarly substituted and
stains darker whereas the other chromatid is bifilarly substituted and stains lighter. In third metaphase chromosomes, only some chromosomes show differential staining (approximately half) and the rest are uniformly stained. The appearance of the different types of staining patterns allowed determination of cell cycle lengths and the proportion of cells in the first, second, etc., cell cycles following the addition of BUdR.

There is considerable inter-individual variation in the frequency of second metaphase chromosome spreads (which are the most suitable for SCE analysis) in in vitro cultures. Crossen and Morgan (1977) showed that in 48 h cultures there were 2-53% second metaphase spreads among 20 individuals. In 72 h cultures, they showed a range of 3-83% second metaphase spreads among 30 individuals. They reported the cell cycle lengths as 12, 16, and 24 h. Craig-Holmes and Shaw (1976) found that lymphocytes had cell cycle lengths of 12-48 h. Tice et al. (1979), using BISACK analysis, mathematical models, and computer simulation models, came up with average cell cycle lengths of 12, 10.6, and 12.3 h, respectively. They also reported that the first metaphases appeared as early as 40 h and as late as 92 h after PHA stimulation. The discrepancy between cell cycle lengths and the varying times for the initial entry of cells into the S phase has been attributed to the following:

1. The heterogeneity of the cells that are stimulated by PHA.
2. The varying culture conditions that are used by different investigators.
3. The state of health of the individual giving blood.
4. Individual variation in PHA response.
In conclusion, human peripheral blood is a complex mixture of heterogeneous cells. Because of the many variables that are involved in the culturing of peripheral lymphocytes, none of the procedures reported in the literature and tested by this investigator gave an adequate yield of differentially stained chromosome spreads for SCE analysis.

**Detection of mutagenic and carcinogenic agents.** Introduction of the differential staining technique has greatly facilitated studies of the incidence and induction of SCEs. In differentially stained chromosomes, SCE can be easily observed as a switch between dark and light chromatids (Figures 1 and 2). Kato (1973 and 1974b) used autoradiography to show that alkylating agents and proflavine, both of which are mutagens, induce SCEs. However, with this method, counting high frequencies of SCEs was difficult. Latt (1974b) used the BUdR technique to show that low doses of alkylating mutagens and carcinogens, such as mitomycin C and nitrogen mustard, induce high frequencies of SCEs in human chromosomes at concentrations far lower than the ones that caused a significant number of chromatid aberrations.

Perry and Evans (1975) confirmed Latt's findings that induction of SCEs is much more sensitive than the induction of chromosomal aberrations. They showed that for 12 known mutagenic/carcinogenic agents, at concentrations that caused a 10-fold increase in SCE frequencies, there was no increase in chromosomal aberrations. The results pertaining to three well known mutagenic/carcinogenic agents (out of the 14 reported) appear in Table 4. In vitro metabolic activation has also been used to activate promutagens/procarcinogens with the SCE technique.
### Table 4

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration Used</th>
<th>Chromosomal Aberrations in 20 M&lt;sub&gt;2&lt;/sub&gt; cells</th>
<th>Total SCEs in 20 M&lt;sub&gt;2&lt;/sub&gt; cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (BUdR only)</td>
<td></td>
<td>3</td>
<td>244</td>
</tr>
<tr>
<td>Ethyl methane-sulfonate</td>
<td>3 x 10&lt;sup&gt;-4&lt;/sup&gt; M</td>
<td>3</td>
<td>2,054</td>
</tr>
<tr>
<td>Methyl methane-sulfonate</td>
<td>3 x 10&lt;sup&gt;-4&lt;/sup&gt; M</td>
<td>1</td>
<td>1,952</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>1 x 10&lt;sup&gt;-7&lt;/sup&gt; M</td>
<td>0</td>
<td>2,555</td>
</tr>
</tbody>
</table>

Source: Perry and Evans, 1975.


Perry and Evans also did some preliminary studies on patients treated with cytotoxic drugs. They showed that SCE analysis provides a tool for monitoring people exposed to known or suspected mutagens/carcinogens. Many such investigations have since been conducted to determine the effect of laboratory reagents on both laboratory workers and rotoprinting factory workers (Funes-Cravioto et al., 1977); the effect of epichlorohydrin and vinyl chloride on workers occupationally exposed to these chemicals (Kučerová et al., 1977; Kučerová et al., 1979). The cytogenetic impact of drugs administered to patients has been assessed by SCE analysis (Nevastad, 1978; Raposa, 1978; Sono &
Sakaguchi, 1978; Littlefield et al., 1980; Ohtsuru et al., 1980). For this purpose, SCEs are determined in peripheral lymphocytes drawn from patients exposed to various drugs. Lambert et al. (1979) found that 1-(2-chlorethyl)-3-cyclohexyl-1-nitrosurea (CCNU) causes a persistent elevation of SCEs after chemotherapeutic treatment.

In vivo analysis systems for differential staining have been developed also. Allen and Latt (1976) employed multiple intra-peritoneal BUdR injections to demonstrate that cyclophosphamide, a drug that needs metabolic activation, can induce SCEs in vivo. Vogel (1976) tested mutagenic agents in vivo by analyzing mice bone marrow cells. Sutou (1981) used Chinese hamster bone marrow cells to determine the incidence of spontaneous SCEs.

Besides chemicals, there are also physical agents that cause an elevation in the baseline incidence of SCEs. Ionizing radiation is a well known mutagenic/carcinogenic agent. However, it causes only a slight increase in SCE frequency in contrast to its remarkable ability to produce chromosomal aberrations (Perry & Evans, 1975; Solomon & Bobrow, 1975). Marin and Prescott (1964) found that 400 R of x-rays double the SCE frequency, whereas it caused more than a 20-fold increase in the aberration frequency.

In contrast to ionizing radiation, ultraviolet irradiation produces a dramatic increase in SCE frequency (Kato, 1973). Wolff et al. (1974) showed that the increase is detectable only after irradiated cells pass through the S phase.

Therefore, chemical agents that induce SCEs can also be divided into the following two categories:
1. X-ray-type agents, that produce a high frequency of chromosomal aberration but a small increase in SCE frequency. (For example, Bleomycin and caffeine.)

2. Ultraviolet-type agents, that induce high frequencies of SCEs and also produce chromosomal aberrations. Most alkylating agents (e.g., mitomycin C and nitrogen mustard) fall into this category as do some DNA intercalating agents. (For example, proflavin and Acridine Orange.)

Thus, in order to ensure that x-ray-type agents are not missed by SCE analysis, one needs to establish culture conditions that will not interfere with their detection.

Factors Affecting Differential Staining and the Incidence of SCE

SCE analyses of human lymphocytes show a great variation in the baseline incidence of SCEs (Table 5).

Some of the culture conditions and the techniques used for differential staining that can cause variation in the baseline incidence of SCE are as follows.

Concentration of BUdR. The BUdR technique to detect SCEs assumed that the SCE events were spontaneous, and not affected by BUdR. Hsu and Somers (1961), Dewey and Humphrey (1965), and Huang (1967) showed that BUdR causes chromosomal aberrations in cultured mammalian cells. Thus, it is possible that it may also cause SCEs. Many investigators have shown that the incidence of SCEs increases with increasing BUdR concentration (Kato, 1974c; Latt, 1974b; Wolff & Perry, 1974; Lambert et al., 1976; Latt & Juergens, 1977; Carrano et al. 1980; Davidson...
Table 5
Frequencies of Baseline SCEs in Human Peripheral Lymphocytes Cultured In Vitro

<table>
<thead>
<tr>
<th>Concentration of BUdR μg/ml</th>
<th>Growth in BUdR (hours)</th>
<th>Frequency of SCEs per cell per chromosome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>27.6</td>
<td>72</td>
<td>6.9</td>
<td>Chaganti et al. (1974)</td>
</tr>
<tr>
<td>6.1</td>
<td>72</td>
<td>14.0</td>
<td>Latt (1974a)</td>
</tr>
<tr>
<td>3.7</td>
<td>74</td>
<td>12.1</td>
<td>Latt (1974b)</td>
</tr>
<tr>
<td>3.07</td>
<td>72</td>
<td>7.9-11.2</td>
<td>Latt et al. (1975)</td>
</tr>
<tr>
<td>200.0</td>
<td>43</td>
<td>27.3</td>
<td>Dutrilliaux et al. (1974)</td>
</tr>
<tr>
<td>20.0</td>
<td>66</td>
<td>13.0</td>
<td>Kim (1974)</td>
</tr>
<tr>
<td>50.0</td>
<td>69-73</td>
<td>15.1</td>
<td>Galloway &amp; Evans (1975)</td>
</tr>
<tr>
<td>27.6</td>
<td>96</td>
<td>5.1</td>
<td>Solomon &amp; Bobrow (1975)</td>
</tr>
<tr>
<td>0.9</td>
<td>72</td>
<td>10.5</td>
<td>Tice &amp; Schneider (1975)</td>
</tr>
<tr>
<td>10.0</td>
<td>72</td>
<td>10.98</td>
<td>Morgan &amp; Crossen (1981)</td>
</tr>
</tbody>
</table>

The average number of SCEs varies from 5.1 to 27.3 per cell in human peripheral lymphocytes (Table 5). The high value (27.3) may be due to the high BUdR concentration (200 μg/ml). But similar SCE frequencies were obtained by different investigators in spite of using various BUdR concentrations; in which case, factors other than the BUdR concentration must have affected SCE induction (Kim, 1974; Latt, 1974a; Galloway & Evans, 1975). BUdR has also been shown to slow down the mitotic cell cycle (DuFrain & Garrand, 1981).

Effect of visible light. Ikushima and Wolff (1974) and Kato (1974b) showed that when BUdR or lUdR-substituted Chinese hamster chromosomes are exposed to visible light, the frequency of SCEs

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increases with increasing intensity of light. Kato also showed that
this increment is detectable only when cells receive visible light
illumination during the S phase, whereas exposure during the G₂ phase
does not affect the incidence of SCEs. Wolff and Perry (1974) showed
that the SCE frequency of cells cultured and handled in complete darkness is about half that of cells cultured under fluorescent lamps
that emit some light at 313 nm. It is this wavelength that causes
photolysis of BUdR containing DNA. Therefore, cells currently are
cultured in the dark.

Effect of the tissue culture medium used on differential staining
and SCE. Sharma and Das (1981) tested various concentrations of BUdR
required for producing differential staining in peripheral lymphocytes
of man, muntjac, and cattle. They grew the cells in three commonly
used culture media, viz., TC 199, Dulbecco's Minimum Essential Medium
(MEM), and Ham's F-10. They concluded that different mammalian species
require different concentrations of BUdR. Even for the same species,
the concentration of BUdR required for differential staining differs,
depending on the type of medium used. The minimum BUdR concentration
required for eliciting differential staining for different mammalian
species was independent of their DNA content. In all three species,
Ham's F-10 medium always required a higher concentration of BUdR as
compared with TC 199 or Dulbecco's MEM for differential staining.
They surmised that this difference was due to the thymidine in the
medium, the concentration being highest in Ham's F-10 and lowest in
TC 199. BUdR has to compete with the available thymidine for incor-
poration into the DNA molecule. Folic acid concentration may also
play an important role because it is essential in thymidine biosynthesis. Folic acid is present in Ham's F-10, although the highest concentration is found in Dulbecco's MEM. TC 199 has no thymidine, and the lowest folic acid concentration of all three media.

Morgan and Crossen (1981) studied the effect of five different culture media on SCE. The five media tested were: McCoy's 5A, Dulbecco's MEM, RPMI 1640, Ham's F-10, and TC 199; and the average SCEs from five subjects in each medium were 11.76, 11.98, 12.97, 13.35 and 14.08, respectively. A two-way analysis of variance demonstrated a significant difference in exchange rates among the five different media ($p < 0.05$). Tukey's method for examining all pairwise contrasts showed that lymphocytes cultured in McCoy's 5A medium had an SCE rate significantly lower than those grown in TC 199 ($p < 0.05$).

**Effect of sera on sister chromatid exchange.** Kato and Sandberg (1977) studied the effects of various batches of calf or fetal calf serum on cultured Chinese hamster cells. They used untreated dialyzed serum from three commercial sources. Their results showed that with some batches the frequency of SCE using untreated serum was significantly higher than with heat-treated serum. The SCE frequency with heat-treated dialyzed serum was lower than with untreated serum, but the difference was not large. They showed that the SCE frequency increased significantly as the untreated serum concentration was raised from 2.5 to 30%. They concluded that the untreated serum has some components that induce SCEs. This might explain the variation in the baseline SCEs reported by other investigators. Morgan and Crossen (1981) also studied the effects of autologous serum, fetal calf serum
(Gibco) and human AB serum (heat inactivated), each in 20% concentration. Tests were done on blood from six people. A two-way analysis of variance failed to indicate that there was a difference in SCEs among cultures grown in these three different sera.

**Effect of the staining technique on sister chromatid exchange frequency.** Morgan and Crossen (1981) compared SCE frequency using the hot phosphate technique (Korenberg and Freedlender, 1974) and the FPG technique (Perry & Wolff, 1974). They tested twelve subjects and showed that the SCE rate was higher with the FPG staining technique than with the hot phosphate technique. With the hot PO₄ technique, the chromosomes swelled and in some instances were removed from the slide.

**Lesions Responsible for Sister Chromatid Exchange**

Because SCEs involve exchange of partners, investigators have suspected that SCE formation depends on DNA repair processes (Kato, 1974a and 1974b; Latt, 1974b; Wolff et al., 1975). At this point, the mechanism of SCE formation is not known. Several mechanisms have been proposed (Galloway, 1977; Kato, 1977; Stetka, 1979; Painter, 1980). The relationship between the induction of point mutations and SCEs was studied by Carrano et al. (1978), who found a linear increase in the number of mutations and SCEs with increasing doses of alkylating agents. But with some agents the rate of mutation was greater than the rate of SCE, whereas it was the other way around with other agents. Thus, they concluded that although mutations and SCEs are caused by DNA modification, the specific lesions may be different. Swenson et al. (1980) studied the relationship between alkylation of specific bases and
SCEs were correlated with $O^6$ guanine alkylations from ethyl methane sulfonate, N-methyl-N-nitrosourea and N-ethyl-N-nitrosourea. Alkylation of $O^6$ guanine also appears to be a critical lesion in the induction of mutations (Singer, 1979). Swenson et al. concluded that SCEs can be considered to be quantitative indicators of potential mutagenic events.

The baseline and induced incidence of SCEs in many genetic disorders have been studied by different investigators in order to understand the mechanism of SCEs. Each disorder predisposes the individual to increased chromosomal aberrations or diminished ability to repair DNA damage. A summary of the results appears in Table 6.

Table 6

<table>
<thead>
<tr>
<th>Human Genetic Disorder</th>
<th>Baseline SCE Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bloom's syndrome</td>
<td>greatly increased</td>
</tr>
<tr>
<td>Fanconi's anemia</td>
<td>normal</td>
</tr>
<tr>
<td>Ataxia telangiectasia</td>
<td>normal</td>
</tr>
<tr>
<td>Xeroderma pigmentosum</td>
<td>normal</td>
</tr>
</tbody>
</table>

Bloom's syndrome, ataxia telangiectasia and Fanconi's anemia are human genetic diseases that are known to produce a high frequency of spontaneous chromosomal aberrations. However, the incidence of SCEs in ataxia telangiectasia (Chaganti et al., 1974; Galloway & Evans, 1975) and Fanconi's anemia (Chaganti et al., 1974; Kato & Stich,
1976) does not differ significantly from normal cells. Only in Bloom's syndrome (Chaganti et al., 1974) is there a large increase (12-fold) in the SCE frequency over the normal SCE rate. In the case of xeroderma pigmentosum, a human genetic disease, patients lack excision repair of DNA damage, but have normal frequency of SCEs (Wolff et al., 1975; Kato & Stitch, 1976). It has been suggested that SCEs may be induced by more than one mechanism, thus some may be related to DNA replications whereas others may be a type of DNA repair (Kato, 1977).

As can be seen from Table 3, many investigators have reported a wide variation in the baseline frequency of SCEs. So far, most of the tests have been done with strong mutagens and carcinogens which induce high levels of SCEs. However, when weak mutagens and carcinogens are tested, the small increases in SCE rate can easily be missed if variables are not carefully controlled. Thus, the need for standardization of the SCE technique in human lymphocytes is evident from the literature.
CHAPTER III

STUDY DESIGN AND METHODOLOGY

Study Design

As was discussed in Chapter II, there are many components of the in vitro culture system that affect the normal cell growth and the baseline incidence of SCEs. To establish a standardized and reproducible SCE technique, the following three objectives were set:

1. Establish culture conditions that yield a high frequency of metaphase spreads.
2. Determine the time of initiation of DNA syntheses.
3. Establish staining conditions that yield an adequate number of differentially stained chromatids for SCE analysis.

Objective 1

To establish culture conditions that yield a high frequency of metaphase spreads, the following components of a cell culture were examined:

1. The medium used to sustain cell growth;
2. The form of blood inoculum used and the initial lymphocyte concentration;
3. The PHA concentration.
4. The FCS concentration.

The medium used to sustain cell growth. There are many culture
media available from commercial firms that can be used for culturing human lymphocytes. For the present study, the following five media from Gibco (Grand Island Biological Company, Grand Island, New York) were tested: Chromosome Medium 1A; Ham's F-10; Medium 199 (IX); Minimum Essential Medium (MEM); and RPMI 1640.

Mitotic Index (MI) was used to determine which of the five media gave optimal cell proliferation.

The form of blood inoculum used and the initial lymphocyte concentration. Human peripheral blood cultures can be initiated using the following blood inoculums: whole blood; leukocyte-rich plasma; and purified lymphocytes.

Because of the need to have clean cultures for measuring cell proliferation with instruments, such as the scintillation counter and flow cytometer, inoculums with leukocyte-rich plasma and purified lymphocytes were used at an initial cell count of $3.5 \times 10^5$ lymphocytes per ml. Purified lymphocytes were obtained by separation on Ficoll-Hypaque (F-H) density gradient medium.

The PHA concentration used. Using the two blood inoculums, the PHA concentration was varied, keeping all the other conditions constant, i.e., the initial lymphocyte concentration and the FCS concentration.

The FCS concentration. The optimal FCS concentration was determined for the three forms of inoculum, using the corresponding optimal PHA, but varying the concentration of FCS. The optimal FCS concentration was determined by comparing the rate of DNA synthesis.
Objective 2

The time of initiation of DNA synthesis was determined for cultures, using optimal growth conditions (see above). The scintillation counter and flow cytometer were used for this purpose since they helped to determine the optimal time for addition of BUdR following PHA stimulation. If BUdR is added at the time of culture initiation, it has a longer time to cause adverse effects, such as, cell cycle delay and SCEs.

Objective 3

Staining conditions that can affect clear differentiation of chromatids are:

1. BUdR concentration.
2. Hoechst 33258 concentration.
3. The time of exposure to black light.
4. The staining time in 3% Giemsa.

BUdR concentration. As discussed in Chapter II, the minimum BUdR concentration needed for differential staining varies with the type of medium used. It is also known that BUdR affects cell kinetics and causes SCEs. Therefore, its concentration should be kept at a minimum. Concentrations ranging from 2.5 to 40 μg/ml were tested to determine the lowest concentration that would give clear differential staining.

Hoechst 33258 concentration. Various Hoechst 33258 concentrations

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(10 to 50 μg/ml) were tested to determine which one gave clear differential staining.

The time of exposure to black light. Various times of exposure to black light were tried to determine the one that would give clear differential staining.

The staining time in 3% Giemsa. Various staining times in Giemsa were tested to determine the time that would give clear differential staining.

Methodology

Subjects

Seven normal, healthy females and males ranging in age from 30 to 52 gave blood voluntarily for this research (a copy of the consent form appears as an Appendix). In order to safeguard the identity of these donors, they are referred to as 'A,' 'B,' etc.

Blood Collection

Blood was collected by venipuncture into a 10 ml evacuated blood collection tube (B-D Vacutainer obtained from A. T. Thomas & Company, Philadelphia, PA). Each tube was coated with 143 USP units of sodium heparin by the manufacturer. Blood was collected in the morning and used to prepare cultures the same day.

Cell Counts

WBC and differential counts were done on whole blood, leukocyte-rich plasma, and F-H-separated blood. The WBC and differential counts
were done by Mr. Marvin Darling, University Health Center laboratory, Western Michigan University. After proper dilution, lysing reagent (Scientific Products, McGaw Park, IL) was added to lyse RBCs. The WBCs were counted in the Hycel Counter-300 (Hycel, Inc., Houston, TX). To do differential counts, a smear made from the sample was stained with Wright's stain. One hundred cells were classified as either lymphocytes, segmented cells, monocytes, eosinophils, or stab cells.

Leukocyte or Lymphocyte Separation

Three methods were used for the separation of leukocytes and lymphocytes:

1. Leukocyte-rich plasma was obtained by allowing whole blood in the heparin tubes to stand at room temperature for one to four hours. The clear plasma on the top was carefully withdrawn, using a sterile Pasteur pipette. The WBC and differential counts were scored and recorded to find out if there was a difference in the concentration of lymphocytes from day to day and also from individual to individual. Cultures were prepared, based on the number of lymphocytes per ml, as calculated from the WBC count and the differential count.

2. Leukocyte-rich plasma was also obtained by adding .2 ml PHA (Wellcome, Research Triangle Park, NC, HA 15, Lot #9689) to 10 ml of whole blood in a 15 ml centrifuge tube. This was refrigerated for 30 min and then centrifuged at 500 rpm for 5 min. The clear plasma layer was removed using a sterile Pasteur pipette. The WBC and differential counts were scored and recorded.

3. Lymphocyte-rich plasma was obtained by density gradient
Three different Ficoll-Hypaque gradient media were tested to determine which one gave a good yield of lymphocytes. These three media were:

a. Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ);
b. Ficoll-Hypaque (Ficoll from Pharmacia Fine Chemicals and Hypaque from Winthrop);
c. Lymphoprep (Nyegaard & Company, Oslo, Norway). For the first two types of F-H media, two types of blood mixture were tried:

(i) Heparinized blood + equal volume of Ham's F-10 (Gibco, Powdered Medium, Cat. #430-1200);
(ii) Heparinized blood + 2 times the volume of Phosphate buffered saline (PBS) at pH 7.4,

The first two media were mixed as follows: Ficoll-29.5 g in 375 ml double distilled water; Hypaque-100.5 ml of 50% solution. The mixture was sterilized in an autoclave. For the third type of media, only the first type of blood mixture was tested. Table 11, included in Chapter IV, describes the conditions used for each type of media and the blood mixture used.

The appropriate type and amount of blood mixture was layered carefully on top of the gradient media in a 15 ml centrifuge tube. It was then centrifuged for the appropriate time.

After centrifugation, four separate bands were obtained. Lymphocytes accumulated in the second, milky-white layer. This layer was removed using a sterile Pasteur pipette into another 15 ml centrifuge tube. The WBC and differential counts were scored and recorded.
The lymphocytes were then washed with 12 ml Ham's F-10 medium. This mixture, after shaking, was centrifuged for 15 min at 1600 rpm at 18° C. The supernatant was discarded and the pellet was resuspended by tapping the tube. The above step was repeated one more time. The WBC and differential counts were scored also on the washed lymphocytes and recorded.

**Cell Culture for Chromosome Spreads**

Cultures were set up in 5 ml or 10 ml Ham's F-10 medium (Gibco, Powdered Medium Cat. #430-1200) in a 25 cm² Corning plastic flask. The medium was made by adding the following reagents to 100 ml medium complemented with 13% FCS (Sterile Systems, Inc., Lot #200214):

1. 3 ml of PHA (Wellcome, HA 15 Lot #9689) after 1:5.25 dilution, i.e., .48%.

2. .5 ml of streptomycin and penicillin mixture (Gibco, 5000 µg/ml and 5000 units/ml, respectively).

5 ml or 10 ml of the above mixture was pipetted into each flask. Plasma containing 3.5 x 10⁵ lymphocytes/ml of culture medium was added and the cultures incubated at 37° C. The appropriate concentrations of BUDR were then added before the initiation of DNA synthesis. The cultures with BUDR were protected from light by wrapping them in aluminum foil. Four hours prior to harvesting, .1 µg/ml colcemid (CIBA Pharmaceutical Company) was added to arrest chromosomes in the metaphase stage. The medium was then transferred to a 15 ml centrifuge tube. The cells were pelleted by centrifuging at 1700 rpm (setting of 70 on Dynac centrifuge) for 6 min. The supernatant was discarded. The pellet was resuspended in the leftover medium by tapping. The flask was washed with
10 ml of .56% (.075 M) potassium chloride warmed up to 37° C. This was transferred to the centrifuge tube containing the harvested cells, the tube shaken 5 times, and kept at 37° C for 7 min. It was then centrifuged at 1700 rpm for 6 min. The supernatant was discarded and the cells resuspended by tapping. Freshly made cold fixative (3 parts absolute methanol:1 part glacial acetic acid) was added in 5 ml aliquot and centrifuged. The above was repeated one more time. The cells in a small amount of fixative (approximately .25 ml) were stored in a refrigerator and were used to prepare slides for a period of up to 10 days.

Preparation of Slides

Slides were cleaned by soaking in a mixture of absolute alcohol and hydrochloric acid (3:1) for 3 to 4 h and then letting them air dry. Approximately 4 drops of the cell suspension were dropped on the clean slide. The fixative was blown to spread it over the slide. The slide was left in a horizontal position to dry.

Staining of Slides

Slides from cultures without BUdR were stained in 3% Giemsa (Harleco) for 11 min. Slides from cultures with BUdR were differentially stained using a slight modification of the florescence plus Giemsa (FPG) technique of Perry and Wolff (1974). In this modification, slides were soaked in Hoechst 33258 (50 μg/ml in sterile water) for 10 min. They were allowed to dry for 15 min. The slides were heated on a slide warmer at 55° C for 10 min and then covered with 5 drops of Sorensen's

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buffer (pH 7). A coverslip was placed on top and the slides placed under two black light tubes (GE F 15T8-BL, 15 watts) at a distance of 3 cm. The slides were exposed to the black light for 50 min. They were soaked in deionized water to let the coverslips slide off. The slides were allowed to dry for 15 min. They were then stained in 3% Giemsa in Sorensen's buffer (pH 6.8) for 15 min.

**Cell Cultures for Determination of DNA Synthesis by Incorporating 3(H)-TdR**

Cultures were set up in 1 ml Ham's F-10 medium supplemented with the following reagents: (a) 13% FCS; (b) 3% of 1:5.25 diluted PHA (Wellcome, HA 15, Lot #K9689); (c) 25 units penicillin; and (d) 25 μg streptomycin. Lymphocytes \(3.5 \times 10^5\) were added to the above mixture in a 3 ml plastic tube. The cultures were incubated at 37°C for 72 h. For the final 7 h, 100 μl of 100 μc/ml \(^3\)H-TdR was added. Cells from each sample were filtered on a glass microfibre filter disc (Whatman, 2.4 cm). Each filter disc was dipped in a saturated phosphate buffer and placed on the sampling manifold (Millipore), which operated under suction. From the 1 ml culture, .4 ml each was placed on 2 filter discs and the medium was removed by suction. The discs were first washed 3 times with 3 ml of cold 10% trichloroacetic acid (TCA). They were then washed 2 times with cold absolute ethanol in 3 ml aliquots. The discs were placed in scintillation vials and .5 ml of .5 N perchloric acid (PCA) was added to each vial. DNA was extracted by heating for 1 h in 70°C water bath. After cooling, 15 ml of cold scintillator fluid (Diotol Scintillator, Burdick and Jackson Laboratories) were added to each vial. Radioactivity counts were obtained.
using a Tri-Carb Scintillation Spectrometer (Packard, Model 3003). From these, counts per minute (CPM) per $1.0 \times 10^6$ lymphocytes were calculated, based on the initial lymphocyte concentration.

**Cultures for Flow Cytometric Analysis**

It was found that lymphocytes after F-H separation did not proliferate well. Yet for flow cytometry the cells had to be cleaned using F-H for clear DNA histograms. Therefore, 5 ml cultures were prepared, as described earlier, for chromosome spreads (without BUdR), i.e., using leukocyte-rich plasma separated after gravity sedimentation. The cultured cells were then separated on the F-H gradient for flow cytometric analysis.

**Fixation of Cells for Flow Cytometer**

Lymphocytes from two 5 ml cultures were pooled, separated on F-H, and kept on ice for 10 min or longer. To these cold cells, 3 ml of cold saline GM + EDTA was added and pipetted vigorously five times to break up clumps. Chilled absolute ethanol (kept in the freezer) was added three times in 3 ml aliquots, i.e., 9 ml altogether, with vigorous pipetting after each addition. The mixture was kept on ice for 10 min and then refrigerated. Flow cytometric analysis was done on FACS-11 (Becton Dickinson FACS Systems, Sunnyvale, CA). DNA was stained with 50 μg/ml mithramycin for 30 min in the dark. Cells in the range of $3-6 \times 10^4$ were analyzed for each sample. DNA histograms were recorded photographically from the cathode ray tube display of an ND-100 multi-channel analyser (Nuclear Data Inc., Schaumberg, IL) and stored on...
cassette magnetic tape. This was analyzed mathematically for cell-
cycle phase distribution on an IBM 370/158 computer.
CHAPTER IV

RESULTS

Determination of the Medium to be Used in the Study

Five tissue culture media, that are very often used for culturing human lymphocytes, were obtained from Gibco. They were tested to assess their ability to support human lymphocyte growth. The culture conditions are given in Table 7 and were the same as reported in the literature. In the case of Chromosome Medium IA, Donor A's whole blood was used. For the rest of the media, Donors C and G's whole blood was used. The mitotic index (Ml) was determined (Table 8). Ham's F-10 gave a high Ml for the blood of the latter two donors. Since Ham's F-10 was available at The Upjohn Company, where most of the present research was done, it was chosen as the medium to be used in all further experiments.

Variation in White Blood Cell Counts of Human Peripheral Blood

Human peripheral blood is a heterogeneous mixture of cells. (Its composition is discussed in Chapter II.) The WBC analysis for the same individual on different days indicates that the WBC concentration varies from day to day (Table 9). It also varies from individual to individual.
Table 7

Conditions Used for Culturing Lymphocytes from Human Blood Using Different Media from Gibco

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>Amount Used</th>
<th>FCS Used</th>
<th>PHA (Gibco)</th>
<th>Penicillin &amp; Streptomycin</th>
<th>Colcemid Used</th>
<th>Amount of Blood Used</th>
<th>Harvest Time</th>
<th>Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome Medium 1A</td>
<td>8 ml</td>
<td>2.5 ml</td>
<td>*</td>
<td>*</td>
<td>5 µg/ml</td>
<td>.5 ml</td>
<td>72 h</td>
<td>A</td>
</tr>
<tr>
<td>Ham's F-10</td>
<td>9 ml</td>
<td>1.0 ml</td>
<td>.3 ml</td>
<td>25 U + 25 µg/ml</td>
<td>5 µg/ml</td>
<td>.4 ml</td>
<td>72 h</td>
<td>C, G</td>
</tr>
<tr>
<td>Medium 199</td>
<td>8 ml</td>
<td>1.0 ml</td>
<td>.2 ml</td>
<td>25 U + 25 µg/ml</td>
<td>5 µg/ml</td>
<td>.5 ml</td>
<td>72 h</td>
<td>C, G</td>
</tr>
<tr>
<td>MEM</td>
<td>3.5 ml</td>
<td>.7 ml</td>
<td>.2 ml</td>
<td>25 U + 25 µg/ml</td>
<td>5 µg/ml</td>
<td>.5 ml</td>
<td>72 h</td>
<td>C, G</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>9.2 ml</td>
<td>2.0 ml</td>
<td>.3 ml</td>
<td>25 U + 25 µg/ml</td>
<td>5 µg/ml</td>
<td>.8 ml</td>
<td>72 h</td>
<td>C, G</td>
</tr>
</tbody>
</table>

* Chromosome Medium 1A is a ready to use medium; the quantities of PHA and antibiotics were not given.
Table 8
Mitotic Index of Various Media for Donors A, C, and G

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>Mitotic Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Donor A</td>
</tr>
<tr>
<td>Chromosome medium 1A</td>
<td>3.7</td>
</tr>
<tr>
<td>Ham's F-10</td>
<td>7.8</td>
</tr>
<tr>
<td>Medium 199 (IX)</td>
<td>3.6</td>
</tr>
<tr>
<td>MEM</td>
<td>1.5</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Table 9
Variation in the WBC and Lymphocyte Count of Two Donors at Various Times of Sampling

<table>
<thead>
<tr>
<th>Donor</th>
<th>Date</th>
<th>WBC/ml</th>
<th>Lymphs</th>
<th>Segs</th>
<th>Eo</th>
<th>Mo</th>
<th>Stab</th>
<th>Lymph./ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12/1/80</td>
<td>8.0 x 10^6</td>
<td>45</td>
<td>53</td>
<td>1</td>
<td>1</td>
<td>3.6 x 10^6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.1 x 10^6</td>
<td>45</td>
<td>52</td>
<td></td>
<td>2.8 x 10^6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/6/81 WB</td>
<td>6.3 x 10^6</td>
<td>39</td>
<td>60</td>
<td>1</td>
<td>1</td>
<td>2.5 x 10^6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.4 x 10^6</td>
<td>50</td>
<td>48</td>
<td></td>
<td>6.2 x 10^6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/20/81 WB</td>
<td>5.8 x 10^6</td>
<td>61</td>
<td>38</td>
<td>1</td>
<td></td>
<td>3.5 x 10^6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.3 x 10^6</td>
<td>45</td>
<td>55</td>
<td></td>
<td>4.2 x 10^6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6/16/81 WB</td>
<td>6.4 x 10^6</td>
<td>46</td>
<td>53</td>
<td>1</td>
<td></td>
<td>2.9 x 10^6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.7 x 10^6</td>
<td>70</td>
<td>29</td>
<td></td>
<td>6.8 x 10^6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1/30/81 WB</td>
<td>5.7 x 10^6</td>
<td>33</td>
<td>63</td>
<td>4</td>
<td></td>
<td>1.9 x 10^6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.4 x 10^6</td>
<td>47</td>
<td>53</td>
<td></td>
<td>6.8 x 10^6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3/27/81 WB</td>
<td>7.0 x 10^6</td>
<td>51</td>
<td>45</td>
<td>2</td>
<td></td>
<td>3.6 x 10^6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.3 x 10^6</td>
<td>72</td>
<td>27</td>
<td></td>
<td>8.7 x 10^6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4/10/81 WB</td>
<td>5.5 x 10^6</td>
<td>45</td>
<td>54</td>
<td>1</td>
<td></td>
<td>2.5 x 10^6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.4 x 10^6</td>
<td>58</td>
<td>52</td>
<td></td>
<td>4.9 x 10^6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4/27/81 WB</td>
<td>7.4 x 10^6</td>
<td>48</td>
<td>46</td>
<td>5</td>
<td>1</td>
<td>3.6 x 10^6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.0 x 10^6</td>
<td>81</td>
<td>19</td>
<td></td>
<td>8.9 x 10^6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* = white blood cells; ** = whole blood
Types of Blood Inoculums

Cells grew well when whole blood inoculum was used. However, the presence of high numbers of RBCs interfered with the study of DNA synthesis on the scintillation counter and the flow cytometer. For the scintillation counter, leukocyte-rich plasma separated by gravity sedimentation was clean enough, but for flow cytometer analysis, clean lymphocyte cultures were needed. Two methods of obtaining pure lymphocytes were tried. They were: (a) PHA separation and (b) Ficoll-Paque (Pharmacia Fine Chemicals Separation).

The yield of lymphocytes, using the PHA method of separation, was 12% whereas using the Ficoll-Paque method it was 23%. Cultures were started with whole blood, PHA-separated lymphocytes, and Ficoll-Paque-separated lymphocytes. After 72 h of culturing, cells were harvested and slides made. The MI was determined. The results appear in Table 10.

Table 10

The Culture Conditions and the Mitotic Index for Three Different Cell Inoculums Using Donor A's Blood

<table>
<thead>
<tr>
<th>Type of Inoculum</th>
<th>MI of Inoculum</th>
<th>Lymphocytes per ml to Initiate Culture</th>
<th>MI %</th>
<th>Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Blood</td>
<td>.4</td>
<td>1.4 x 10^5</td>
<td>7.1</td>
<td>A</td>
</tr>
<tr>
<td>PHA-separated lymphocytes</td>
<td>2.0</td>
<td>2.2 x 10^5</td>
<td>0</td>
<td>A</td>
</tr>
<tr>
<td>Ficoll-Paque-separated lymphocytes</td>
<td>1.5</td>
<td>1.3 x 10^5</td>
<td>0</td>
<td>A</td>
</tr>
</tbody>
</table>

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The results in Table 10 indicate that whole blood cell cultures grew well whereas PHA-separated and Ficoll-Paque-separated lymphocytes did not show any proliferation at all. Since the yield of lymphocytes with PHA separation was poor, the F-H separation was tried again with a view to improving the yield of lymphocytes. Three different types of F-H media were tested. The different conditions used for the separation appear in Table 11.

Tubes #2 and #4 did not evidence any separation (Table 12). The percentage recovery of lymphocytes from tubes #1, #3, and #5 were 50, 20 and 43, respectively (Table 12).

Although Ficoll-Hypaque gave the highest recovery of lymphocytes, Lymphoprep was used because it gave adequate recovery and was available ready-made in sterile vials.

Having determined the method of separating lymphocytes, cultures were started with different types of inoculums to determine the proliferation of cells.

Determination of the Optimal Level of PHA for Three Types of Inoculums

The following three blood inoculums were used:

1. Leukocyte-rich plasma.
2. Lymphoprep-separated lymphocytes: (a) with, and (b) without autologous cell-free plasma.

The highest level of DNA synthesis was seen with (1). Lymphocytes from F-H-separated blood did not proliferate well, irrespective of whether autologous cell-free plasma was added or not.

The three types of inoculums listed above were added to the culture
### Table 11

Conditions for Lymphocyte Separation with F-H

<table>
<thead>
<tr>
<th>Tube #</th>
<th>F-H type &amp; Amount</th>
<th>Blood Mixture* and Amount</th>
<th>Centrifuge** Setting</th>
<th>Temperature</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ficoll-Hypaque 3 ml</td>
<td>(A) 4 ml</td>
<td>140 x g</td>
<td>room</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>Ficoll-Hypaque 3 ml</td>
<td>(B) 7 ml</td>
<td>140 x g</td>
<td>room</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>Ficoll-Paque 3 ml</td>
<td>(A) 4 ml</td>
<td>400 x g</td>
<td>18° C</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>Ficoll-Paque 3 ml</td>
<td>(B) 7 ml</td>
<td>400 x g</td>
<td>18° C</td>
<td>35</td>
</tr>
<tr>
<td>5</td>
<td>Lympho-prep 5 ml</td>
<td>(A) 5 ml</td>
<td>400 x g</td>
<td>18° C</td>
<td>35</td>
</tr>
</tbody>
</table>

* Mixture (A) = heparinized blood + equal volume of Ham's F-10
  Mixture (B) = heparinized blood + 2 times the volume of phosphate buffered saline (PBS) solution at pH 7.4.

** The centrifuge used was Beckman's J-6B.
Table 12
Percentage Recovery of Lymphocytes after Separation Using Three Types of F-H Media

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Type of F-H Media*</th>
<th>Result after Centrifugation</th>
<th>% Recovery of Lymphocytes Without Washing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ficoll-Hypaque</td>
<td>clear separation</td>
<td>56</td>
</tr>
<tr>
<td>2</td>
<td>Ficoll-Hypaque</td>
<td>no separation</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>Ficoll-Paque</td>
<td>clear separation</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>Ficoll-Paque</td>
<td>no separation</td>
<td>--</td>
</tr>
<tr>
<td>5</td>
<td>Lymphoprep</td>
<td>clear separation</td>
<td>43</td>
</tr>
</tbody>
</table>

* The source of these media is given on page 31.

medium with various concentrations of PHA and an FCS concentration of 21.7% (Figure 3). (Various dilutions of PHA were made and 3% of each dilution was added to the culture medium.)

Higher concentrations of PHA (.36-.84%) were tested on leukocyte-rich plasma with 13% FCS in the medium. As the near-horizontal curve in Figure 4 indicates, any concentration of PHA between .36% and .84% gives optimal DNA synthesis and, therefore, optimal cell proliferation for cultures in which the initial lymphocyte concentration is approximately $3.5 \times 10^5$ ml.
Figure 3
Counts per Minute per $1.0 \times 10^6$ Lymphocytes at Different PHA Concentrations

The FCS concentration used was 21.7%. The initial lymphocyte concentration used was approximately $3.6 \times 10^5$ lymphocytes per ml. Blood from Donor B was used. In culture 0, leukocyte-rich plasma was used. In culture 1, F-H-separated blood with autologous cell-free plasma was used. In culture 2, F-H-separated blood without autologous cell-free plasma was used. $^3$(H)-TdR was added for the final 7 h of culturing. DNA was harvested at 71 h.
Leukocyte-rich plasma from Donor B was used. Optimal FCS concentration (13%) was used. The initial concentration of lymphocytes was approximately $3.5 \times 10^5$ lymphocytes per ml. $^3$(H)-TdR was added for the final 7 h of culturing and DNA was harvested at 71 h.
Optimal PHA concentration was also determined using a different lot (Wellcome, HA 15, Lot #K1340). Donor A's blood was used. Along with the scintillation counts, M1 was also determined. Figure 5 presents the results, with M1 given in parentheses. Both methods of determining cell proliferation, viz., DNA synthesis rate and M1, indicated the same optimal range of PHA concentration: .75-3%. For Lot #K9689, the optimal range was .36-.84%, though it could be higher (this was not tested). A different PHA lot (Lot #K9786) was tested at .48% concentration (a concentration within the optimal range for Lot #K9689). The M1 showed a very low yield of chromosome spreads (Table 13). Upon increasing the PHA concentration to 1%, a large increase in the M1 was observed.

Table 13

<table>
<thead>
<tr>
<th>% PHA Concentration (Lot #K9786)</th>
<th>M1 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>.48</td>
<td>.85</td>
</tr>
<tr>
<td>1.00</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Determination of Optimal FCS Concentration

Three inoculums were used:
1. Leukocyte-rich plasma (PHA -.48%).
Donor A's blood was used. An FCS concentration of 13% was used, and the initial lymphocyte concentration was kept at \(3.5 \times 10^5\) ml. Cell cultures were grown for 72 h. PHA was tested at .38, .75, 1.5, 3, and 6% concentrations. CPM per \(1.0 \times 10^6\) lymphocytes are plotted here against PHA concentrations. The percentage M1 appears in parentheses. \(3(H)\)-TdR was added for the last 6 h of incubation.
2. Lymphoprep-separated lymphocytes with autologous cell-free plasma (PHA - .24%).

3. Lymphoprep-separated lymphocytes (PHA - .16%).

The initial lymphocyte concentration was kept at $3.6 \times 10^5$ lymphocytes per ml. Each type of inoculum was added to three culture flasks, each of which had 5 ml Ham's F-10 medium complemented with 6.5, 13, and 21.7% FCS. The results appear in Figure 6. Again, Lymphoprep-separated lymphocytes with and without autologous cell-free plasma did not proliferate well. FCS concentration of 13% was used in all further experiments.

**Determination of the Initiation of DNA Synthesis**

The initiation of DNA synthesis was determined by two methods: (a) by following the uptake of $^3$(H)-TdR at different time intervals using a scintillation counter; (b) by determining entry into S phase, using a flow cytometer.

**Method 1.** Donor B's blood was used to obtain leukocyte-rich plasma. Cultures (5 ml) were set up in duplicates. From these, 1 ml portions were removed and $^3$(H)-TdR (100 μl of 100 μc/ml) was added for the last 2 h before harvesting the cells. Two discs, each with .4 ml culture, were prepared. The results shown in Figure 7 indicate that DNA synthesis starts around 30-40 h.

**Method 2.** After removing 1 ml for $^3$(H)-TdR addition, the remaining 4 ml portions from duplicate cultures were pooled. After centrifuging, the pellet was resuspended in 5 ml Ham's F-10 medium.
Blood from Donor B was used. In cultures ● and □, leukocyte-rich plasma was used. In culture ○, Ficoll-Hypaque-separated blood with autologous cell-free plasma was used. In culture ▲, Ficoll-Hypaque-separated blood without autologous cell-free plasma was used. The initial concentration of lymphocytes was approximately 3.6-3.8 x 10⁶ lymphocytes per ml in each culture. The PHA (Lot #K9689) concentration in each culture ● and □ was .48%, in culture ○ it was .24%, and in culture ▲ it was .16%. DNA was harvested at 69½ h in cultures ■, ○ and ▲ and at 71½ h in culture ●.
Figure 7

Counts per Minute per 1.0 x 10^6 Lymphocytes at Different Culturing Times

Plasma from Donor B was used. The optimal concentrations of PHA (.48%), Lot #K9689, and FCS (13%) were used with the initial lymphocyte concentration of 3.5 x 10^5 per ml. 3(H)-Tdr was added for 2 h prior to harvesting cells at 0 + 2, 24 + 2, 38 + 2, 44 + 2, 50 + 2, and 64 + 2 h.
This was layered over Lymphoprep and the lymphocytes were separated as described on pages 31-32. The lymphocytes were fixed and then stained with mithramycin (50 μg/ml) for analysis on the flow cytometer. The results in Table 14 show the percentage of cells in different phases, i.e., G₁, S, or G₂, present at various times of culture growth. Figure 8 shows the increase in the number of cells in the S phase as a function of time. Figure 8 also shows that DNA synthesis starts around 40-50 h.

Table 14
Flow Cytometer Analysis of Human Lymphocytes from Donor A's Blood

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% Cells in G₁</th>
<th>% Cells in S</th>
<th>% Cells in G₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>92.10</td>
<td>6.00</td>
<td>1.9</td>
</tr>
<tr>
<td>28</td>
<td>88.30</td>
<td>7.20</td>
<td>4.5</td>
</tr>
<tr>
<td>38</td>
<td>86.40</td>
<td>8.00</td>
<td>5.5</td>
</tr>
<tr>
<td>44</td>
<td>85.50</td>
<td>12.70</td>
<td>1.8</td>
</tr>
<tr>
<td>50</td>
<td>66.85</td>
<td>26.35</td>
<td>6.8</td>
</tr>
<tr>
<td>64</td>
<td>46.20</td>
<td>39.80</td>
<td>14.0</td>
</tr>
</tbody>
</table>
Flow Cytometer Analysis Showing Percentage of S Phase Cells at Various Time Periods

Plasma from Donor B was used. The optimal FCS concentration of 13% and the optimal PHA concentration of .48% (Lot #K9689) was used for $3.5 \times 10^3$ lymphocytes per ml. Cells were harvested at 0, 28, 38, 44, 50, and 64 h. Flow cytometric analysis was used to determine the percentage of cells in S phase.
Determination of the Lowest Concentration of BUdR and Hoechst 33258 that Would Give Clear Differential Staining

Cultures were set up with various concentrations (2.5, 5, 10, 20, 30, and 40 µg/ml) of BUdR. The MI and the percentage of differentially stained chromosome spreads appear in Table 15. Donor B's blood was used. BUdR was added 21 h after culture initiation. The lymphocyte concentration was $3.5 \times 10^5$ per ml. Cells were harvested at 72 h. As the BUdR concentration was increased, the MI decreased. The staining conditions and results appear in Table 15.

In this experiment, 10 µg/ml BUdR, 50 µg/ml of Hoechst 33258 with a staining time of 10 min, black light exposure of 50 min, and Giemsa staining of 15 min, gave clear differentiation and also the highest percentage of differentially stained chromosome spreads.
Table 15

Staining Conditions, Ml, and the Percentage of Differentially Stained Chromosome Spreads Using Donor B's Blood

<table>
<thead>
<tr>
<th>BUDR μg/ml at 21h</th>
<th>Hoechst 50 μg/ml Exposure Time (min)</th>
<th>Slide Warmer 55° C min</th>
<th>Black Light min</th>
<th>Giemsa min</th>
<th>Ml %</th>
<th>% Differentially stained spreads</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>10</td>
<td>10</td>
<td>45</td>
<td>13</td>
<td>5.3</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>10</td>
<td>50</td>
<td>15</td>
<td>4.6</td>
<td>48</td>
<td>clear differential staining</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>10</td>
<td>50</td>
<td>15</td>
<td>4.2</td>
<td>55</td>
<td>very clear differential staining</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>10</td>
<td>50</td>
<td>15</td>
<td>3.1</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>10</td>
<td>10</td>
<td>50</td>
<td>15</td>
<td>3.1</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>10</td>
<td>50</td>
<td>15</td>
<td>3.3</td>
<td>39</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER V

DISCUSSION

The need to identify genotoxic chemicals and estimate their potential somatic or genetic damage to humans calls for a sensitive and reliable bioassay as well as a readily available human tissue. As Carrano et al. (1980) have pointed out, the SCE assay, applied to human peripheral cells, fulfills these requirements. The SCE techniques available at the time when this study was begun, gave generally poor yields of differentially stained chromosome spreads and a poor person-to-person reproducibility. Often the yield of differentially stained metaphase spreads varied in cultures established from the same person at different times.

Lymphocyte Proliferation is Affected by the Type of Inoculum Used

For differential staining and SCE analysis, investigators have used whole blood, leukocyte-rich plasma, and defibrinated gelatine-sedimented blood to set up lymphocyte cultures. The present investigator used whole blood, leukocyte-rich plasma, and F-H-separated blood. Cultures started with whole blood (Table 7) and leukocyte-rich plasma (Figure 3) gave better cell proliferation than lymphocytes collected by F-H separation. The F-H separation may have altered lymphocytes in such a manner that they became insensitive to mitogenic activation with PHA (Nelson & Gatti, 1976). The addition of autologous cell-free plasma did not reconstitute the ability of F-H-separated
lymphocytes to respond to PHA. Since the leukocyte-rich plasma was free of RBCs, cultures from it were somewhat easier to handle than cultures started with whole blood; therefore, leukocyte-rich plasma was used as the inoculum in most experiments.

Lymphocyte Concentration Varies from Individual to Individual As Well As from Day to Day

Investigations reported in the literature invariably used a fixed volume of inoculum to initiate cell cultures from different individuals, as well as from the same individual at different times. Yet there is a wide variation in the concentration of lymphocytes from one individual to another and indeed from one day to another (Table 9). Therefore, as Carrano et al. (1980) have emphasized, there is a need for carefully controlling the amount of BUdR available per lymphocyte even for repeat experiments with the same individual's blood. They found that over a specific dose range, either doubling the BUdR concentration or halving the lymphocyte concentration can affect a 20 to 40% increase in SCE frequency.

Keeping the lymphocyte concentration uniform ensures that the same amount of PHA and BUdR per lymphocyte was available in each culture. Bernheim et al. (1978) showed that high doses of PHA resulted in suboptimal cell proliferation by causing excessive cell death. They also showed that at lower lymphocyte concentrations (2 x 10^5 per ml) cell proliferation was better than at higher lymphocyte concentrations (2 x 10^6 per ml). In the present research, a lymphocyte concentration of 3.5 x 10^5 per ml was used in all cultures. This
concentration gave a reasonably high MI and enabled this investigator to avoid drawing large quantities of blood from the donors.

**PHA Has a Wide Optimal Range which Varies from One Lot to Another**

The three PHA lots from the same manufacturer exhibited optimal mitogenic activity at different concentrations. Therefore, for the best results, each new lot of PHA should be tested for optimal mitogenic concentration.

**There is an Optimal FCS Concentration**

Investigators have used different type of sera, i.e., fetal calf serum, calf serum, autologous serum, and human AB serum for lymphocyte cultures. Kato and Sandberg (1977) showed that, in Chinese hamster cells, the frequency of SCEs was significantly different, depending on whether the serum used was heat-inactivated or not. They also showed that increasing the concentration of serum also increased the SCE frequency. Morgan and Crossen (1981) reported that there was no significant difference in the frequency of SCEs when they used autologous serum, fetal calf serum, or human AB serum. In the present study, 13% FCS concentration proved to be optimal. There may be a variation in the optimal concentration of FCS from one batch to another, but this was not tested. Since, as Kato and Sandberg have surmised, there may be some protein in the non-heat-inactivated that causes an increase in SCEs, the FCS used in this study was heat-inactivated.
Cell Proliferation is Affected by the Type of Culture Medium Used

Out of the five Gibco media tested, Ham's F-10 gave the highest MI. Sharma and Das (1981) found that for TC 199, Dulbecco's MEM, and Ham's F-10, the minimum BUDR needed for good differential staining was .17, 1.0 and 5.0 μg/ml, respectively. Another study, by Morgan and Crossen (1981), showed that the baseline SCE rate was similar for these three media at a BUDR concentration of 10 μg/ml. Thus, the correctness of choosing Ham's F-10 in this study was supported by studies reported later. Yet, if in future experiments other media yield as high or higher MI, and prove to be at least as efficacious in other respects at lower BUDR concentrations, the continued use of Ham's F-10 will need to be reconsidered.

The Time of Initiation of DNA Synthesis Varies from 30-50 H from the Beginning of PHA Stimulation

BUDR is needed in a lymphocyte cell culture just prior to the initiation of DNA synthesis. Adding it at culture initiation may add to adverse effects on the cell culture (DuFrain & Garrand, 1981). Tice et al. (1979) found that DNA synthesis may start as early as 40 h in some cells and as late as 92 h in others. There may be various subpopulations of lymphocytes with varying times of activation with PHA. The present study showed that the first DNA synthesis starts around 30-40 h (using flow cytometric analysis, Figure 8). Therefore, BUDR can be added around 30 h after culture initiation to make sure that it will be available for cells that start DNA synthesis earlier than others.
In conclusion, in the present investigation, a standardized protocol has been developed for optimizing the proliferation of human lymphocytes and for obtaining maximal number of differentially stained chromosome spreads. The standardization of culture conditions in this manner should contribute to the utility of the SCE bioassay.
CHAPTER VI

CONCLUSIONS

The three objectives of the present study outlined in Chapter III, and discussed in the last chapter, were accomplished.

Objective 1: Establish Culture Conditions that Yield a High Frequency of Metaphase Spreads

The following culture conditions were determined to give optimal cell proliferation:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>Ham's F-10 (Gibco)</td>
</tr>
<tr>
<td>Form of inoculum used</td>
<td>Leukocyte-rich plasma</td>
</tr>
<tr>
<td>Initial lymphocyte concentration</td>
<td>$3.5 \times 10^5$/ml</td>
</tr>
<tr>
<td>PHA concentration (Wellcome, HA 15, Lot #K9689)</td>
<td>.48%</td>
</tr>
<tr>
<td>FCS concentration (Sterile Systems, Inc., Lot #200214)</td>
<td>13%</td>
</tr>
</tbody>
</table>

Objective 2: Determine the Time of Initiation of DNA Synthesis

This was done by using two methods which, with the time ranges indicated by them, were:

<table>
<thead>
<tr>
<th>Method</th>
<th>Time Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scintillation counter analysis (Tri-Carb Scintillation Spectrometer)</td>
<td>30-40 h</td>
</tr>
<tr>
<td>Flow cytometer analysis (FACS-II)</td>
<td>40-50 h</td>
</tr>
</tbody>
</table>
Objective 3: Establish Staining Conditions that Yield Differentially Stained Sister Chromatids

It was found that the following staining conditions gave the clearest differentiation and optimal number of differentially stained chromosome spreads:

- BUdR concentration: 10 µg/ml
- Hoechst 33258 concentration (Aldrich Company, Inc.): 50 µg/ml
- Staining time in Hoechst 33258: 10 min
- Slide warmer (@ 55° C): 10 min
- Black light exposure (GE, F 15T8-BL, 15 watts): 50 min
- Staining time in Giemsa (3%)(Harleco): 15 min.

Differential staining and SCE analysis of human lymphocytes is a useful technique to test mutagenic-carcinogenic agents. Its use is marred by three factors: (a) an insufficient number of chromosome spreads obtained from in vitro culturing of cells; (b) difficulty in obtaining clear differential staining; and (c) variation in the baseline incidence of SCEs reported by different investigators.

The present study provides a procedure for standardizing the differential staining technique. This procedure ensures optimal growth conditions for human lymphocytes in vitro. The use of standardized differential staining techniques should bring about greater uniformity in methods as well as consistency in results; this in turn should ameliorate the present disarray surrounding the baseline incidence of SCEs. Such standardization should enhance the usefulness of SCE analysis as a diagnostic tool in the detection of mutagenic/carcinogenic agents.
APPENDIX

INFORMED CONSENT FORM

I am aware that: (1) Kamlesh Sharma is currently engaged in research involving the in vitro culturing of human lymphocytes; (2) the lymphocytes will be cultured along with cigarette smoke condensate and/or alcohol; (3) the effect of these agents on human chromosomes will be determined by looking at the change in the incidence of sister chromatid exchange; (4) more particularly, an investigation will be conducted as to the sister chromatid exchange-inducing ability and hence the potential carcinogenicity and mutagenicity of cigarette smoke condensate and alcohol individually and together; (5) I will be one of several volunteers who will donate blood for the aforementioned purposes; (6) it will entail my giving no more than 10 ml. of venous blood at any one time to be drawn at regular intervals no less than a week apart by a qualified person at the Western Michigan University Health Center or by a qualified medical technologist, and (7) the results of this investigation will be reported using a confidential code to safeguard the identity of the persons donating blood.

Consenting: .................................................. (Signature)

Date: ..................................................


Chaganti, R. S. K., Schonberg, S., & German, J. A many-fold increase in sister chromatid exchanges in Bloom's syndrome lymphocytes. Proceedings of the National Academy of Sciences, USA, 1974, 71, 4508-4512.


Csukas, P., Guagli, E., Anoni, F., Vida, G., & Solymosy, F. Role of metabolic activation in the sister chromatid exchange-inducing activity of ethyl carbamate (urethane) and vinyl carbamate. Mutation Research, 1981, 89, 75-82.


Galloway, S. M. Ataxia telangiectasia: The effects of chemical mutagens and x-rays on SCE in blood lymphocytes. Mutation Research, 1977, 45, 343-349.


Ikushima, T., & Wolff, S. Sister chromatid exchanges induced by light flashes to 5-bromodeoxyuridine and 5-iodo-deoxyuridine substituted Chinese hamster chromosomes. Experimental Cell Research, 1974, 87, 15-19.


Kihlman, B. A. Sister chromatid exchanges in vicia faba. II. Effects of thiotepa, caffeine and 8-ethoxycaffeine on the frequency of SCEs. Chromosoma, 1975, 51, 11-18.


Kučerová, M., Políková, A., & Bátora, J. Comparative evaluation of the frequency of chromosomal aberrations and the SCE numbers in peripheral lymphocytes of workers occupationally exposed to vinyl chloride monomer. Mutation Research, 1979, 67, 97-100.


Sharma, T., & Das, B. C. Culture media and species-related variations in the requirements of 5-bromodeoxyuridine for differential sister chromatid staining. Mutation Research, 1981, 81, 357-364.


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