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Comparison of Electron and Light Microscopy of Chromosome Aberration Induced in Human Lymphocytes by Adriamycin and Mitomycin C

Abbas Parsian
Western Michigan University

COMPARISON OF ELECTRON AND LIGHT MICROSCOPY OF CHROMOSOME ABERRATION INDUCED IN HUMAN LYMPHOCYTES BY ADRIAMYCIN AND MITOMYCIN C

by

Abbas Parsian

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COMPARISON OF ELECTRON AND LIGHT MICROSCOPY OF CHROMOSOME ABERRATIONS INDUCED IN HUMAN LYMPHOCYTES BY ADRIAMYCIN AND MITOMYCIN C

Abbas Parsian, Ph.D.

Western Michigan University, 1986

The present study was designed to develop a technique to prepare human chromosomes for sequential light and electron microscopic observation and to compare detectability of chromosome aberrations induced by adriamycin and mitomycin C by the two procedures. The technique developed preserved the morphological and structural organization of chromosome while allowing observation of the cell's entire chromosome complement. It was rapid and reproducible and chromosomes could be treated and stained for banding.

Light microscopic data showed that in cultures of human lymphocytes both drugs induce chromosome aberrations. In comparison with controls both drugs produced significantly more chromosome and chromatid fragments. Electron microscopy revealed greater numbers of chromosome aberrations in both drug groups at higher levels of statistical significance.

The differences between chromosome and chromatid fragments observed at the light and electron microscope levels were statistically significant. However, with mitomycin C, only the number of chromatid fragments scored at electron microscope was significantly greater than at light microscope. In mitomycin C and control groups chromosome fragments failed to show significant differences

between electron and light microscopy. However, the number of chromosome fragments scored was small. It is also possible that length measurement accuracy is not increased at the greater resolution level.

The present study showed the advantage of high resolution in detecting minute chromosomal aberrations. Should a reproducible banding technique applicable to electron microscope be developed, the use of high resolution in cytogenetics could be greatly extended. The technique developed could also be used for studying the ultrastructural organization of chromosome and/or chromosomal fibers.

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Western Michigan University PH.D. 1986

DEDICATION

To: All scientists who have spent the best period of their lives in research.

To: My mother--and in memory of my father.

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I wish to express my appreciation to my major professor, Dr. Darwin A Buthala, to members of my committee, Drs. George G. Mallinson, and Leonard C. Ginsberg, for their encouragement and guidance throughout this study; and to Dr. Robert H. Poel, coordinator of The Science Education program, Western Michigan University, when I enrolled.

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Abbas Parsian

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CHAPTER I

INTRODUCTION

Over the past decade it has become increasingly evident that mankind is being exposed to a wide variety of clastogenic agents, i.e., physical or chemical agents that are capable of breaking chromosomes. The production of visible chromosome aberrations in eukaryotic cells is known to be a sensitive indicator of damage to the genetic apparatus (Kihlman, 1966; Lea, 1946). A direct relationship between dose and effect has been demonstrated in man for radiation-induced aberrations both in vivo (Fischer et al., 1966) and in vitro (Evans, 1962). This relationship has also been shown for a large number of mutagenic and carcinogenic substances, in particular cytostatic drugs used in the treatment of malignant diseases (Hampel, Kober, Rosch, Gerhartz & Meining, 1966) and in a number of nonmalignant conditions (Jensen, 1967; Jensen & Soborg, 1966; Locher & Franz, 1967; Ryan & Baker, 1969).

Several lines of evidence indicate that chromosomal rearrangements may be one of the steps in carcinogenesis, although it is not completely known which types of genetic alteration are more relevant in this process (Cairns, 1981; Radman, Jeggo & Wagner, 1982). However, the mechanisms of production of chromosomal aberrations following treatment with mutagens or carcinogens are not fully understood. Chromosome breaking agents induce a variety of DNA lesions, such as, among others, single- and double-stranded breaks, apurinic and

apyrimidinic sites and cross-links. The primary lesions do not necessarily give rise to chromosomal aberrations, since they are subject to cellular repair. However, unrepaired or misrepaired lesions lead to chromosomal aberrations (Evans, 1977; Evans & Scott, 1969).

Virtually all the work on chromosome banding and chromosomal aberrations has been done at the resolution of the light microscope (LM). Although the electron microscope (EM) can provide a wealth of information not attainable with the LM, few attempts have been made to study banded chromosomes by this means (Bahr, Mikel & Engler, 1973; Burkholder, 1974, 1975; Comings, Avelino, Okada & Wyandt, 1973; Green & Bahr, 1975). No evidence was found of any attempts to study chromosome aberrations at high resolution transmission EM up to this date. The resolution of about 10 to 20 nm should be attainable with EM, whereas the practical limit of resolution in LM is about 200 nm. This represents approximately a 10 to 20 fold improvement. Such high resolution studies are important because they may reveal previously unidentified bands, pinpoint more precisely the exact location of breaks and exchanges, improve our knowledge of the structure of chromosome, and finally provide ideas about the mechanisms of banding and aberrations.

There are two conventional methods of observing chromosomes by EM: thin sectioning and whole mounts. The most common method for the ultrastructural analysis of metaphase chromosome is the whole mount technique (DuPraw, 1965), in which unfixed cells are spread on the surface of distilled water. This method has provided most of our current knowledge of chromosome structure (Comings & Okada,

1972; DuPraw, 1965, 1970). Burkholder (1974, 1975) using fixed Chinese hamster Don cells, made chromosome spreads on plastic film floated on distilled water. These techniques have several disadvantages: they produce various degrees of stretching and/or dispersion of the chromosomes and chromatin fibers; they do not provide consistently good chromosome spreads; and they are not rapid and reproducible. Xu and Wu (1983) used a technique in which chromosome spreads were transferred from glass slide to EM grids by coating slide with thin layer of parlodion film. With this technique it is frequently not possible to pick up all the chromosome within a spread.

It is generally considered that most of the cell injury is caused by damage to the genetic material that may be studied by observing chromosome aberrations. To compare chromosome aberrations (chromatid and chromosome types) induced in human lymphocytes at LM and EM level, adriamycin (ADM) and mitomycin C (MMC) were used. ADM is a glycoside antibiotic that was isolated from Streptomyces peucetius var. cesius. Reports on ADM in 1969 indicated its antitumoral potential. It has since been employed as an effective chemotherapeutic agent in the treatment of solid tumors and leukemia. Cytogenetic investigations of ADM reveal chromosomal lesions of both the chromatid and chromosomal type in phytohemaglutinin (PHA) stimulated lymphocytes. The frequency of "altered mitosis" (abnormal metaphase cells) was as high as 74% when doses as low as 0.05 or 0.1 μg/ml were present throughout a 72 hour culture period (Massimo, Dagna-Bricarelli, & Fossati-Guglielmoni, 1970).

Antibiotic MMC was first isolated from Streptomyces caespitosus

in 1956 and shown to possess antitumor and antibacterial properties. Shiba, Terawaki, Taguehi and Kamawata (1959) demonstrated that the antibiotic was a specific inhibitor of DNA synthesis, and the action of the chemical was suggested to be due to its alkylating properties (Schwartz, Sodergren & Philips, 1963). MMC produces only chromatid-type aberration whereas ADM has been shown to produce both chromatid and chromosome aberrations. One striking effect of MMC is the induction of chromatid exchanges involving homologues at corresponding points. It also produces quadriradial configurations in the treated cells.

There are problems with techniques that have been developed for studying human chromosomes with EM. Due to high resolution power of EM, more knowledge regarding the chromosomal structure, breakages and exchanges may be obtained. The purpose of this study was threefold: (1) to develop a technique to prepare human chromosomes for LM and EM, (2) to induce chromosome aberrations in human lymphocytes by the ADM and MMC, and (3) to compare chromosome aberrations at LM and EM levels to find out if there are minute aberrations that could not be identified by LM.

CHAPTER II

LITERATURE REVIEW

Electron Microscopy of Chromosome

The success of the EM in interpreting cell ultrastructure began with the introduction of better fixatives and methods for the preparation of ultrathin sections. In a few years the structure of cytoplasmic organelles was worked out in amazing detail. But the same methods proved most unrewarding for the study of the nucleus. With the best techniques, it remained a monotonous jumble of granules and nondescript fibrils with no evidence of chromonemata, chromosomes, or in fact, any continuous structures. Some blamed it on the fixation and looked for a special fixative to preserve chromosomal continuity. Others who had doubted the genetic continuity of chromosomes all along and saw, in the interphase nucleus, a bag filled with colloidal particles, hailed it as proof for their views (Makarov, 1960). But perhaps this unsatisfactory picture was not the fault of buffered osmium tetroxide, but rather the outcome of faulty interpretation of the data presented by thin sections. How can these essentially two-dimensional pictures be translated into the three-dimensional reality of the fixed nucleus? Ris (1961) proposed two methods that could be useful to accomplish this. First, the preparation for electron microscopy of intact chromosomes isolated from cells and secondly, the use of stereoscopic electron micrographs of

relatively thick sections. Together, these two methods are useful if the concern is to study the structural organization of small regions of a chromosome.

There are two conventional methods of observing chromosomes by EM: thin sectioning and whole mounts. There are pros and cons for each method for studying the ultrastructure of the chromosome.

Thin Sectioning

At this time, electron microscopy of the chromosome showed it consists of delicate fibers, whose reported diameters varied from 20-500 Å (Hay & Revel, 1963; Ris, 1961). Gall (1966), based on his work, believed that further characterization of the chromosome fiber, using conventional embedding and sectioning techniques, was difficult since only short segments appear in a given thin section. Burkholder (1977) stated that "since chromosomes are relatively large structures, thin sectioning only permits the examination of a wafer of the chromosome in one section and this method has provided little information on chromosome structure or organization" (p. 324). Ris (1978) restated his idea of 1961 about thin sectioning of chromosome by saying that in usual thin sections, it is, however, quite impossible to get information on the spatial arrangement of chromatin fibers.

Hozier, Furcht and Wendelshafer-Grabb (1981) are supporters of thin sectioning method. He and his associates developed an embedding procedure called "EM Transfer Technique." Briefly, fixed cells were spread in 60 mm plastic tissue culture dishes. Good spreads were

identified by LM and were fixed with 2.5% glutaraldehyde and post-fixed with 1% osmium tetroxide. They were dehydrated by ethanol and washed with ethanol: epon 812. Epon was poured to depth of 3 mm in a dish and short pieces of polyethylene tubing were centered over marked areas. After overnight incubation at 60 degrees C, the tubings were filled with epon and incubated for 48 hours. The tubings with specimen were separated by immersion in liquid nitrogen. Sections were made using a diamond knife and picked up by carbon-formvar coated grids. Hozier et al. (1981) published several EM micrographs of longitudinal cross section of whole chromosomes. But, it is important to notice that producing this kind of section is not easy, especially for long chromosomes. So, this technique poses a problem for studying the cells entire chromosome complement.

Mitotic cells in suspension were used for embedding by Lampert and Lampert (1970). Cells were fixed with glutaraldehyde and post-fixed with osmium tetroxide. After dehydration, the cells were embedded in epon. Thin sections with gray color were stained with uranyl acetate (UA). A reduction in the site of fiber diameter was noticed in sections, of course, as compared with the fiber size of whole mount chromosomes. This reduction is explained by shrinkage secondary to fixation and embedding (Schwarzacher & Schnedl, 1967; Wolfe, 1968).

Some investigators embedded isolated chromosomes for thin sectioning. Bak, Zeuthen and Crick (1977) isolated human fetal fibroblast chromosomes by a method based on the procedure of Wray and Stubblefield (1970). Chromosomes were embedded in vestopal and

60-80 nm sections were produced. Adolph (1980) isolated Hela cell chromosomes by four different methods. The methods differed in divalent cations (Mg 2+ and Ca 2+) and polyamines (Spermine and Spermidine) in HEPES and CAPS buffers. The isolated chromosomes were fixed with glutaraldehyde and postfixed with osmium tetroxide. Spurr medium was used for embedding and 60-90 nm sections were cut using a diamond knife. Laughlin, Wilkinson-Singley, Olin and Olin (1982) used Chinese hamster ovary cells in their studies. The chromosomes were isolated by three techniques, namely, Adolph, 1980; Rattner and Hamkalo, 1978; Wray and Stubblefield, 1970. The chromosome suspension was fixed and postfixed with glutaraldehyde and osmium tetroxide respectively. Chromosomes were embedded in Spurr's resin and 60-80 nm sections were produced. Laughlin et al. (1982) believed that ultrastructural studies of metaphase chromosomes fixed in situ have been largely uninformative due to the dense packing of chromatin fibers. In the above studies, the thin sections were cut for the most part across the chromatid arms. Again, the sections are representative of a small region of chromosome. Therefore, they are not informative for structural studies of whole chromosomes although they provide information regarding the structure and organization of chromatin fibers in small areas.

Whole Mount

There are many investigators who have supported the whole mount techniques, but there have also been detractors of these techniques. For example, Hozier et al. (1981) stated that in whole mount chromosomes, little detail regarding the internal arrangement of chromosomal components can be seen. He believed there were two main disadvantages: (1) difficulty in visualizing internal structure when it is necessary to penetrate the entire body of the chromosome; and (2) difficulty in comparing standard cytogenetic staining patterns with the electron microscopic view. He also believed that formvar is fragile and does not stand up to banding and staining procedures.

The studies done with whole mounts area are divided into four categories based on the principles behind the techniques. Chromosomal spreading on a water surface for whole mount EM studies was first developed by Gall (1963, 1966). Similar spreading techniques had been previously used by Kleinschmidt and Zahn (1959) to study DNA preparations and by Fernandez-Moran (1948), Parsons (1963) to observe cytoplasmic structures. Materials were spread at an air-water interface, using a "Langmuir trough" built according to instructions by Stong (1961). The trough was filled with distilled water. Control over the density of material on the final EM grid is achieved by compressing the spread film. The bulk of the film is assumed to be protein, with the chromosomal material being merely enmeshed in the film or suspended from it. Materials were picked up by touching conventional carbon coated grids to the surface, followed by fixation

and critical point drying. Gall (1966) reported that chromosomal fibers were not clearly evident. Groups of chromosomes from dividing cells were recognizable on the basis of their gross morphology. However, many chromosomes showed extreme stretching and distortion. Ris (1978) reported that the chromosomes prepared in this manner were generally considerably distorted and the procedure was not recommended where the arrangement of chromatin fibers in higher order structure was to be preserved. However, the procedure has been used successfully, with some modification, by several investigators (Comings & Okada, 1972; DuPraw, 1965, 1970; Lampert & Lampert, 1970; Okada & Comings, 1980; Ris, 1978, 1981; Ris & Chandler, 1963; Wolfe, 1965a, b).

Burkholder's (1974, 1975) spreading technique is a modification of Gall's (1966) procedure. He coated a slide with a formvar film and evaporated carbon. A stiff wire screen was placed in the bottom of a tray. The film was released on the surface of the water in a tray. A drop containing mitotic cells suspended in 6:1, methanol: acetic acid was put on top of the floating film. The wire screen with grids on it, was lifted out of the water with forceps so that the formvar-carbon film is spread over the grids. The grids were then air-dried and stained with Giemsa by floating grids on drops of stain. Even though this technique is an improvement of Gall's (1966) technique, it still causes some chromosome stretching and dispersion.

The second category of whole mount preparation of chromosome for EM is the transfer of chromosomes to formvar coated grids by gradient centrifugation. The technique, developed by Stubblefield (1975),

uses chromosomes isolated by the technique of Wray and Stubblefield (1970). Goyanes and Mendez (1981) have used this technique with some modification. A 12 mm circular coverslip (No. 1) is placed in the bottom of a 15 ml round bottom glass centrifuge tube. Two hundred mesh coated grids are put on top of the coverslip and covered with 2.5 ml of 10% sucrose in chromosome buffer. The chromosome suspension was layered over the sucrose and sedimented by centrifugation at 1500 g for 15 minutes. Grids with adhering chromosomes were washed in chromosome buffer, dehydrated and critical point dried. Goyanes and Mendez (1981) have used this technique with some modification. Chromosomes were preserved but the technique required considerable time, was difficult to reproduce and did not exhibit the entire chromosome complement.

The third category of chromosomal preparation for EM is the direct transfer of cell (Hozier et al., 1981) or chromosome (Bak et al., 1977; Ris, 1981) suspension onto EM grids. Either fixed or unfixed cells or chromosomes were spread on formvar-carbon coated grids, then fixed, stained, dehydrated and critical point dried. Hozier et al. (1981) stated that little detail regarding the internal arrangement of chromosomal components can be seen by this technique. He also reported that his chromosome preparations appeared quite similar to Chinese hamster chromosomes prepared in 1974 and 1975 by Burkholder. Burkholder's technique is quite different from that of Hozier's. The limitations of these techniques are that good chromosome spreads are difficult to produce and a significant number of cells or chromosomes are wasted during the transfer.

The fourth whole mount chromosome category for electron microscopy can be divided into two groups based on differences in methods of transferring chromosome spreads from the glass slide to EM grids. In the first group, glass slides are coated with plastic (Burkholder, 1981; Haapala & Nokkala, 1982; Parsian & Buthala, 1985) or carbon (Ruzicka, 1977) and the chromosome spreads are produced on these slides by air-dry techniques universally used for light microscopy. Good spreads are located with a LM, marked, and the supporting film and chromosomes floated or pulled off the slides on surface of water or water containing hydrofluoric acid. EM grids are positioned over or under the chromosome spread and the films with grids picked up using parafilm or nonwetable cardboard or stiff wire screen and air-dried. At this stage, chromosomes can be treated with stains, enzymes or any agents.

In the second group under category four, chromosome spreads are produced on plain glass slides by the squash technique (Ris, 1978, 1981) or by the air-dry technique (Xu & Wu, 1983). The chromosome preparation is then stained and coated with a thin layer of parlodion by dipping the slides in a solution of parlodion in amyl acetate. Good chromosome complements are marked, scoring the plastic with a razor blade or other markers. The parlodion film with or without grids is floated off on the surface of water or water containing hydrofluoric acid. The chromosomes are then air-dried and/or can be critical point dried or coated with carbon. The disadvantage of this technique is that not all the chromosomes within a spread are recovered.

The techniques described were developed primarily to study the structure and organization of mammalian and human chromosomes. Different techniques reveal different information regarding the organization of chromosome chromatin fibers and proteins. These procedural differences have lead to several models for chromosome organization. However, it is generally accepted that chromosomes are uninemic (Comings & Okada, 1972; Kavenoff & Zimm, 1973; Prescott, 1970; Wolfe & Perry, 1975), that is, a single DNA molecule runs from end to end through a mitotic chromatid. Human chromosomes prepared by surface spreading and critical point drying appear composed of 200-300 Å wide fibers (Abuelo & Moore, 1969; DuPraw, 1965; Gall, 1966; Ris & Chandler, 1963; Wolfe, 1965). Similar fibers have been seen in thin sections but with smaller dimensions (Wolfe, 1968). The deoxyribonucleoprotein component of the chromosomes resides in this fiber. As revealed by autoradiography (Hay & Revel, 1963) and enzyme digestion (DuPraw, 1965) this single, irregularly folded fiber that makes up a chromatid (DuPraw, 1965, 1966) contains the DNA components. Based on dry mass determinations, DuPraw and Bahr (1969) proposed that a fully packed chromosome fiber consists of a second order supercoiled fibril that contains the DNA-double helix as a first order DNA-protein supercoil.

Several investigators have used whole mount of chromosomes collected by spreading unfixed cells on the surface of distilled water for EM study of chromosome banding (Bahr, Mikel & Engler, 1973; Comings, Avelino, Okada & Wyandt, 1973; Green & Bahr, 1975). This method commonly produces various degrees of stretching and/or

dispersion of the chromosomes and chromatin fibers. This can be a distinct disadvantage for investigations of chromosome banding since the banding methods are normally performed on condensed and fixed chromosomes. Several others have used whole mount electron microscopy of chromosome by spreading fixed cells on surface water (Burkholder, 1974, 1975) or on coated slide (Burkholder, 1981; Haapala & Nokkala, 1982; Ruzicka, 1977) or on plain slide (Xu & Wu, 1983) for studying banded chromosomes.

Techniques for banding chromosomes for EM commonly employed G-banding procedures. It has been observed that the various G-banding methods yield similar results with some differences in the quality of the ultrastructure of the banding pattern (Burkholder, 1974, 1975; Ruzicka, 1973). Bahr et al. (1973) reported that for the distribution of chromatin mass (46xy) they have found 225 bands that were considered major (EM) and 453 minor (em). It appears that, on the average, two EM bands contribute to one Q band, whereas the ratio of Q to G bands is 1:1.7 in their assessment. A distinctly discontinuous distribution of chromosomal matter along chromatids suggest a basis for all banding observations. Such detailed banding description may be useful in the mapping of the human genome, in defining new cytogenetic markers, and in identifying small marker chromosomes in cancer cells as well as chromosomal fragments in microchromosomes.

As mentioned in Chapter I, there has not been any study regarding the chromosomal aberrations at transmission EM level. Therefore, the literature related to ADM and MMC and their effects on human chromosomes is at LM level.

Induction of Chromosome Aberrations

The study of chemically-induced chromosomal abnormalities in humans is relatively recent, the first being carried out by Pollini and Colombi (1964a, b) and Vigliani and Saita (1964). These published papers concerned abnormalities in the peripheral lymphocyte chromosomes of benzene-exposed workers. Studies of other chemical effects on human chromosomes tend to be more limited than those of benzene. Several anticancer drugs have been shown to produce such abnormalities after treatment. These include azothioprine (Jensen, 1967), daunomycin (Whang-Peng, Leventhal, Adamson & Perry, 1969), cytosine arabinoside (Bell, Whang, Carbone, Brecher & Bloek, 1966), methotrexate (Jensen, 1967) and 6-mercaptopurine (Bischoff & Holtzer, 1967). In view of the general cytotoxic and carcinogenic effects of some of these anticancer drugs it is not surprising that they produce clastogenic effects.

In this study designed to compare chromosome aberrations (chromatid and chromosome types) induced in human lymphocytes at LM and EM level, ADM and MMC were used. These antibiotics are antineoplastics agents. Biochemically, their significance resides primarily in the fact that both are capable of interacting with DNA and can inhibit synthesis of macromolecules of genetic importance. This, and related properties, are apparently responsible for their potency in causing chromosome damage in both plant and animal cells. However, both of these chemicals do not appear to have the same potentials for being general mutagens. MMC is a definite mutagenic compound whereas less

is known about the potentials of ADM, although preliminary data do not suggest its general mutagenic properties. Despite the fact that both these chemicals induce sister chromatid exchanges (SCE), alter recombinational patterns, and induce chromosome aberrations, their modes of action in terms of their effects on DNA, RNA and protein synthesis are clearly different. The concentrations of these chemicals required to induce aberrations are generally very low (Fishbein, Flarn & Falk, 1970) and the chemicals appear to be capable of expressing delayed-type effect to different degrees. As discussed in detail in the following pages, MMC produces only chromatid-type aberrations whereas ADM has been shown to produce both chromatid as well as chromosome types of aberrations, sometimes in the same cell given only a pulse treatment.

Adriamycin (ADM)

ADM is a glycoside, made up of an aglycone chromophore (adrimycinone) linked to an amino sugar and it is 14-hydroxydaunomycine. The anthracyclines are antibiotics with anticancer properties against a variety of tumors. The best studied member of the group, ADM, has potential against several carcinomas, among others soft tissue sarcomas, pediatric solid tumors, malignant lymphomas and acute leukemia (Carter, 1975).

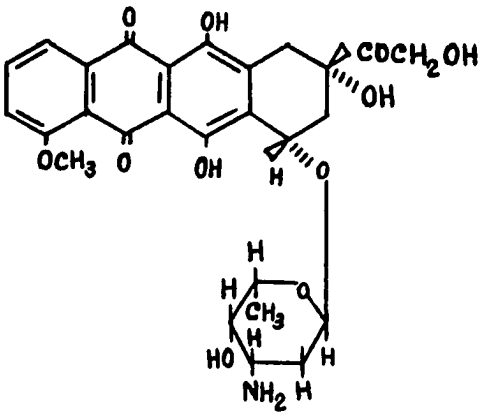

Figure 1. Structure of Adriamycin (ADM)

ADM was isolated from Streptomyces peucetius var. caesius in 1967 by Arcamone, Franceschi, Teno and Selva at the Farmitalia Research Laboratories, in Milan, Italy. Studies on its antitumor activity have been carried out mainly on mice and rats bearing Ehrlich ascites as well as solid tumors. A comparison of the results obtained with the antibiotics daunorubicin and ADM, showed that the latter is a more active drug (DiMarco, Gaetani & Scarpinato, 1969). The microscopic findings demonstrated that the ADM promptly stops the proliferation of a tumor. A clinical trial has been made by Bonadonna, Monfardini, DeLena and Fossati-Bellani (1969) in adults and children, suffering either from leukemia, hematosarcoma or solid tumors, and by Massimo, Cottafava, Mori and Fossati-Guglielmoni (1969) in children with solid tumors in an advanced stage.

Effects on Chromosomes

ADM has been investigated for its cytogenetics effects. Two laboratories independently reported on chromosome aberrations induced by ADM in cultured human lymphocytes (Massimo et al., 1970; Vig, 1971) with results similar to those obtained with daunomycin (DNM). Massimo et al. (1970) proposed that chromosomal damage induced by the chemical can produce an inhibition of blastogenesis and cellular aberrations observed in the treated cells. The frequency of "altered mitosis" (abnormal metaphase cells) was as high as 74% with doses as low as 0.05 or 0.1 μg/ml when added throughout the culture period of 72 hours. But, most of the mitosis had only minor aberrations and the chromosomes maintained their regular morphology. Under these conditions, doses of 1 μg/ml or higher were apparently lethal or completely inhibited cell division. At all the concentrations used, (0.05 to 5 μg/ml), ADM inhibited the transformation of small lymphocytes into blast cells. The inhibitory effect increased in a linear relationship, with the increase in the dose. The cytogenetic damage produced by ADM on lymphocytes, was detectable at doses lower than 0.5 μg/ml, whereas at higher concentrations mitotic figures were absent. The severity of the chromosome lesions varied with the dose. The most frequent effect seen was the fragmentation of the chromatids, the extremities of which tended to link either between themselves, forming dicentric chromosomes and rings, or with the chromatids of other chromosomes forming rackets, rearrangements, translocations or polyradial figures. Longitudinal fragmentation of centromeres with

consequent division of the chromatids between themselves; the fusion of the satellites of two acrocentric chromosomes; alteration of the morphology of the chromosomes such as "erosion," lysis, stickiness and agglutination; polyploidy, due to either endoreduplication or to lack of cell division; and aneuploidy with hypo- and hyperploidy were also observed. Massimo et al. (1970) concluded that ADM mainly induces chromosome lesions on normal lymphocytes grown "in vitro" with PHA, an effect usually assumed as mutagenic.

A more detailed account of aberrations was provided by Vig (1971) who reported a high percentage (71%-92%) of abnormal cells from cultures treated for only 4 hours (44-48 hrs.) with concentrations ranging between 0.02 to 0.15 μg/ml. In this study some cells appeared totally "demolished" or pulverized whereas in others, the morphology of the chromosomes was deformed. The chromosomal aberrations included fragments and exchanges of both chromatid and chromosome types. The most predominant aberration (37.6%) was chromatid-type exchanges. Next in order were the chromosome fragments (28%), that together with the chromatid-type fragments made up 17.7% of the total. The frequency of asymmetrical (U-type) exchanges was higher in the material treated with 0.02 μg/lm than that of symmetrical (X-type) exchanges from the same material (63% to 37%). These observations confirm the large deviation from expected 1:1 as observed in almost every instance dealing with such analyses. Also, about 73% of the cells had chromatid-type exchanges. To confirm the chromosome damaging potential of ADM and to determine whether continuous treatment as given in above experiments yields different results from a

short-term treatment for a few hours, Vig (1971) treated the cells with ADM at 0.05 and 0.10 μg/ml of culture between 20 to 24 hours and also with 0.05 μg/ml during 44 to 48 hours of culturing. Cells were harvested 24 hours later. The results confirmed the observations made earlier (Vig, 1971). The type and frequency of aberrations and the relationship between aberrations were also studied. The results indicated that the frequency of aberrations is dose dependent. An increase in concentration of the drug caused a rapid increase in the proportionate frequency of chromatid-type exchanges. Although there were as many as 37.5% of the exchanges observed that involved homologous or apparently homologous chromosomes, only 8 out of 120 exchanges involved exactly corresponding loci. Vig (1971) believes that these data do not express preferential breakage and reunion at corresponding points on homologous chromosomes and may not thus support the idea that somatic crossing over had occurred. The frequency of chromosome breaks was always higher than the frequency of chromatid breaks but in general parallel with the frequency of chromatid exchanges. There were very few, if any, exchanges of chromosome type.

Newsome and Singh (1977) studied the cytologic effects of ADM on human peripheral lymphocytes by exposure of cells to ADM for a very short period of time. During hour 44-45 and 67-68 of 72 hours series of cultures were treated for one hour with ADM in a final concentration of 0.00, 0.01, 0.03, 0.05 and 0.10 μg/ml. All the treated cultures showed chromosomal structural changes. The aberrations recorded five and 24 hour posttreatment were mostly of the

chromatid-type, including simple breaks and deletions. Other structural abnormalities observed were sister chromatid reunions, despiralization, binucleate cells and dicentric chromosomes. Chromatid exchanges were rare. The overall aberration frequency decreased from 52% to 10% with an increase in duration of recovery and concentration of ADM. Most of the aberrations that recovered were chromatid breaks in cells harvested five and 24 hours posttreatment. Such breaks were higher in cells treated with lower concentrations (.01 and .03 μg/ml) and harvested five hours later. The 44 chromatid breaks scored failed to show specificity in terms of the chromosomal breaking action of ADM within or among chromosome groups. ADM also induced a paucity of both chromosome type lesions and chromatid exchanges even in cells with the highest frequency of aberrations. No chromosome breaks were recovered in cells treated with higher concentrations of ADM (0.10 μg/ml) and harvested five and 24 hours posttreatment. Dicentric chromosomes were very rare and were recovered only in cells treated for one hour with 0.05 μg/ml ADM and harvested 24 hours later. Newsome and Singh (1977) concluded that preliminary data in their laboratory showed clinical preparations of ADM cause chromosomal structural changes in human lymphocytes. But the spectrum of aberrations failed to show that ADM induces aberrations as drastic and extensive as earlier studies reported (Vig, 1971). Previous studies reported a percentage aberration frequency of 50% to 83% in cells treated with 0.02 μg/ml for 24 hours or 0.05-0.15 for 3 to 4 hours (Vig, 1971). Newsome and Singh (1977) believed that this differential effect may well be due to some cells escaping the effect of ADM, a limited

exposure time, and a different genetic background of the exposed cells.

Nevstad (1978) studied the effects of ADM on chromosomal aberrations in human lymphocytes in vitro and in vivo. In an in vitro study, ADM treatment lasted for 48 or 24 hours. Only a few chromosomal aberrations (break events) were observed at low concentrations of ADM (0.001 μg/ml). Maximal frequencies of SCE (24.5/cell) and chromosomal aberrations (5.5/cell) in the experiment treated with ADM for 48 hours were observed at 0.1 μg ADM/ml. Higher concentrations of ADM inhibited mitosis and the number of second divisions necessary for scoring SCE was extremely low. In another set of experiments ADM lasted for 24 hours. Here, at a concentration of 0.1 μg/ml, the frequency of SCE was lower than when treatment lasted for 48 hours; and chromosomal aberrations were rare. Higher frequencies of SCE and chromosomal aberrations were observed at 0.2 μg/ml. Even though Nevstad (1978) showed the relationship between duration of exposure and concentration of ADM with chromosomal aberration, he failed to report what percentages of cells were affected or had chromosomal aberrations. He only showed the chromosome aberration/cell for different ADM concentrations on a graph. So, it is difficult to compare his results with previous data that show percentages of aberrant cells. As far as the chromosomal aberrations are concerned, Nevstad's (1978) study supports the previous data but again fails to indicate the types of chromosomal aberrations induced in the cells.

Triradial structures have been observed after radiation and chemical treatments. These structures are generally interpreted to be

the results of isochromatid-chromatid type exchanges. As expected, triradials generally are rare. Some triradials have two centromeres, suggesting their origin from two chromosomes. Others have only one centromere, and these monocentric triradials sometimes have one very small arm. The monocentrics are, however, less frequent than dicentrics. Some of these monocentric triradials appeared to have originated from intrachromatidal rearrangements of one chromosome; a fact supported by the rare presence of cells with a monocentric triradial as the only aberration. Vig (1971) reported that one class of interest in human lymphocytes treated with ADM, was the triradials with single, two, or very rarely three centromeres in the whole complex. Usually, centromeres in excess of one were located one on each chromosome participating. Some of the monocentric triradials were extremely small and appeared to have originated by intrachromosomal manipulation. Massimo et al. (1970) reported about polyradial figures but Newsome and Singh (1977) and Nevstad (1978) did not mention anything regarding triradial or polyradial figures in lymphocytes treated with ADM.

Position of Chromosome Breaks

Positions of chromosome breaks induced by ADM along the length of human genome have been investigated and nonrandomness was observed both between and within chromosomes. Vig (1971) analyzed the positions of chromosome breaks and exchanges from three samples treated with 0.05 μg/ml ADM between 44 and 48 hours of culturing. It was found that the break positions, at least qualitatively, were similar to one

another. Hence, the data were pooled to give composite diagrams for the points of breaks inferred from exchanges and for breaks inferred from fragments. Chromosome 2 was involved more often, in absolute frequency, than any other chromosome, but it was group G (21/22) that was involved most often per unit length. Y chromosome was the only one that was not involved in any exchange. Group C was engaged maximally in intragroup exchanges, i.e., exchanges involving both chromosomes from the same group. The involvement of various chromosomes was not uniform or dependent on their length but, nevertheless was somewhat nonrandom. The distribution of the points of exchanges gave impression of being nonrandom, with a certain region along the length of chromosome being much more susceptible to breakage. A total of 112 points of chromosome breaks were analyzed from fragments. The distribution of points of breaks along the length of chromosomes or groups thereof, did not support the conclusion that breaks are nonrandom. Also, the frequency of breaks per unit of chromosome length in this case did not compare well with the respective values for exchanges. In both cases, the Y chromosome and chromosome 3 and 19/20 were the least involved. It is a matter of interest that the breaks were not localized only in the heterochromatic part of the chromosomes but the aberrations were found in and around the areas of centromeres (proximal heterochromatin) also (Vig, 1971).

As previously discussed, MMC induces quadriradial configurations involving breakage and rejoining between homologous chromosomes at apparently corresponding regions as observed in human lymphocytes and in Vicia faba. From the later studies, the idea was

developed that homologous chromosomes, at least in some species, are paired in interphase of somatic cells. ADM does induce exchanges between homologous chromosomes, but rarely, if ever, induces quadriradials at corresponding points. Thus, in one study (Vig, 1971) with ADM, 37.5% of total exchanges involved homologous or apparent homologues, but only 8 out of a total of 120 such exchanges met the criteria of region-specific reciprocal recombination. The chromatid exchanges induced by this chemical were most frequently of the asymmetric type. For instance, in one experiment in which cells were treated with 0.02 μg/ml of ADM (Vig, 1971) for four hours, 63% of all exchanges were of U-type leaving only 37% as X-type exchanges.

Effect of Temperature

Hahn, Braun and Har-Kedar (1975) and Hahn and Strand (1976) have demonstrated a striking synergism between ADM and hyperthermia in relation to cytotocicity to HA1 Chinese hamster cells and EMT-6 mammary sarcoma of mice. In one experiment HA1 cells treated with 0.02 μg of ADM per ml at 43 ^{o}C showed survival of only 4 X 10^{-2} in contrast with 90% for heat control and 60% for ADM control at 37 ^{o}C. Although the mechanism of such synergistic effects is not yet clear, fluorescence measurements showed an increased concentration of the ADM in cells treated at 43 ^{o}C, presumably because of increased permeability of the cells' plasma membranes. However, this situation of increased permeability can be reversed if cells are exposed to a high temperature for much longer than 30 minutes.

Although synergism between chemicals and hyperthermia has been

previously demonstrated (Johnson & Pavelec, 1973), the experiments mentioned above raise questions of practical importance for chemotherapy as well as for basic cell biology, specifically, because ADM is one of the most promising antitumor drugs available. Vig (1978) studied the effects of hypothermia and hyperthermia on the induction of chromosome aberrations in human leukocytes by ADM. All cultures were started at 37 ^{o}C and ADM solution was added at room temperature for a desired length of time. For comparing the effect of ADM at 37 ^{o}C versus 4 ^{o}C, the cells were treated during 22 to 23.5 hours after culture initiation at 0.04 μg/ml of ADM. The frequency of aberrations after 48 hour recovery was only 0.02 per cell in the 4 ^{o}C treatment compared with 0.07 per cell in the 37 ^{o}C treated material. In another similar experiment in which the recovery period was reduced to 24 hours, no scoreable metaphases were available, indicating the delay induced by the ADM. Thus, in spite of a very low frequency of aberrations per cell, there is little doubt that ADM treatment at 37 ^{o}C produces more aberrations than at 4 ^{o}C. The frequency of aberrations was also increased by raising the concentrations of ADM. The drastic reduction in the relative frequency of aberrations requiring rejoining of broken ends of chromosomes was noticed. Thus, whereas chromatid exchanges had shown a highly reduced frequency in the 4 ^{o}C population, aberrations like rings and triradials were totally absent.

The data on chromosome aberrations studied after recovery of 44 or 62 hours, exhibited a far greater potential of ADM in causing aberrations in synergism with hyperthermia (43 ^{o}C) than at 37 ^{o}C

(Vig, 1978). The number of aberrant cells at 43 °C was three times the number observed at 37 °C, but the frequency of aberrations was about ninefold greater at 43 °C than at 37 °C. The largest relative increase was found in the frequency of those aberrations that required rejoining, especially chromatid exchanges. In a series of experiments (Vig, 1978), cells were treated at 24 to 25 hour postculture initiation period and were treated with 0.2 μg/ml of ADM at 4 °C, 23 °C, 37 °C and 43 °C. A recovery of 45 hour was allowed. The data confirm the earlier findings with a slightly more than twofold increase in the frequency of aberrations at 43 °C as compared with that at 37 °C. Also, a large increase (about fourfold) was observed in the cumulative frequency of all types of exchanges at 43 °C. At temperatures lower than 37 °C fewer aberrations (about 30%) were observed.

It is possible that the effect of hyperthermia is an inhibition of restitution of ADM-induced damage to the chromatin material. Distinction between immediate effectiveness of ADM and inhibition of repair at the posttreatment period can be made if cells are treated at 37 °C and then postincubated for a few hours at various temperatures. In case "delayed" effectiveness due to the repair process is involved, the end results should be similar to those in data for 4 °C or 43 °C posttreatment in Vig's (1978) work. A series of experiments was conducted by Vig (1978) and the conclusion was that the major effect of ADM is during the period when the chemical is in contact with the cells, or, alternately, that any changes in enzymatic activity, brought about by temperature fluctuations in the post-ADM treatment period, do not have any serious effect on the frequency of

aberrations induced by ADM.

Vig, Cornforth and Farook (1982) studied the hyperthermic potentiation of chromosome aberrations by three anticancer antibiotics. One with a nondelayed type of effect (adriamycin), one with a delayed type of effect (mitomycin C), and one with a truly radio-mimetic effect (bleomycin) were selected for study on human lymphocytes and Chinese hamster K-1 cells. The data showed increased potential of these chemicals to induce chromosome aberrations when applied at temperatures higher than 37 ^{o}C, irrespective of the phase of cell cycle. The potentiation may be synergism (bleomycin) or the facilitation of entry of larger quantities of the drug (ADM).

Hyperthermia increases the effectiveness of ADM in inducing cell death and chromosome damage. The mechanism is not synergism, if it is the increased influx of the ADM molecules into the cell (nucleus) as indicated by studies of Hahn et al. (1975) and Hahn and Strand (1976). It might well be called facilitation resulting in apparent synergism or pseudosynergism. If hyperthermia does result in an increased quantity of ADM in the cell, then one simply expects a higher degree of damage to cellular components. True synergism shall be demonstrable in case the quantity of ADM in the cell is kept constant, as might be possible at doses that saturate the cell with ADM. Alternately, one may compare the effect of hyperthermia and ADM with ADM alone if, in both instances, the quantities inside the cell can be shown to be equivalent. Such experiments, although possible, do not appear in the literature.

Data from Newsome and Littlefield (1975) with skin fibroblasts

differ from those of Vig (1971) using leukocytes. In the former case few exchanges were induced in ADM-treated materials. The high incidence of exchanges induced in the Vig (1978) experiments at 43 ^{o}C, but fewer exchanges at 4 ^{o}C, indicate that exchange frequency depends on the total frequency of aberrations and may well be related positively with the increased uptake of ADM by the cell. It can also be true for reduced quantities of ADM in the cell at 4 ^{o}C treatment. Among others, the length of the recovery period and time of treatment also promote higher frequency of exchanges.

Mechanism of Action

Because of the clinical importance of ADM in the treatment of many common tumors, extensive studies have been performed to determine the possible antitumor mechanisms of ADM and other related antitumor anthracyclines. The major biochemical effects of anthracyclines (ADM & DNM) are claimed to be related to nucleic acid synthesis through interference with template DNA function. Distortion of the DNA structure and uncoiling of supercoiled, double helix of DNA have been taken as evidence of intercalation (Waring, 1970). However, the data on DNA-anthracycline binding (DiMarco, Areamone & Zunino, 1975) suggest at least two types of DNA binding sites for the anthracyclines. One of these is the site of intercalation for DNA. The other represents electrostatic interactions involving DNA phosphate groups and anthracycline amino acid (Pigram, Fuller & Hamilton, 1972). Kersten (1968) has provided evidence that DNM-DNA complex formation depends only slightly, if at all, on the G:C content of DNA. It is, however,

significant that an alternate dG-dC sequence binds to ADM tenfold better than alternate dA-dT (Tsou & Yip, 1976).

ADM and DNM cause inhibition of DNA, as well as RNA, synthesis. This inhibition of DNA shows a peculiarly severe effect in the late S phase (Silvestrini, DiMarco & Dasdia, 1970; Theologides, Yarbro & Kennedy, 1968). Also, of the two types of RNA studied, in one case nucleolar RNA synthesis was found to be much more sensitive than extranuclear RNA and some reports (Evans, Lindstead, Rhodes & Wilkies, 1973) provide evidence of high sensitivity of a particular fraction of mitochondrial RNA. Detailed studies have shown that the inhibition of the two types of nucleic acids results from the inhibition of DNA polymerase and DNA-dependent RNA polymerase (DiMarco et al., 1975). The inhibition of nucleic acid synthesis by anthracyclines in tissue culture experiments may be similar to inhibition of DNA synthesis reported for proliferating cells of different organs in mice given ADM intravenously (Zedeck, Formelli, Sternberg & Philips, 1976). Both DNM and ADM have, in addition, been reported to inhibit DNA polymerase in mutant and wild type T4 bacteriophage (Goodman, Bessman & Bachur, 1974). But, when compared with DNA polymerase of wild type phage, antimutator enzymes were inhibited to a far greater extent than the mutator enzymes. The stronger inhibition of viral DNA polymerase than of cellular DNA polymerase has been suggested to result from interaction of anthracyclines with the primer template-DNA (Zunino, Gambetta, DiMarco, Zaccara & Luoni, 1975).

The studies dealing with shifts in relative frequencies of aberrations of different types suggest that G_1 chromosomes may be the

target of ADM action. This does not imply that post G_1 chromosomes are not affected by the drug. In fact, S phase appears to be more vulnerable to the action of ADM (Hittleman & Rao, 1975). Chromosome aberrations have also been reported by Sinkus (1972) to appear after treatment of G_2 population of cells. This has been additionally demonstrated by Hittleman and Rao (1975) who used the premature chromosome condensation (PCC) technique. It is interesting to note that contrary to the results obtained with x-rays and phleomycin, mitotic G_2 cells exhibited higher frequencies of exchanges than those observed in PCC cells treated with ADM. The S cells are much more sensitive than G_2 cells to ADM induced breaks but the ADM induced prolongation of G_2 phase is about three times greater than that of S phase (Hittleman & Rao, 1975), an observation contradicted by some other studies (Krishan & Frei, 1976).

Observations of experimental tumor systems have shown that ADM promptly inhibits DNA and RNA synthesis and to a lesser extent protein synthesis (Kim & Kim, 1972; Zunino et al., 1975). The interaction of ADM with the DNA of the chromosomes induces initial lesions in the DNA that precipitate visible structural changes in the chromosomes at metaphase. First, the initial lesions induced along the DNA of the chromosomes by ADM may fail to repair. Thus, the affected chromosomes may show discontinuous strands that are released as fragments at metaphase. Second, terminal or end point breaks may become involved in exchanges between chromosomes. A paucity of exchanges were observed as compared with breaks. Similar phenomena were observed in ADM treated human fibroblasts. Since exchanges were

rare, it is reasonable to infer that S cells are blocked in their progression through the cell cycle. Newsome and Singh (1977) concluded that since chromatid breaks predominated in cells harvested 5 and 24 hours posttreatment, it is plausible to suggest that ADM induced chromosomal aberrations and DNA inhibition are not causally related. Based solely on cytologic evidence, G_1 and S cells appear to be more sensitive to ADM.

ADM binds tightly to DNA and so interferes with many DNA-related functions such as DNA replication and RNA synthesis (Crooke & Reich, 1980; Myers, 1982). It has been shown that, when linked to agarose beads, ADM can exert its cytotoxic effect without entering cells (Tritton & Lee, 1982). ADM can also be reduced to a semiquinone radical that damages macromolecules such as DNA and cell membranes. Whether this damage is related to toxic side effects, such as cardiac toxicity or to the antitumor effect, has not been established. Many intercalative antitumor drugs, including ADM, induce protein-linked DNA breaks in cultured mammalian cells by a mechanism that is probably independent of radical formation. The nonintercalative antitumor drugs VP-16 and VM-26 also induce protein-linked DNA breaks (Wozniak & Ross, 1983).

To test whether protein-linked DNA breaks induced by ADM and other antitumor drugs also involve mammalian DNA topoisomerase II, Tewey, Rowe, Yang, Halligan and Liu (1984) conducted in vitro studies using purified calf thymus DNA topoisomerase II. Two interesting features of topoisomerase II-mediated cleavage were noted.

1. At higher concentrations of intercalators, DNA cleavage

was actually inhibited. ADM (0.01 μg/ml) was one of the most potent drugs in stimulating topoisomerase II-mediated DNA cleavage at low concentrations. At doses of more than 0.25 μg/ml of ADM, topoisomerase II-mediated DNA cleavage was not observed.

2. Although antitumor anthracyclines (ADM, DNM, & IDR) stimulated cleavage at similar sites, antitumor drugs of different chemical classes showed strikingly different cleavage patterns. Several pieces of evidence indicated that DNA double-strand breaks, induced in vitro by topoisomerase II and anthracycline antitumor drugs, are protein-linked. For example, in the absence of DNA topoisomerase II, anthracyclines alone did not produce any DNA double-strand breaks. On the basis of the known properties of mammalian DNA topoisomerase II, Tewey et al. (1984) favor the hypothesis that, like m-AMSA, ellipticines, and epipodophyllotoxins, these anthracycline antitumor drugs affect the breakage-reunion of DNA topoisomerase II by stabilizing the cleavage complex formed between topoisomerase II and DNA (Nelson, Tewey & Liu, 1984).

To investigate the effect of ADM on the catalytic activity of mammalian DNA topoisomerase II, Tewey et al. (1984) monitored the strand-passing activity of mammalian DNA topoisomerase II. Like ellipticine, ADM strongly inhibited the unknotting activity of calf thymus DNA topoisomerase II in the P4 unknotting assay. Thus, it seems possible that the inhibition of activity by intercalative antitumor drugs may result from both drug stabilization of the cleavage complex and drug intercalation into DNA. Intercalation may block the binding of topoisomerase II to DNA and thus inhibit

the strand-passing activity. This interpretation is also consistent with the observations that, at higher concentrations of intercalative antitumor drugs, DNA cleavage was actually inhibited (Tewey et al., 1984). Tewey et al. (1984) results are consistent with the earlier reports that the antitumor activity is closely related to drug stabilization of the cleavable complex rather than to drug inhibition of the enzymatic activity of mammalian DNA topoisomerase II (Nelson et al., 1984). Nalidixic acid selectively kills growing bacterial cells presumably by a mechanism related to its specific stabilization of the gyrase-DNA complex (Gellert, 1981). Many potent antitumor drugs affect the breakage-reunion reaction of mammalian DNA topoisomerase II by stabilizing the cleavable complex. Whether this unusual DNA damage is related to drug-induced cytotoxicity, sister chromatid exchange, or chromosomal aberration is still not clear. Tewey et al. (1984) studies suggest a possible role of topoisomerase II in the action of these antitumor drugs.

Mitomycin C (MMC)

MMC is a chemically reactive antibiotic derived from streptomyces caespitosus (Fig. 2). The drug selectively inhibits DNA synthesis (Ben-Porat, Reissig & Kaplan, 1961; Iijima & Hagiwara, 1960; Shiba et al., 1959) and degrades cellular DNA but does not affect the synthesis of RNA or protein (Reich & Franklin, 1961). MMC induces bacteriophage production in lysogenic bacteria (Levine, 1961), increases the rate of genetic recombination among mutant forms of E. Coli and possesses antitumor activity (Iijima & Hagiwara,

1960). In tissue culture systems, MMC inhibits mitosis, reduces cell viability and produces nuclear disorganization and giant cells (Kuroda & Furuyoma, 1963).

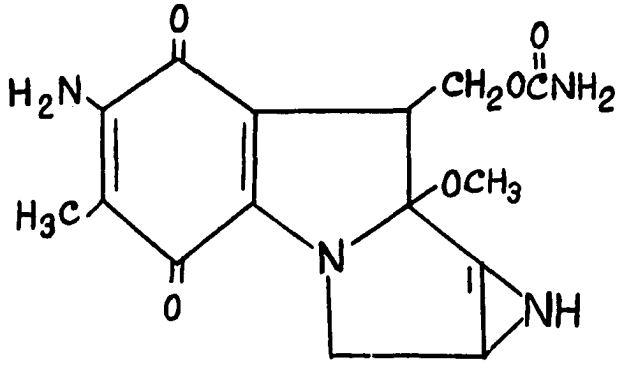

Figure 2. Structure of Mitomycin C

The effects of MMC on the cell-cycle traverse have been varied, and contradictory results have appeared from various laboratories. In general, the cells can be affected both in the S-phase and out of S-phase. There are reports of the Hela cells showing high sensitivity to MMC in the latter half of G_1 and the first half of S-phase (Doi, Taki, Aoki, Higashi & Kosaka, 1967) as well as the findings that MMC is generally more toxic to non-S cells than to the cells in the S-phase (Djordjevic & Kim, 1968). On the colony forming units of mouse bone marrow, the antibiotic appears to have more pronounced lethal effects on the S-phase than in the G_1 phase, and the latter

is not significantly less sensitive than is the GO phase. As an additional example of inconsistency, Stein and Rothstein (1968) have reported that the drug inhibits mitosis by reducing RNA synthesis in the G_2 phase. Thus, it appears difficult to present a generalization of specificity of action of MMC on the cell cycle traverse.

Effects on Chromosomes

Chromosome studies using MMC were first carried out by Merz (1961) on the root-tip chromosomes of Vicia faba. In treatments lasting only for one hour with 0.001% solution of antibiotic, he observed chromosome aberrations including gaps, chromatid exchanges and deletions. The effect of MMC is delayed; rejoining occurs, and aberration frequency is not affected by anoxia or changes in pH and temperature (Cohen & Hirschhorn, 1971; Kihlman, 1960; Merz, 1961). Treatment of human leukocytes in GO, G_1- or S-phase has also been known to produce chromatid aberrations (Nowell, 1964). The cells treated during GO or G_2 are not inhibited in their development to the first mitosis, though inhibition is observed for cells in G_1 or S at the time of treatment. Chromosome breaking effects of MMC have been attributed to its property to link complementary DNA strands together by the formation of covalent bonds (Iyer & Szybalski, 1963) and results exclusively in the production of chromatid-type aberrations. The embryonic fibroblasts appear to be much more sensitive than leukocytes from peripheral blood (Simard, 1967).

The chromosome aberrations induced by MMC are localized mainly

in secondary constriction in cultured human leukocytes (Cohen & Shaw, 1964; German & LaRock, 1969; Morad, Jonasson & Lindstan, 1973; Nowell, 1964; Sinkus, 1969). The breaks are maximal in their frequency in secondary constrictions of chromosomes 1, 9 and 16 in man (Bourjeois, 1974; Cohen & Shaw, 1964; Morad et al., 1973; Shiraishi & Sandberg, 1979). MMC is known to function as an alkylating agent, even though the response of MMC treated human cells is different from those with ethyl methanesulfonate (Brogger, 1974).

Shiraishi and Sandberg (1979) reported that normal human lymphocytes, exposed to MMC (0.3 μg/ml) during 48 hours of culture, exhibited an average of 58.6 SCE per cell, i.e., about ten times as high as the control frequency (4.1 SCE/cell). A large number of chromosome aberrations was observed, including exchanges, breaks and dicentrics. In another study done by the same group (Shiraishi, Minowada & Sandberg, 1979) to compare the effects of MMC on normal and abnormal human lymphocytes, the chromosome aberrations observed in normal cells were of exchange- and break-type and included chromatid as well as isochromatid-breaks, with the heterochromatic regions of chromosomes being particularly affected. The frequency of these exchange configurations increased with MMC concentration. The number of induced exchanges and breaks observed in normal cells were higher after 24 hour treatment with MMC at 0.5 μg/ml than at 0.1 μg/ml. Chromosome damage in the normal cells was less marked at and below 0.05 μg/ml. As evidenced by the remarkably high reduction of viable cells, the neoplastic cell lines appeared to be much more sensitive to MMC than were the normal cells. Therefore, there is a

possibility that cells with chromosome damage (e.g., exchanges) cannot survive at concentrations of MMC higher than 0.05 μg/ml or cannot enter into metaphase. This agrees with previous findings that chromosome aberrations are associated with a high rate of cell death (Shiraishi, Minowada & Sandberg, 1976; Wolfe, Rodin & Cleaver, 1977).

Chromosome aberrations were scored simultaneously with SCE after 72 and 96 hours of cultivation (Novotna, Goetz & Surkova, 1979). The normal human lymphocytes showed a statistically significant increase of aberrant cells only after 0.160 μg/ml of MMC that was added after 24 hours of cultivation. The statistically significant differences showed a dependence on the time of cultivation. High doses (0.16 - 1.0 μg/ml) of mutagen resulted in significantly increased numbers of aberrant cells after 96 hours cultivation compared with those found after 72 hours. A high percentage of severely damaged mitoses, i.e., multiple breakage and rearrangements, was observed. Chromosome damage occurred almost exclusively in the first mitoses.

Miura, Morimoto and Koizumi (1983) reported that in cultures exposed to increasing concentrations of MMC, normal lymphocyte cells showed a clearly dose-dependent delay in cell turnover times. For example, an exposure of cells to 0.03 μg/ml MMC gave a distribution of 10% X1 (first division), 20% X2, and 70% X3 cells. Chromosomal aberrations were examined separately in X1, X2, and X3 metaphase cells. It was noted that chromosomal aberration frequencies decreased with successive divisions by about 60% in normal cell cultures. This reduction has been shown by others (Carrano & Heddle, 1973; Conger, 1965; Sasaki & Norman, 1967) to be 50% through subsequent mitoses.

Another effect of MMC and other related agents is attenuations (Brogger, 1974a, b; Cohen & Shaw, 1964; Hoehn & Martin, 1972), or even induction (Brown, Palmer & Yu, 1972), of secondary constrictions. In man, the paracentromeric area in chromosome 9 is the most sensitive area and the effect might be due to the presence of a particular type of satellite DNA present in this region (Arrighi & Hsu, 1971). Even though marked aberrations in the morphology of constrictions have been observed in treated materials, a total lack of these in some chromosomes appear to be incompatible with the life of the cell (Hoehn & Martin, 1972). Perhaps, the suggestion that longer viability of MMC induced micronuclei in Vicia than that induced by radiation is due to higher amounts of heterochromatin (Arora, Shah & Rao, 1969). This may be interpreted in terms of this function of heterochromatin.

One striking effect of MMC is the induction of chromatid exchanges involving homologues at corresponding points. In one study as many as 50% of all exchanges produced were of this type (Shaw & Cohen, 1965). Such cross-configurations have been observed in treated human leukocytes (German & LaRock, 1969; Nowell, 1964; Shaw & Cohen, 1965; Shiraishi et al., 1979; Shiraishi & Sandberg, 1979) and are reminicent of chromosome configurations considered responsible for producing meiotic recombination. German and LaRock (1969) observed such exchanges in human blood cells and suggested these as evidence of possible somatic crossing over in mammalian cells. Generally, quadriradial configurations in the treated cells utilize the parts of chromosome containing repetitive DNA possibly associated with

secondary constrictions and hence late replicating regions of chromosomes (German, 1964). Models for the induction of chromosome aberrations by MMC have been proposed that explain the origin of quadriradials in the areas of repetitive DNA (Brogger & Johansen, 1972; Comings, 1975) but production of free fragments in other regions also (Brogger & Johansen, 1972). These views challenge the traditional concepts, like those formulated by the study of homo/hetero ratio of MMC induced exchanges in Vicia (Reiger, Michaelis, Schubert & Meister, 1973). The somatic chromosomes in interphase are paired together, and the fusion tendency of heterochromatin (Natarajan, Ahnstrom & Sharma, 1974) perhaps is the only factor contributing to the production of complementary exchanges.

Position of Chromosomal Breaks

MMC, a bifunctional alkylating agent, is known to effectively induce nonrandom chromosome aberrations and SCE in normal human lymphocytes (Brogger & Johansen, 1972; Cohen & Shaw, 1964; Morad et al., 1973; Nowell, 1964). The majority of the former are involved preferentially in the constitutive heterochromatin of homologous chromosomes, especially in the secondary constriction regions of chromosomes number 1, 9, 16. This may be a result of the association of these chromosome regions during interphase, either through somatic pairing (Rao & Natarajan, 1967) or a unique "gathering" effect of MMC (Kobayashi, 1960). Aberrations are not always found in all heterochromatic areas. For example in man, all centromeric regions with constitutive heterochromatin are not particularly affected by MMC in

chromosomes other than 1, 9 and 16 (Brogger & Johansen, 1972). And, heterochromatic segments from chromosomes numbers 1 and 9 have been found to be translocated by MMC in the parts of the genome devoid of any heterochromatin (Hoehn & Martin, 1973). Also there is no evidence of involvement of telomeric regions of chromosomes that may contain repetitive DNA (Bourjeois, 1974).

The availability of both facultative and constitutive heterochromatin in the same cell has been exploited by Natarajan and Schmid (1971) in differentiating the effect of MMC on two types of heterochromatin. Chinese hamster cells offer this possibility with the added advantage that the short arm of the inactivated (lyonized) X chromosome in the female cells is facultatively heterochromatic whereas the long arm is constitutively heterochromatic. The experiments convincingly demonstrated that the effect of MMC is related to the structural rather than functional properties of heterochromatin. Interestingly, however, the long arm of inactivated X was affected much more frequently than the long arm of the active X.

Distribution of chromosome aberration sites has also been found to be related to the banding patterns. Thus, Morad et al. (1973) found that interchanges are overrepresented in the C-bands of homologues in quinacrine mustard banded preparations. As expected, the secondary constriction areas of chromosome 1, 9 and 16 were most often involved. However, the breaks were preferentially localized in the regions of R-bands. Studies carried out by Rieger, Michaelis, Schubert, Doebel and Janak (1975) using Vicia faba have indicated that clustering of aberrations occur within or adjacent to G-bands

and that MMC induced exchanges and chromatid deletions do not share the "hot spots" to a similar extent. Sinkus (1969) and Funes-Cravioto, Yakovienko, Kuleshov and Zhurkov (1974) have analyzed, segment wise, the distribution of chromatid aberrations in man. Generally, segment 3 on chromosome 1 and 3 on C (apparently No. 9) are among the best responding, and segments 2 on chromosome 1, 3 on chromosome 3, 4 on B, 4 on C, and 1 on G, are some of the least. However, no segment of any chromosome or group was totally unaffected. The distribution of MMC induced aberrations in cells differ from what is expected from poison distribution and confines more closely to geometric pattern (Bochkov, 1972). In human cells, this pattern has been found to be independent of the stage of cell cycle and the time of fixation (Bochkov, 1972).

Among 200 human lymphocyte cells observed (Shiraishi et al., 1979) at 0.5 μg/ml MMC, 101 cells (about 50%) had exchange configurations. Sixty percent of the figures occurred between homologs or apparent homologs and the majority of exchanges at the secondary constrictions of chromosomes number 1, 9 and 16 or in the so-called C-bands. Fifty percent of the exchange figures included chromosomes possessing secondary constrictions (No. 1, 19%; No. 9, 25%; No. 16, 6%). A large number of homologous chromatid exchanges were also observed in the centromeric heterochromatin segments of chromosomes number 1, 9 and 16. This agrees with the findings of other reports (Brogger & Johansen, 1972; Cohen & Shaw, 1964; Morad et al., 1973; Nowell, 1964).

Vogel and Schroeder (1974) have suggested that such nonrandomness

may reflect a spatial or temporal organization of chromosomes during interphase. Schaap, Sagi and Cohen (1980) investigated MMC-induced rearrangements to study the spatial vs. temporal hypothesis. The lack of a positive relationship between the frequency of aberrations and the measured length of chromosomal segments at metaphase has been repeatedly demonstrated (Aurias, Prieur, Dutrillaux & Lejune, 1978; Mattei, Ayme, Mattei, Aurran & Giraud, 1979; Mitelman & Levan, 1978; Nakagome & Chiyo, 1976; Sagi, Cohen & Schaap, 1978; Vogel & Schroeder, 1974; Yu, Borgaonkar & Boiling, 1978). Therefore, the conventional method of calculating expected frequencies of chromosomal rearrangements from measured lengths at metaphase was not employed in Schaap et al. (1980) study. Instead, the expected frequency of rearrangements between two given chromosomal regions was based on the total number of rearrangements involving each of the given regions.

In approximately 1400 metaphases analyzed (Schaap et al., 1980), 613 open breaks and 353 rearrangement configurations were observed. The latter were scored twice, once for each partner, thus yielding a total of 706 rearrangements. Even though the aberration could be unequivocally assigned to specific bands, their total number in most chromosomal regions was too small to make such assignment meaningful. Therefore, assignments to specific bands was practiced only for aberrations occurring at highly breakable regions (e.g., the centromeric regions, including the secondary constriction of chromosomes 1, 9 and 16 and the centromeric region of 5). The remaining aberrations were assigned to chromosome arms (1p, 1q, 16p, and

16q) or to entire chromosomes.

The centromeric regions, including the secondary constrictions of chromosomes 9 (9C), 1 (1C), and 16 (16C), with 193, 139 and 47 rearrangements respectively, were most often involved in rearrangements. Chromosomes 19 and 17 followed, with 28 and 27 rearrangements, respectively. These five highly "rearrangeable" regions were involved in 67% (474/706) of the scored rearrangements. A pronounced "preference" for rearrangements between homologous regions was apparent for all but the rarely rearranging (rr) regions. The latter "preferred" members of their own group, barring homologs, or acrocentric chromosomes, and strongly "avoid" rearrangements with the 1C and 9C regions. The acrocentric chromosomes "preferred" members of their own group, including homologs, and "avoided" region 9C. The combinations 1C X 17, 9C X 17, and 9C X 19 were rare than expected (Schaap et al., 1980). Weaker trends of "avoidance" and "preference" were apparent from comparisons of the observed and expected numbers of rearrangements. Thus, both 1C and 9C seem to "avoid" chromosomes 2 and 6, whereas rearrangements between homologs seem to be "favored" by 5C, 6, and 20. However, these numbers of rearrangements are too small to draw any firm conclusions.

Schaap et al. (1980) calculated the correlation coefficient for all chromosomes and regions except 9C and 1C and found not to differ significantly from zero ($r = 0.28$; 27 df). Hence, the probability of an open break seems to be independent of that for a rearrangement in the same region. This low correlation may be due to heterogeneity in the analyzed group. They concluded that "the obvious lack of

correlation between open breaks and rearrangements indicates that the three possible outcomes of a chromosomal break (open break, restitution, and rearrangement) have different probabilities in different chromosomal regions (p. 247)." Some observations of this study contradict several previous results. For example, Bourgeois (1974) suggests that exchanges between the acrocentric chromosomes and 1, 9 and 16 are more frequent than expected. Schaap et al. (1980) data demonstrated that rearrangements between the acrocentrics and 9C are certainly less frequent than expected, rearrangements between the acrocentrics and 16C probably occur less often than expected, and rearrangements between the acrocentrics and 1C surely do not occur more often than expected. These contradictions may stem, however, from Schaap et al. (1980) method of calculating expected exchange frequencies. Schaap et al. (1980) beleive that the concept of a predesignated spatial organization of interphase chromosomes as underlying the pattern of "choosing" partners for rearrangements can hardly account for some of their results. An exceptionally complex topological model is necessary to explain the observation that rarely rearranging (rr) chromosomes do not "prefer" their homologs as partners, in view of the pronounced homolog "preference" displayed by all other analyzable chromosomal regions. Therefore, a temporal model provides a more plausible explanation for the "preference" and "avoidance" of specific partners, as well as the different rates of involvement in rearrangements. Any structural differentiation determining the specific pattern of availability and choice of partners for rearrangements may also underlie the pattern of open break:

rearrangement ratios.

Mechanism of Action

The selective action of the antibiotic MMC on DNA, together with its reported antineoplastic, mutagenic and phage-inducing activities (Otsuji, 1962; Reich & Tatum, 1960; Shiba et al., 1959), have stimulated several investigations on the mechanism of its action. The preferential inhibition of the bacterial DNA synthesis by MMC, accompanied by progressive and extensive breakdown of the DNA, indicated that DNA is the principal target. However, the rapidity of MMC-induced "death" seemed to be "out of step" with the relatively much slower process of DNA breakdown. This suggested that the effects observed might be secondary to an earlier action of the antibiotic on DNA.

Several models for the mechanism of MMC activity have been suggested. Reich and Tatum (1960) proposed that MMC acts through the splitting of the DNA strands, thereby preventing replication. This implies that the template competence of the DNA is destroyed (Reich & Franklin, 1961). The depolymerization of DNA and the accumulation of acid-soluble fragments implicates the DNA polymerase system as a possible target of MMC activity (Reich, 1961). Iyer and Szybalski (1963) suggested that the primary action of MMC is the "cross-linking" of the complementary strands of the DNA molecule and that the degradation of DNA may be of a secondary nature.

After uptake and enzymatic reduction of MMC (Iyer & Szybalski, 1964) the substance reacts with DNA by alkylation, either

monofunctionally so that one mitomycin residue attaches to a single base, or bifunctionally, cross-linking the two strands in the DNA (Iyer & Szybalski, 1963; Weissbach & Lisio, 1965). It is not known whether the whole MMC molecule is involved in the final reaction product. If the MMC-DNA complex is left unrepaired, it may inhibit DNA and RNA functions so that the cell never enters mitosis. If the chromosomes are replicated and the cell divides, the damage may be invisible at the level of the metaphase chromosome, or it may interfere with the packing of DNA into a metaphase chromosome resulting in an attenuation, constriction or gap. Brogger (1971) considered such aberrations to be packing or folding changes.

The existence of cross-links, a phenomenon of genetic significance, was demonstrated by heating the DNA to 100 ^{o}C in the presence of MMC and then rapidly cooling to 0 ^{o}C. The MMC treated cells "retained" their double stranded form in contrast to the single strandedness of the untreated cells. The cross-linking can, however, be achieved only after apparent reduction of the molecule of MMC to its hydroquinone derivative through the mediation of an NADPH-dependent quinone-reductase (Samuels, 1964). This reduction has also been found to be necessary for DNA cross-linking in vitro, since nothing happens when MMC is added to purified DNA and allowed to react for as long as one to two hours (Waring, 1968). The cross-linking property is a peculiarity of the MMC molecule, and derivatives decarbamoyl MMC and 7-methoxymitosene do not evidence this phenomenon in spite of their action in producing single strand DNA breaks (Otsuji & Murayama, 1972). The cross-linking efficiency of MMC depends on

the presence or absence of proteins associated with DNA. In human tissue, about a third of the DNA shows such cross-linking with the addition of large amounts of the antibiotic (Szybalski, 1964). This is far less than in bacterial DNA that has no associated proteins and can have as high as 100% of its molecule altered (Waring, 1968) in spite of the fact that only 10-20% of all MMC is associated with DNA to form cross-links (Szybalski & Iyer, 1964).

The binding of MMC to DNA affects the physical properties of the latter, viz., UV hypochromicity, specific transforming activity and buoyant density. However, reassociation of complementary nucleotide chains under appropriate conditions suggests the existence of some sort of covalent bonding. It has been shown that the antibiotic molecule becomes bound to two neighboring sites on opposite DNA strands. This interpretation has helped to explain most of the data of biological significance obtained with MMC; even though the question of MMC binding at the G-C moiety (Goldberg, Rabinovitz & Reich, 1962) is still a matter of debate.

The formation of cross-links appears to be positively related to cell killing. It has been advocated that the difficulty experienced in the separation of sister strands of DNA during replication is the cause of lethal action of MMC. This would mean that a single cross-link in some cells may lead to the death of the cell (Szybalski & Iyer, 1964) especially in akaryotes. In early studies (Kersten, Kersten, Leopold & Schneider, 1964) it was suggested that MMC, by acting on RNA, may liberate an RNA-bound DNAase, supporting the observation that DNA is rapidly degraded in bacteria and tumor cells

treated with the antibiotic (Reich, 1963). Thus, DNAase in MMC-induced degradation of DNA was suggested to be released by the action of MMC on ribosomes (Kersten et al., 1964; Weissbach & Lisio, 1965). The concept that cell death after MMC treatment may not necessarily result from cross-linking of DNA, has also been supported by the finding that most of MMC residues attack single bases of DNA through monofunctional alkylation (Weissbach & Lisio, 1965).

Several studies have suggested that the effects of MMC are generally, although not always, similar to those produced by ultraviolet radiation. As a matter of fact, cell resistance to MMC are cross resistant to UV (Otsuji & Murayama, 1972). Also, cells from patients with Fanconi's anemia show enhanced sensitivity to MMC (Miura et al., 1983; Novotna et al., 1979) and UV but not 8-rays or ethyl methanesulfonate (Finkelberg, Thompson & Siminovitch, 1974). This suggests that the enzymatic background for inducing cross-links in these cells may be similarly affected by UV and MMC.

According to Kihlman (1977), chromosome breaking agents can be classified operationally into "S-dependent" agents, like UV and alkylating agents and "S-independent" agents like x-rays and bleomycin. The S-independent agents produce chromosomal aberrations independently of chromosome and semiconservative DNA replication, whereas the S-dependent agents produce lesions that must be replicated in order to give rise to chromosomal aberrations. Evans and Scott (1969) suggested that chromosomal aberrations involved a misrepair process if induced by S-independent agents and a misreplication process if induced by S-dependent agents. Recently another

hypothesis has been suggested by Kihlman, Hanson, Palitti, Andersson and Hartly-Asp (1982): namely, the damage produced by S-dependent agents has to be replicated before mistakes in connection with a repair process occur and this process takes place in G_2 giving rise to chromatid-type of aberrations. Palitti et al. (1983) recent results have confirmed the existence of unrepaired lesions in DNA of mutagen-treated cells that persist until the cells enter mitosis and that the repair of these lesions can be inhibited effectively at this stage. Palitti, Tanzarella, Degrassi, DeSalvia and Fiore (1984) believe that at least three processes are relevant in the formation of chromatid type of aberrations, operating in G_2. These include (a) DNA repair, (b) replicative DNA synthesis, and (c) chromatin condensation.

Palitti et al. (1984) data showed that both Chinese hamster cells and human lymphocytes, treated only with inhibitors together with colchicine, did not respond with increased frequencies of chromatid aberrations or SCE over the controls. With MMC-treated hamster cells, after a G_2 posttreatment with the three inhibitors (HU, 3AB, Caffeine) there was an increased frequency of both chromatid aberrations and SCE. The MMC-treated human lymphocyte cultures showed an increased frequency of both chromatid type of aberrations and SCE after a G_2 posttreatment with caffeine or APC, whereas only a slight increase follows the posttreatment with HU. These data confirmed Palitti et al. (1983) results and demonstrated that if, after a G_2 posttreatment with replication or repair inhibitors, there is an increase in the frequency of chromosomal aberrations, there is also

a potentiation of SCE frequency. MMC-induced SCE and chromatid aberrations are enhanced.

As can be seen in EM section of the literature review, there are very few techniques in the area of whole mount electron microscopy of chromosome. These techniques are difficult to reproduce and they are not rapid. The greatest disadvantage of them is that they are not suitable for study of the whole chromosome complement in a cell. Therefore, these techniques, as human cytogenetics is concerned, can not be used in study of chromosomal aberrations in human or other mammalian cells in culture. So, there is a need for development of a technique that allows the study of chromosomal aberrations at both LM and EM level. As noticed in the induction of chromosome aberrations section of the literature review, no evidence was found of any study in this area at transmission EM level. Almost all the works are at LM level of resolution. Again, there is a need to do such study at EM and show if the developed technique can be used in this area. It is also important to compare the chromosomal aberrations induced by the drugs at LM and EM levels to determine any advantage of EM.

CHAPTER III

MATERIALS AND METHODS

Blood Collection

Blood was collected by venipuncture into five ml evacuated blood collection tubes (B-D vacutainer obtained from Becton Dickinson, Rutherford, NJ) from three normal and healthy individuals. Each tube was coated with 100 USP units of sodium heparin by the manufacturer. The blood was used the day of collection to prepare cultures.

F-H Gradient Medium

9.2% ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden) solution was prepared with distilled water and sterilized by autoclaving at 121 ^{o}C for 15 minutes. 43.4 ml of above solution was mixed with 6.6 ml of hypaque-M (75% brand of sodium and meglumine diatrizoates, Winthrop Laboratories, New York, NY) and shaken vigorously. This F-H gradient medium was stored at 4 ^{o}C.

Cell Culture Medium

The culture medium was RPMI-1640 with added L-glutamine (Flow Laboratories, McLean, VA). To culture human lymphocytes, the medium was prepared as follows:

RPMI-1640	10.38 grams (gr)
Glass distilled water	858.7 ml
PHA (from Phaseolus vulgaris) (Sigma Chemical Co., St. Louis, MO)	5 milligrams (mg)
HEPES buffer (Flow Laboratories, McLean, VA)	2.5 gr
7.5% Sodium Bicarbonate	26.7 ml
Fetal Bovine Serum (Hyclone Laboratories, Logan, UT)	100 ml
Antibiotics: Gentamycine Sulfate (United States Biochemical Corp., Cleveland, OH)	1 ml (25000 mg/ml
Nystatin (mycostatin) (U.S. Biochemical Corp.)	1 ml (25000 IU/ml)

After thorough mixing the medium was filtered through a 0.20 μm metricel membrane (Gelman Instrument Co., Ann Arbor, MI) and stored at 4 °C.

ADM and MMC Solutions

One mg of ADM (Doxorubicin HCl: Distributed by Adria Laboratories, Wilmington, DE; manufactured by Farmitalia, S.P.A., Italy) was dissolved in 100 ml of glass distilled water to give a final concentration of 10 μg ADM/ml. The ADM solution was agitated thoroughly and filtered through 0.20 μm metrical membrane (Gelman Instrument Co.). Two mg of MMC (Sigma Chemical Co., St. Louis, MO) was dissolved in 100 ml of glass distilled water to obtain a final concentration of 20 μg/ml. The rest of the procedure was same as for ADM. Both solutions were kept in the dark at 4 °C.

Lymphocytes Separation

Two ml of F-H gradient medium was transferred to each of three dilution tubes (Falcon Plastic, Oxnard, CA) under the laminar flow hood (Labconco, Ann Arbor, MI). Five ml of blood was slowly layered on top of the gradient. The mixture was centrifuged at 400 g for 30 minutes at room temperature. The band containing the lymphocytes was collected and transferred into dilution tubes. Cells were washed in two ml of RPMI-1640 medium and centrifuged at 400 g for 15 minutes at room temperature. The supernatant was removed except for 0.5 ml, into which cells were resuspended. Four cultures were prepared from each blood sample and were started in tubes (Polystyrene, from Corning Glass Works, Elmira, NY) containing five ml media. All cultures were incubated at 37 ^{o}C for 72 hours.

Administration of Drugs

After 48 hours of incubation the 12 cultures were randomly divided into three groups, namely, ADM, MMC, and control. The time of administration and drug concentration were determined by a series of pilot experiments. In one series, 0.1 ml of working ADM solution (1 μg/ml) was added to each of the four cultures to give a final concentration of ADM at 0.02 μg/ml; in another 0.1 ml of working MMC solution (15 μg/ml) was added to each of the four cultures for a final concentration of 0.3 μg/ml, and each of four control cultures received 0.1 ml sterile glass distilled water. All cultures were immediately returned to the 37 ^{o}C incubator.

Chromosome Preparation

After 70 hours of incubation, all cultures were treated with colcemide (Demecoline, Sigma Chemical Co.) at final concentration of 0.2 μg/ml. The cultures were then reincubated for two hours. At the end of colcemide treatment, the cultures were agitated gently and centrifuged for three minutes at 250 g. The supernatant fluid was withdrawn leaving the cell pellet and 0.5 ml media. The cells were resuspended and four ml of 0.075M KCl added, gently shaken, and held at room temperature. After 30 minutes, all tubes were centrifuged at 250 g for three minutes. The supernatant fluids were drawn off leaving about 0.5 ml on top of the cell pellet. The cells were resuspended and four ml fresh fixative (3 parts ethanol: 1 part glacial acetic acid) was added dropwise. After 20 minutes, all tubes were centrifuged at 250 g for three minutes. The supernatant fluids were drawn off and last step was repeated two more times, using freshly made fixative each time. After the last sedimentation and removal of the supernatant fluids, the contents of four tubes of each group were suspended and mixed to produce one rich cell suspension.

Slide Preparation

Standard microscope slides were cleaned with acetone and ethanol, wiped with Kimwipe and air-dried. Each slide was dipped into a 0.5% solution of formvar (Ernest F. Fullman, Inc., Schenectady, NY) in ethylene dichloride. The dipped slide was removed quickly and suspended for 20 seconds in a wide-mouthed jar with an atmosphere

saturated with ethylene dichloride. The slides were then removed and air-dried.

Preparation of Chromosomes for EM and LM

The fixed suspension of lymphocytes were applied dropwise to plastic filmed slides that were previously chilled in 4 ^{o}C 70% ethanol. The slides were stood at a 45^{o} angle to allow the excess fixative to drain off. They were then air-dried at room temperature. Six slides were made from each group and the quality of chromosome spreads checked using phase microscopy. The slides were then randomly coded from one to 18 by a second party. The film on each slide was scored using the edge of a standard microscope slide. Each of the 18 slides were transferred individually to petri dishes and 30 ml of a 0.15% (V/V) hydrofluoric acid solution with distilled water were poured into the dish. The film was pulled off the slide by a sharp end forceps, and was then positioned so that the chromosome spreads were facing the fluid. The film was picked up on a wet slide and checked under phase microscope. Where good chromosome spreads were located a 50 mesh copper grid (Ernest F. Fullman, Inc.) was positioned on the film. The film with positioned grids was floated on the surface of glass distilled water. The film with grids and chromosome spreads was picked up by nonwetable cardboard and air-dried. This procedure was repeated until all of the usable pieces were recovered. Each piece of cardboard was numbered according to the code on the slide and stored in dry and dust free containers. The grids were later separated from the cardboard and checked under low power phase

microscopy (40x) for quality and position of chromosome spreads. From each group grids were randomly selected that had 20 to 25 chromosome spreads. One hundred chromosome spreads were expected from each of the three groups.

Staining

For LM, chromosomes were stained with solution of one part giemsa (Fisher Scientific Co., Fair Lawn, NJ) and 25 parts of glass distilled water. Grids were floated on drops of giemsa solution for 10 minutes. The grids were then washed with two changes of glass distilled water and air-dried.

After light microscopic observation the chromosomes were stained for EM with 1% uranyl acetate (Polysciences, Inc., Warrington, PA) in glass distilled water. Followed three minutes of stain, the grids were rinsed in two changes of cooled freshly boiled glass distilled water and air-dried.

LM and EM Photography

The chromosome spreads on each grid were photographed at 1000x magnification (American Optic LM) using Tri-X pan film (Eastman Kodak Co., Rochester, NY). Negatives were developed for 15 minutes in a solution of one part microdol-X (Eastman Kodak Co.) and three parts distilled water. They were washed in distilled water for five minutes, fixed for eight minutes in Kodak fixer, and washed for 25 minutes in running tap water. The film was then dipped in Photo Flow (Eastman Kodak Co.). After air-drying the negatives were cut and matched

according to the code.

The same chromosome spreads were photographed with Siemens transmission EM model 1A at 80 KV and 1600 magnification. A Kodak electron microscope film 4489 (Eastman Kodak Co.) was used for this purpose. The negatives were developed four minutes in D-19 developer (Eastman Kodak Co.), rinsed for two minutes in running water and fixed for six minutes in Kodak fixer solution. After fixation, the negatives were rinsed in running water for 20 minutes and dipped in Photo Flow prior to air-drying.

Printing

Both LM and EM negatives were printed on Kodak photographic paper (Kodabromide F-3, Eastman Kodak Co.) using a Laborator 138 S, Durst (made in Italy) enlarger. Following the correct exposure for 10 to 20 seconds, the paper was developed in dektol (Eastman Kodak Co.), rinsed in stop bath (Eastman Kodak Co.) for two to three minutes and fixed in Kodak fixer for four to five minutes. The prints were then rinsed in running water for one hour, placed in pakosol (Pako Corp., Minneapolis, MN) solution for a few minutes, drained and dried on a drying drum (Arkay Corp., Milwaukee, WS).

CHAPTER IV

RESULTS AND DISCUSSION

Data Collection

The sources of data were 589 metaphase cell micrographs that were taken with both LM and EM. From this pool of micrographs, 316 were at LM and 273 at EM levels of resolution. These micrographs were subjected to chromosome aberration analysis and were classified using standard criteria. Gaps were small achromatic discontinuities seen along the length of chromatid or chromosome without disturbing the continuity of the chromosome. Isolated fragments (or breaks) were of chromatid type if only one of the two chromatids was broken or deleted at any one point. Where both chromatids were affected, the two sister fragments were classified as one chromosome fragment. Exchanges and intrachromosomal reunions were classified as chromosome or chromatid type, depending on whether the origin could be traced to single-stranded or double-stranded chromosomes. Thus, dicentrics were considered as chromosome exchanges (or reunions) whereas quadriradials were classified as chromatid exchanges. For the analysis of fragments with respect to their points of origin along the chromosome, only chromosomes or fragments were used that could be definitely identified with a particular chromosome or a group of chromosomes. The analyzable fragments were in the vicinity of the parent chromosome and their points of origin. The points of

chromatid breaks were easily recognized because of the intact position of the sister chromatid. Approximate positions of breaks were marked along the lengths of reference lines drawn to represent the lengths and positions of centromeres of the chromosomes (or groups) in the complement.

After all metaphase cells were classified for chromosome aberrations, the codes were broken. The metaphase cells in LM and EM levels were pooled into three groups, namely, control, ADM and MMC. In every group, the data were tabulated in specific classes of aberrations.

Statistical Analysis

For statistical analysis of the data double precision Fisher exact test for one and two-tailed tests were used. These tests were accomplished by using a computer program developed for the IBM-PC by Forbes (1985). The results appear on the following pages. Dicentrics, gaps and quadriradials were not included in the statistical analysis due to few numbers. The probability value of 0.05 was accepted.

Adriamycin (ADM)

The data for ADM at LM and EM levels of resolution appear in Table 1. At LM level, 118 metaphase cells were scored for chromosomal aberrations. The aberrant cells were 53 (44.91%) of the total. The numbers of chromosome and chromatid fragments (breaks) were 62 (52.54%) and 43 (36.44%) respectively. The numbers of

cells scored for aberrations in control group was 116 from which the numbers of aberrant cells, chromosome and chromatid fragments were 9 (7.75%), 6 (5.17%) and 4 (3.44%) respectively. In the case of chromosome fragments, the differences were statistically significant, the values for one and two-tailed tests were p1 = 0.000 and p2 = 0.000. The same values for chromatid fragments were p1 = 0.000 and p2 = 0.000 that proved also to be statistically significant. Therefore, the data at LM level showed that ADM may be related to chromosome and chromatid breaks. Massimo et al. (1970) reported that after treatment of human lymphocytes with 0.05 to 0.1 μg/ml ADM for 72 hours, 74% of the cells showed "altered mitosis." The differences between percentages of aberrant cells in Massimo's and the present study is that in the former the percentage is for "altered mitosis." Massimo et al. (1970) did not report what percentage of cells had chromosome or chromatid aberrations. The other sources of this difference are the ADM concentrations (0.05-0.1 vs. 0.02 μg/ml) and the duration of treatment (72 vs. 24 hours).

Table 1

Frequency of Chromosome Aberrations Induced in Human Lymphocytes by ADM at LM and EM Resolution Levels

Resolution level	Treatment	Cells scored	Aberrant cells (%)	Chromosome Aberrations			
				Chromosome fragments (%)	Chromatid fragments (%)	Dicentrics	Gaps
LM	Control	116	9 (7.75%)	6 (5.17%)	4 (3.44%)	-	-
	ADM	118	53 (44.91%)	62 (52.54%)	43 (36.44%)	-	-
EM	Control	115	26 (22.60%)	13 (11.30%)	30 (26.08%)	-	3
	ADM	93	69 (74.19%)	75 (80.64%)	134 (144.08%)	-	13

Vig (1971) treated human lymphocytes with 0.02 μg/ml ADM for the last 24 hours of 72 hours of cultivation time. The percentages of aberrant cells reported were 83.06% of which 37.06% were chromatid exchanges. The high percentage of aberrant cells in his study may have been due to chromatid exchanges that were not classified in the present study. Excluding the chromatid exchanges, the number of aberrant cells in both studies were quite similar. Vig (1971) also reported that the numbers of chromosome fragments (breaks) were always higher than chromatid fragments. This is in agreement with data in the present study.

In another study (Newsome & Singh, 1977) human lymphocytes were treated with 0.03 μg/ml of ADM for one hour between 44-45 or 67-68 hours of cultivation. The percentages of aberrant cells were 40%-52% with few chromosome and chromatid breaks. In this study the numbers of chromosome breaks were higher than chromatid breaks. The percentage of aberrant cells in Newsome's and Singh's (1977) study is in agreement with that for the present study even though in the former the chromatid exchanges are included. It should be noticed that Newsome and Singh treated the cells for very short period of time.

At the EM level, total of 93 metaphase cells were classified for aberrations that were treated with ADM. The number of metaphase cells with aberrations was 69 (74.19%). The numbers of chromosome and chromatid fragments (breaks) were 75 (80.64%) and 134 (144.08%) respectively. In the control group, from total of 115 metaphase cells, 26 (22.06%) had aberrations in which there were 13 (11.03%)

chromosome and 30 (26.08%) chromatid fragments (breaks). The differences between chromosome fragments in control and ADM groups were statistically significant (p1 = 0.000, p2 = 0.000). Also, the differences in chromatid fragments were statistically significant (p1 = 0.000, p2 = 0.000).

In comparing the data of LM with that of EM, the differences between the chromosome fragments (breaks) induced by ADM (62 and 75) were statistically significant (p1 = 0.0074, p2 = 0.0119). In the same group, the difference between chromatid fragments at LM and EM level (43 and 134) was very significant (p1 = 0.000, p2 = 0.000). In control group, the numbers of chromosome fragments at LM and EM level were 6 and 13 but the difference was not statistically significant (p1 = 0.080, p2 = 0.113). However, the difference between chromatid fragments at LM and EM level (4 and 30) was statistically significant (p1 = 0.000, p2 = 0.000). In the ADM group the advantage and resolution power of EM over LM are not only clearly obvious but are statistically significant. The number of chromatid fragments (breaks) induced by ADM, at EM level was more than threefold greater than that of LM level. The differences between chromosome fragments at EM and LM levels in control groups were not significant. The reason may be that for detection of chromosome fragments, the lengths of chromosomes were measured. Therefore, higher resolution may not play an important role in length measurements. Of course, the numbers of chromosome fragments in the control group at both level of resolution were quite low. At EM level, it was noticed that the numbers of chromatid fragments were higher than

chromosome fragments in both ADM and control groups. This is opposite the data at LM level. Indeed, this may show the advantage of EM over LM.

Mitomycin C (MMC)

The results of experiment with MMC at LM and EM levels are presented in Table 2. As can be seen the same control was used for both ADM and MMC. At the LM level of resolution, in MMC group, of 82 metaphase cells that were classified for aberrations, 37 (45.12%) were aberrant. In these cells 19 (23.17%) chromosome fragments, 50 (60.97%) chromatid fragments and three quadriradials were observed. The differences between the numbers of chromosome fragments in control and MMC groups (6 vs. 19) were statistically significant ($p1 = 0.000$, $p2 = 0.000$). The values for chromatid fragments were also statistically different ($p1 = 0.000$, $p2 = 0.000$). Therefore, the data at LM level showed that MMC may be related to chromosome aberrations in human lymphocytes. It was also noticed that most of the chromosome aberrations were of the chromatid type.

Table 2

Frequency of Chromosome Aberrations Induced in Human Lymphocytes by MMC at LM and EM Resolution Levels

Resolution level	Treatment	Cells scored	Aberrant cells (%)	Chromosome Aberrations				
				Chromosome fragments (%)	Chromatid fragments (%)	Dicentrics	Gaps	Quadriradials
LM	Control	116	9 (7.75%)	6 (5.17%)	4 (3.44%)	-	-	-
	MMC	82	37 (45.12%)	19 (23.17%)	50 (60.97%)	-	-	3
EM	Control	115	26 (22.60%)	13 (11.30%)	30 (26.08%)	-	3	-
	MMC	65	45 (69.23%)	25 (38.46%)	66 (101.53%)	5	15	5

Morad et al. (1973) treated human lymphocytes with 0.25 µg/ml of MMC for 72 hours. He observed that 72% of cells had chromosome aberrations including exchanges. In this study the high percentage of aberrant cells could be related to the long treatment period (72 hours) and chromosome exchanges. In another study done by Novotna et al. (1979), human lymphocytes were treated with 0.16 µg/ml of MMC for the last 24 hours of cultivation. From a total of 311 cells classified only 9 breaks were observed. They did not mention the type of chromosome breaks that were observed. It is obvious that the number of chromosome aberrations in the present study is much higher than Novotna's study. The sources of this difference could be the MMC concentration, duration of treatment and genetic background of the cells.

Shiraishi and Sandberg (1979), in one of their experiments, treated human lymphocytes with 0.3 µg/ml of MMC for 48 hours. The percentage of aberrant cells was 37% from which 29 chromosome and chromatid breaks were recovered. Although, the MMC concentration was the same in the Shiraishi and Sandberg and present study, the duration of treatment was not. It has been shown that the chromosome aberrations were reduced by 50% in subsequent cell division following drug treatment (Carrano & Heddle, 1973; Conger, 1965; Sasaki & Norman, 1967). The difference in chromosome breaks in these two studies may well be due to the time of treatment.

A recent study was done by Palitti et al. (1984) in which human lymphocytes were treated with 0.6 µg/ml of MMC. Of the 30% aberrant cells, 21% had chromosome or chromatid breaks. However, it is

difficult to compare this study with the present study because the duration of MMC treatment was not reported in the former. In all the studies with human lymphocytes, the differences may be related to differences in genetic background of the cells.

At EM level, 65 cells were scored in MMC group from which 45 (69.23%) were aberrant cells. The numbers of chromosome and chromatid fragments in aberrant cells were 25 (38.46%) and 66 (101.53%) respectively. The difference between the numbers of chromosome fragments (breaks) in control and MMC groups (13 and 25) was statistically significant ($p1 = 0.0001$, $p2 = 0.0002$). In case of chromatid fragments, the difference in control and MMC group (30 and 66) was statistically very significant ($p1 = 0.0000$, $p2 = 0.0000$). At EM level, like LM, the number of chromatid fragments was more than twofold the number of chromosome fragments.

To determine potential advantage of EM over LM, the data for MMC at the two levels of resolution were compared. The numbers of chromosome fragments at LM and EM levels were 19 and 25 respectively. The difference between these two values was not statistically significant ($p1 = 0.062$, $p2 = 0.095$). The reason for the lack of a significant difference may be the low number of cells classified at EM level (65 cells). In addition, since chromosome fragments were determined by measuring the chromosome length, high resolution power of EM could not be a major factor. The numbers of chromatid fragments at LM and EM levels were 50 and 66 respectively. The difference was statistically significant ($p1 = 0.003$, $p2 = 0.006$). Therefore, the advantage of EM over LM in detecting minute fragments (chromatid

breaks) seems obvious.

A reduction in number of metaphase cells classified from LM to EM was observed. This reduction was very small for the control group, namely, from 116 cells to 115 cells at LM and EM respectively. But, this reduction increased to 17 cells in MMC and 25 cells in ADM groups. There are some explanations for this phenomenon. During the development of technique, it was noticed that the cells close to the margins of EM grids could not be observed and photographed under transmission EM. This caused reductions of observable cells on grids from two slides in MMC group from 17 for LM to 9 for EM and from 8 LM to 2 EM. Therefore, in MMC group, only half of the cells observed at LM could be observed at the EM level. The same reduction was noticed in two slides in ADM group; in one slide the reduction was from 21 to 10 and in the other 16 to 8. The other reduction in total cell number happened during the transferring of film to EM grids. In one slide in MMC group, only eight cells were recovered during the transferring step. This was the lowest number of cells that were recovered from a slide during the course of the study. It is believed that the film on that particular slide was very thin and difficult to pull off the slide. In future studies the number of recoveries could be increased by making more slides. Overall, the total numbers of cells or spreads recovered in three groups were sufficient to show statistical differences between control and treated groups, and at LM and EM levels.

CHAPTER V

CONCLUSIONS

The objectives outlined for the present study were accomplished. The first objective was to develop a technique for preparing human chromosomes for sequential LM and EM. The technique described preserves the morphology and structural organization of chromosome while allowing observation of the cell's entire chromosome complement. Chromosomes thus prepared can be stained for banding and aberration studies, two important procedures in areas of cytogenetics and genetic toxicology. Most of the techniques described in the literature for studying mammalian chromosomes at the EM level either failed to preserve all the chromosome complement, caused chromosome dispersion or stretching.

The second objective aimed at inducing chromosome aberrations in human lymphocytes by ADM and MMC and observing them by LM and EM was also accomplished. At the LM level, differences between chromosome and chromatid fragments in MMC and ADM groups in comparison with control were found to be statistically significant. However, at the EM level, the numbers of chromosome aberrations observed in both drug groups were much greater than that for the control and were found to be statistically significant.

The third objective was to determine whether EM observations might reveal chromosomal aberrations not observable by LM. The objective was accomplished. In control groups the number of chromatid

fragments observed by EM was significantly greater statistically than by LM. However, the difference between the numbers of chromosome fragments observed was not statistically significant. In the ADM group, the differences between both chromosome and chromatid fragments observed at the LM and EM levels were statistically significant. With MMC, the number of chromatid fragments scored at EM was significantly greater than that at LM. The numbers of chromosome fragments recovered at EM were also greater than that at LM level but the difference was not statistically significant. There were two cases of chromosome fragments whose differences at EM and LM were not significant, namely, in MMC and control groups. However, the number of chromosome fragments scored was small. It is also possible that length measurement does not discriminate well between resolution levels.

Overall, the present study showed the advantage of high resolution in making observations in cytogenetics. Should a reproducible banding technique applicable to EM be developed, the use of EM could be greatly extended. An EM banding technique might increase accuracy in detecting minute chromosome or chromatid deletions or duplications. The technique described in the present study could be used for evaluating banding procedures and for studying the ultrastructural organization of chromosome and/or chromosomal fibers.

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