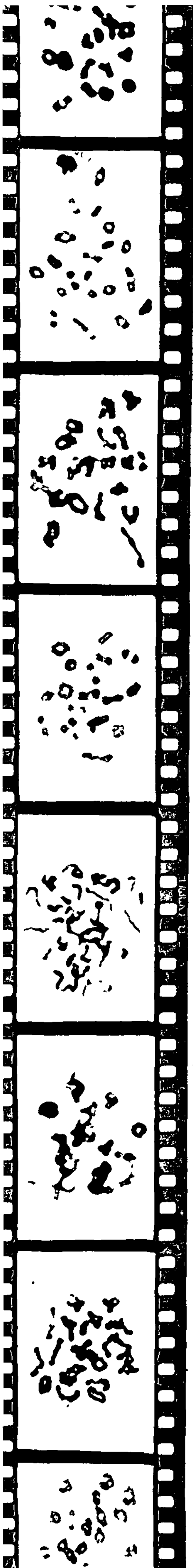
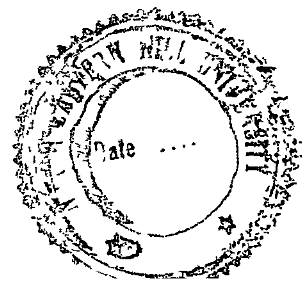


SOME ASPECTS OF GERM CELL CYTOGENETICS IN S180 TUMOUR BEARING MICE SUBJECTED TO COMBINATION CHEMOTHERAPY

Ph. D. Thesis : 1989
The University of Burdwan



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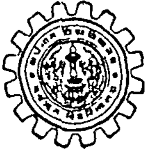

THE UNIVERSITY OF BURDWAN

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SAMAR CHAKRABARTI, Ph. D

Certified that the thesis entitled, 'Some Aspects of Germ Cell Cytogenetics in S·80 Tumour Bearing Mice Subjected to Combination Chemotherapy', is a record of original research work, done under my supervision and guidance by Mr. Debasish Dey, M.Sc., during the period of his stay at the University of Burdwan, and it has not previously formed the basis for the award of any degree, diploma or any other similar title.

Mr. Dey shares equal authorship for the papers & the abstracts of papers published on the related topics during the period of his research.

Burdwan
2 May 1989

(Samar Chakrabarti)

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study of cancer is regarded as an important branch of general biology both biology and medicine will profit—Julian Huxley

Dedicated to

my well wishers who

first taught me to

READ , WRITE & THINK

contents

CONTENTS

PREFACE ——— *** i

ACKNOWLEDGEMENT ——— ***

INTRODUCTION ——— *** 1

MATERIALS & METHODS – *** 34

a. Experimental animal ... 34

b. Tumor model ... 35

c. Tumor transplantation ... 36

d. Meiotic chromosome preparation from tumor bearing mice ... 36

e. Drug selection ... 39

f. Therapeutic dose ... 47

g. Treatment & experimental protocol ... 51

h. Maintenance of Control ... 52

i. Scoring of aberrations ... 59

j. Fertility potential ... 54

k. Epididymal sperm count ... 56

l. Sperm head morphology ... 57

m. Simple statistics ... 61

Cont..

OBSERVATIONS ——— *** 62

a. Tumor regression ... 62
b. Mean Survival Time (MST) ... 62
c. Meiotic chromosomes of Normal mouse ... 63
d. Spermatogenesis of mouse ... 66
e. Meiotic chromosomes of tumor bearing mouse ... 69
f. Negative Control ... 71
g. Treated series : ... 71
 i. CP single Therapy 71
 ii. Combination I Therapy 74
 iii. Combination II Therapy 76

h. On Fertility Potential : ... 79
 i. Sperm head assay ... 79
 ii. Epididymal sperm count ... 80
 iii. Testis weight ... 81

DISCUSSIONS ——— *** 83

SUMMARY ——— *** 105

REFERENCES ——— *** i

LIST OF PUBLICATIONS ——— *** xxiv

Preface

PREFACE

During the past few years chemotherapy has become established as a form of treatment for a majority of malignant tumors. A large number of antineoplastic agents have been isolated, designed and tested, but only a few have found acceptance. Many that showed initial promise were later abandoned because of their less effectiveness or more toxic side effects than was anticipated earlier. Most of these agents have not, as a rule, been approved after systematic study of their cytotoxic potential, particularly on germ cells of the patients or appropriate test systems. It is known that an increase in chromosomal abnormalities in somatic cells may enhance the chance of neoplasia while in germinal cells any such abnormalities may lead to serious congenital malformation. Hence

investigations on cytotoxic and genotoxic potentials of established and promising anticancer drugs are of continuous importance both from fundamental as well as applied point of view.

For identifying substances hazardous to human beings, test data from *in vivo* mammalian germ cell assays carry much greater weight than those from *in vitro* short term tests. Despite its toxic potential and certain other limitations, chemotherapy has become established as most effective and popular form of treatment for a wide variety of cancer. For certain types of malignancies, however, it is a treatment of choice.

Cyclophosphamide is an important antineoplastic and immunosuppressive agent used either singly or in combination with various mitostatic (eg.vincristine) drugs to combat as many as twenty five different human malignancies. Recent studies indicate that the drug may cause secondary neoplasia in cancer patients as well as cancer in noncancer patients, and also in experimental animals [IARC 1982, Shelby 1988]. Although some of the hazardous potentials of CP are well documented, the drug is still used rather extensively particularly in different parts of the Third World.

A variety of cytotoxic effect of CP on somatic and germinal cells of man,mouse and other mammals is know known. But the precise way by which the drug affects meiotic chromosomes and influences germ cell cytogenetics is not clearly understood.One of the pathways of drug action obviously involves interference with cellular DNA synthesis. A review of existing literature indicates that most of the studies made in this regard were designed with an assumption that the drug exclusively affects spermatogonial stem cells hence post-exposure cytogenetic evaluation was made day- 19 onwards. During this period the surviving spermatogonia, as per time sequence established by Oakberg(1956, 1957) would enter spermatocytic phase of spermatogenesis and the effect of the drug can be perceived by scoring the cells at diakinesis-metaphase.I stage for aberrations.

Our pilot studies on the effect of certain alkylating anticancer agents indicated that even a 'short term exposure of these drugs may produce meiotic abnormalities in normal and tumor-bearing mice (Chakrabarti _et al_.1986, Dey _et al_.1989, Dey and Chakrabarti 1989). Moreover, thee reports of the effects of CP and VC on germinal cells and testis weight of different mammalian test systems are, to some extent, conflicting.

Published reports on the influence of the drugs on the fertility potential of man and laboratory mammals too are contradictory.

The main objectives of the present study was to expand the existing pool of information in the field of cyclophosphamide related cancer chemotherapy and to give a fresh insight on the controversy of short-term effect of the drug and drug combinations on germinal cells of tumor bearing mouse in course of therapy.

Acknowledgement

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He is indebted to Profs.JN.Medda, S.Roy, MC. Mukherjee and PK. Choudhuri for placing laboratory facilities at his disposal. He is also grateful to Professor Deb Kumar Choudhuri for encouragement.

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Finally, the author acknowledges the contribution of his supervisor, Dr.Samar Chakrabarti, Lecturer of the department who introduced him to this interesting field of cancer cytogenetics. His guidance and inspiration have been of inestimable value.

Debasish Dey 25/8

Debasish Dey

introduction

INTRODUCTION

Cytogenetic assays on the genotoxic potential of drugs and chemicals are of continuous importance. Hsu(1982) advocated that an increase in chromosome abnormalities in somatic cells may enhance the chance of developing neoplasia while in germinal cells any such abnormalities may lead to a high frequency of spontaneous abortion, birth defects and heritable chromosome rearrangements.

Since the present project is oriented on germ cell cytogenetics of tumor-bearing animal model in response to single agent and combination chemotherapy, a brief review of literature on various aspects of cancer chemotherapy will be of value. The ultimate goal of present day cancer therapy is to remove all malignant cells with least toxic side effects on host's normal body cells. Since the neoplastic

cells are, in general, heterogenous population consisting of different 'mutant clones',it often becomes difficult to select appropriate therapeutic measures to stop their multiplication.

Of the two possible approaches to cancer treatment, ie., the reversal of the neoplastic state, and the removal of the neoplastic cells from the system,present-day cancer therapy is based almost exclusively on the second approach. Surgery, radiotherapy,chemotherapy,immunotherapy are all effective measures for the removal of the tumor cells from the system. During the past forty years chemotherapy has become established as a form of treatment for a wide variety of malignant tumors. A large number of antineoplastic agents have been tested, but only a few have found acceptance;many that showed promise initially were later abandoned because of their less effectiveness or more toxic nature than was originally anticipated. Hence investigations on the toxic potentials of anticancer drugs are continuing along with the search for more potent drugs and more reliable therapeutic modality. Despite its toxic potential and certain other limitations chemotherapy has become established as most effective and popular form of treatment for a majority of types of cancer. The socio-economic implications of chemotherapy are also too many.

Tumors are, in general, heterogenous population of cells consisting essentially of three compartments : i. a proliferating compartment consisting of cells in the process of division, ii. a non-clonogenic compartment comprising of cells which lost their capacity to divide, and iii. the clonogenic compartment which includes cells that although not in division at the time but may divide if an appropriate stimulus is available. Chemotherapeutic agents act mainly on the dividing fraction of tumor cell population. Thus the response obtained by a particular drug against a tumor depends to a great extent on the size and accessibility of the proliferating compartment. In fact one of the main obstacles to a complete cure of advanced tumors is the persistence of these clonogenic cells in the system.Besides the clonogenic cells,the existence of hypoxic cells also offer troubles in the treatment of cancer (Goldacre 1977, Tannock 1982). Hypoxic cells in solid tumors are known to be resistant to treatment with radiation. They may also escape chemotherapy because of limited diffusion capacity of many antineoplastic drugs. Moreover, hypoxic cells are in general, slowly proliferating cells, while most anti-cancer drugs are more active (with possible exceptions to certain tumors) against rapidly proliferating cells.

The clinical effectiveness of any anticancer drug requires selective toxicity to malignant cells in vivo at doses that allow enough cells in the patient's critical tissues to be least affected. Since most tumors are heterogenous and sometimes cells of different origin(bi- or multi-clonal) respond differently to the same anticancer agent,no specific target at the molecular level could be identified for effective drug therapy. Almost all chemotherapeutic agents are cytotoxic and interfere either in a direct way or in an indirect means with various phases of the cell cycle(Table1.) The classification of chemotherapeutic agents depends mostly on their mode of mechanism of action at molecular or macro-molecular level and the source from where they have been isolated (Brule et.al.1973, Krakoff 1977, Pratt and Rudden 1979).

TABLE. 1 : Classification of chemotherapeutic agents according to their mode of action & source

I. ALKYLATING AGENTS

A. Classic alkylating agents

(Inhibitor of DNA synthesis)

i. Bio(chloroethyl)amines :

i. Asaley
ii. Chlorambucil
iii. Cyclophosphamide
iv. Melphalan
v. Mechlorethamine
vi. Uracil mustard
vii. Ifosfamide

2. Ethyleneimines
   i. Thio-tepa(THIO)

3. Alkylsulfonate
   i. Busulfan
   ii. Yoshi 864

B. Nitrosoureas :
   i. Carmustine
   ii. Estramustine
   iii. Lomustine
   iv. Semustine
   v. Streptozocin
   vi. Chlorozotocin

C. The Antibiotics :

a. impair DNA synthesis:
   i. Mitomycin C (MC)
   ii. Bleomycin

b. impair RNA synthesis:
   i. Actinomycin D (ACT)
   ii. Mithramycin
   iii. Daunomycin
   iv. Adriamycin (ADR)
   v. Streptonigrin
   vi. Chromomycin
   vii. Dactinomycin
   viii. Doxorubicin
   ix. Daunorubicin (DNR)
   x. Carminomycin

c. impair protein synthesis:
   i. Puromycin

d. impair both DNA and RNA synthesis:
   i. Rubidazone

D. Miscellaneous Alkylators:

a. impair DNA synthesis:
   i. Cisplatin

b. impair both DNA and RNA synthesis :
   i. Decarbazine

c. impair DNA,RNA and Protein synthesis:
   i. Galactitol

II. Antimetabolites :

(inhibitors of Nucleic acid biosynthesis)

A. Folate antagonists :

i. Ethanesulfonic acid compound
ii. Methotrexate (MTX)
iii. Dichloromethotrexate

B. Purine antagonists :

i. Azathioprine
ii. 6-Mercaptopurine (6-MP)
iii. Thioguanine
iv. Bromo-deoxyuridine

C. Pyrimidine antagonists:

i. 5-Fluorouracil (5-FU)
ii. 5-Fluoro-deoxyuridine
iii. 5-Azacytidine
iv. Cytosine arabinoside
v. Cyclocytidine

D. Glutamine antagonists

i. Azaserine

E. Polynucleotide antagonists:

i. Hydroxyurea

III. Plant Alkaloids :

Mitotic inhibitors :

i. Vincristine (VC)
ii. Vinblastine (VN)
iii. Vindesine
iv. Etoposide
v. Teniposide (VM-26)
vi. Maytansine

IV. Enzymes :

Substitute for defective synthesis:

i. L-asparaginase

V. Hydrazine :

inhibitor of DNA replication and RNA synthesis:

i. Procarbazine (PCB)

VI. Steroids :

inhibitors of protein synthesis:

i. Adrenocortical hormones
ii. Progestational steroids
iii. Estrogen
iv. Antiestrogen
v. Androgen
vi. Antiadrenal agents

VII. Miscellaneous agents:

A. Multiple inhibitory action (enzyme inhibitors,DNA biosynthesis etc.)

i. Cytembena

B. Inhibitor of DNA synthesis;

i. Razoxane
ii. Hydroxyurea (HU)*

*abbreviations used in the text are kept in parenthesis

Recently, Matney et al.(1985) have classified seventeen different antineoplastic agents according to their ability to cause gene mutation in excision repair proficient test bacteria (Table. 2).

In the DNA-RNA-Protein kinetic cycles the anticancer drugs act differently at different steps to regulate the abnormal proliferation of neoplastic cells. For example, antifolate,

(eg.,Methotrexate), purine antimetabolites (eg.6-mercaptopurine) and pyrimidine analogues (eg.,5-FU and FUdR)act at the same step to inhibit the enzyme thymidylate synthetase which converts deoxycytidylic acid to thymidylic acid— a must for the synthesis of DNA (Heidelberger 1965). Cytosine arabinoside, hydroxyurea and many other anticancer agents are known to inhibit the activity of ribonucleoside diphosphate reductase to impair the reduction of cytidylic acid into deoxycytidylic acid necessary for the biosynthesis of DNA (Krakoff *et al.*,1968). However, cytosine arabinoside is also known to block DNA polymerase responsible for polymerisation of nucleic acid (Furth and Cohen 1968).

TABLE. 2 : Genotoxic classification of some anticancer drugs

Commercial Drugs	Experimental Drugs
Class.I (Mutagenesis in Uvr$^+$strains only)	
i. Bleomycin, ii. Mitomycin	
Class.II(Major mutagenesis in Uvr$^+$strains)	
i. Cisplatin	i. Etoposide ii. Mitoxantrone iii.PCNU iv. Teniposide
Class.II(Minor mutagenesis in Uvr$^+$ strains)	i. Isophosphamide ii. Mercaptopurine
i. Carmustine, ii.Decarbazine iii.Mechlorethamine	
Class.III (No mutagenesis in Uvr$^+$strains)	
i. Cyclophosphamide,ii.Thiotepa, iii. Doxorubicin,iv.Daunorubicin	i,AZQ

Antibiotic mitomycin C interferes with DNA synthesis by cross-linking its base pairs while other antibiotics like actinomycin D, daunomycin and adriamycin are known to affect the synthesis of specific enzymes required for nucleic acid synthesis (Reich 1963).

Bruce et al. (1966) explored the kinetics of antineoplastic drug induced cytotoxicity of normal and malignant cells and classified these agents into several groups :
Class I/'nonphase specific' agents include nitrogen mustard which is toxic for proliferating G_0 cells, and showed no difference in toxicity against the normal hematopoietic stem cells and tumor cells.
Class II or 'phase specific' agents include MTX, vinblastine azaserine etc. that kill cells during only a specific part of the cell cycle and did not appear to affect G_0 cells if the exposure time was short. These agents exhibited increasing cell kill with the increase in dose until a plateau was reached; after which there is no further increase in cell kill frequency (Fig.1).
Class III or 'cycle specific' agents affect both proliferating as well as non-proliferating cells. However, dividing cells were more sensitive than G_0 cells and were killed throughout the cell cycle. These agents include 5-FU, actinomycin D

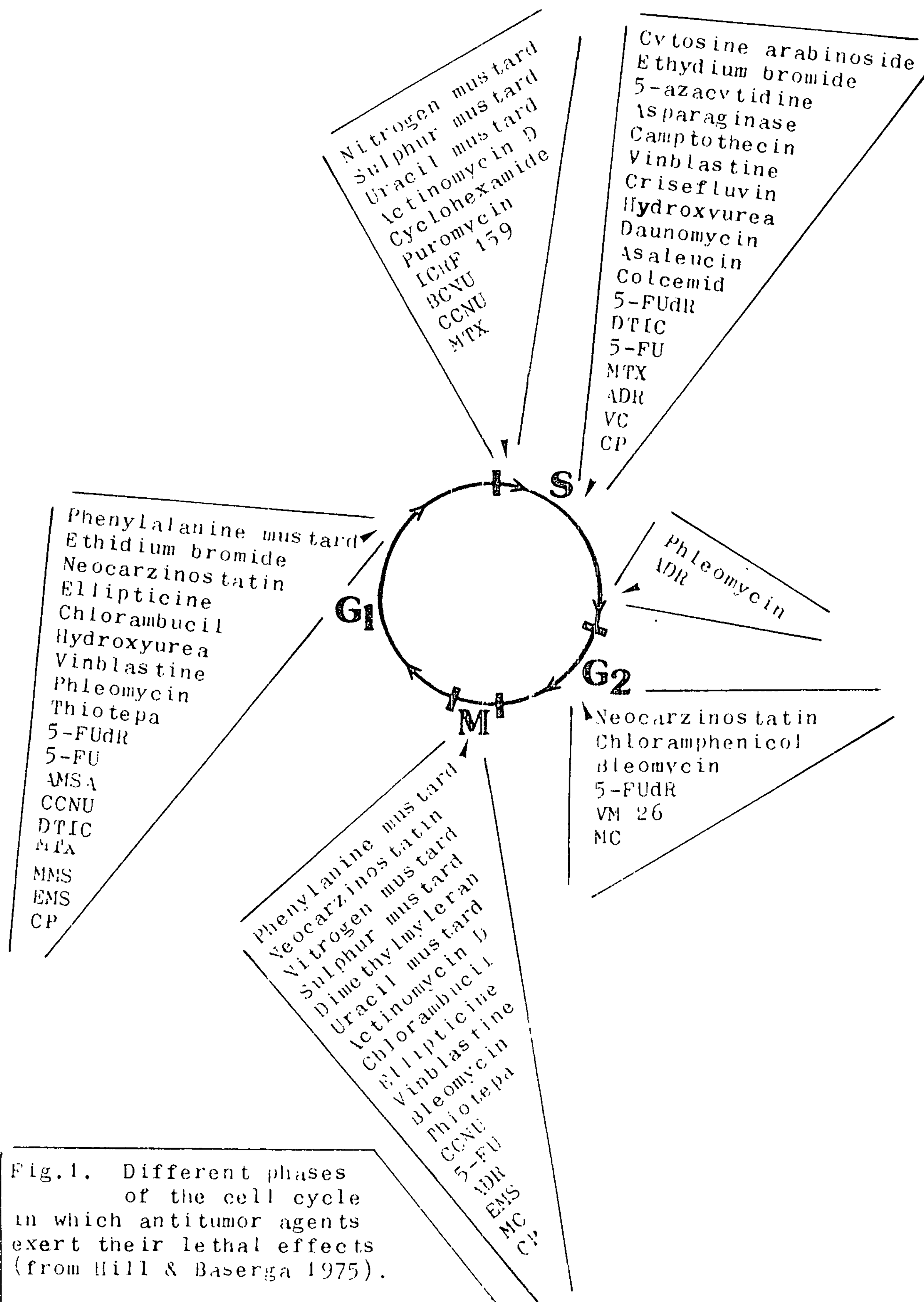


Fig.1. Different phases of the cell cycle in which antitumor agents exert their lethal effects (from Hill & Baserga 1975).

and Cyclophosphamide. The greater kill of tumor cells as opposed to normal marrow stem cellswith both phase and cycle specific agents was felt to be due to the fact that most stem cells were in a state of G_0 , and most tumor cells were in actively dividing phase. Studies by Valeriote and Bruce (1967) showed increased sensitivity of normal stem cells with increased exposure to drug, and loss of specificity for malignant cells vs. normal stem cells if the stem cells were rapidly proliferating to repair previous marrow damage.

Combination Chemotherapy

The introduction of combination chemotherapy has extended the horizon of cancer treatment to a great extent. Combination chemotherapy has been found to be effective for a variety of tumors particularlyfor those which are known to possess a multiclonal genetic make-up (Symington 1980). For certain tumors, combination of drugs have produced results clearly superior to those obtained with the use of a single anticancer agent. Not only is the percentage of complete remissions increased, but also the probability of long term disease free survival and cure appears to be reality for certain patients with wide spread disseminated disease.

General principles :

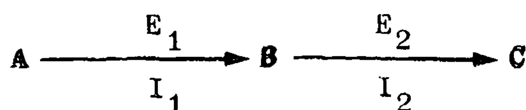
i. Drugs individually effective against the tumor: With

several exceptions, the choice of agents for combination chemotherapy is restricted to those drugs that have demonstrated efficacy against the tumor when used alone.Arguments against this temet are that all available drugs have not been tested individually against all kinds of tumors or even the most common types of tumors (Livingston and Carter 1970). In addition, effectiveness may also dependent on dose or schedule or both,and proper manipulation of either or both may yield significant activity of a heretofore relatively 'inactive' agent.

ii. Minimal overlapping toxicity : Most anticancer drugs possess some degree of toxicity to normal tissues. By selecting active agents with minimal to non-overlapping toxicity, full effective doses of the components can be used with tolerable toxicity.

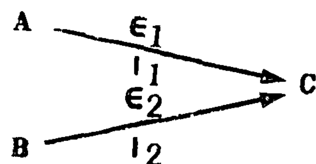
iii. Pharmacological considerations : a) Biochemical mechanism of action :- Most of the currently available anticancer drugs are grouped in a chemical classification indicating their mode of action (vide page 4-6). Within each category, the toxicity of individual agents is also comparable. Thus if an alkylating agent such as CP is used at its maximal doses, it makes little sense to add another alkylating agent in the list of therapy. As a rule, combination of agents with dissimilar mechanism of action is followed. This principle also needs further thought,however, since with certain combinations,

one drug may create metabolic and cytokinetic conditions so as to minimize or completely antagonize the therapeutic effectiveness of the second agent. These interactions are called schedule dependence. The terms sequential, concurrent and complementary inhibition have been proposed (Sartorelli 1969, Harrap and Jackson 1975). Potter (1951) applied the term sequential inhibition to the simultaneous action of two inhibitors acting on different stages of a metabolic pathway. This type of inhibition may be represented as :



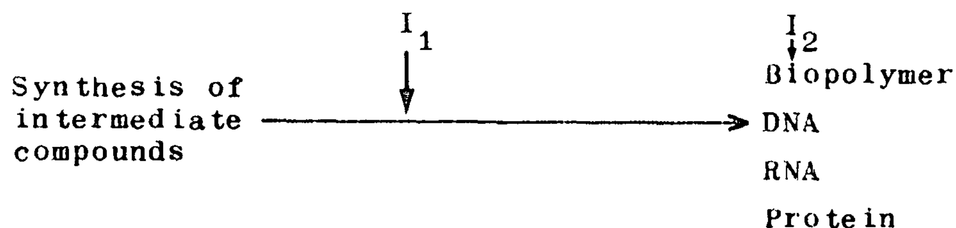
where E_1 and E_2 are the enzymes that catalyze the reactions from A to B and from B to C, and I_1 and I_2 are the inhibitors of these reactions.

A scheme of concurrent inhibition (Elion *et al*.1954) may be represented as follows:-



Clinically useful examples of this inhibition are not available at present. However, it has been suggested that if a potent inhibitor (I_1) of thymidine kinase (E_1) was available, it might be of considerable use in combinations with an

inhibitor (I_2) of thymidylate synthetase (E_2) such as 5-FU. This combination would provide a concurrent inhibition of the synthesis of thymidylate (Harrap and Jackson 1975). The concept of complementary inhibition is the other useful model (Sartorelli 1969) which involves the use of drugs that act at different loci involved in the formation of certain polymeric molecules. This can be depicted as follows:



In metastatic breast carcinoma, the combination of the alkylating agent CP with the antimetabolites 5-FU and MTX (Canellos et al. 1976) has shown significant improvement over the results obtained using each agent alone (Livingston and Carter 1970).

Transport : Passage of a drug across the cell membrane and entry into the cell is another prerequisite for an optimum drug effect. The only anticancer drug that exerts its effect extracellularly is asparaginase. Interference with this transport mechanism through the action of a second drug can significantly alter the therapeutic outcome. The combination of anticancer agents with membrane active agents is useful because it can overcome drug resistance and inhibit the

development of drug resistant tumors. To obtain clinically useful combinations suitable potentiating agents are also required. Vitamin A or isoprenoids carrying an isoprene chain have been found to potentiate the activity of antitumor agents (Cohen and Carbone 1972, Tomita et al.1982, Yamaguchi et al. 1984). Adriamycin, Bleomycin and Cisdichlorodiamminoplatinum with squalene showed significantly potentiated antitumor activity in an in vitro system against S180 tumor model (Nakagawa et.al. 1985).

Biotransformation :- Antineoplastic drug induced alteration of the enzymatic activation of a second anticancer drug administered concurrently or sequentially has not been demonstrated to be of clinical importance, but remains theoretically possible. The alkylating agent CP requires 'activation' by the mixed function of oxidase enzyme present in the hepatic microsomal fraction (Slodek 1971).

iv. Cytokinetic considerations: The S phase showed a marked difference in duration between tumor cells and normal cells. In normal cells S is generally 11 to 13 hrs., depending on the cell type, while in benign tumor S phase lasts 11 to 17 hours, and in malignant tumors S phase varies inbetween 18 and 25 hours (Bleiberg and Galand 1976, Fabricant 1970).

Both phase and cycle specific agents are usually given in short,intensive courses using maximally tolerated doses.

The use of small daily doses of, phase and cycle specific agents is avoided, since it results in less tumor cell kill and greater hematologic toxicity. Combinations of cycle or phase specific agents or both given concurrently showed additive toxicity. Agents that exert their maximum toxic effects in the same phase may give additive toxicity, but not necessarily additive or synergistic tumor effects.

In a given combination, therefore, agents that exert their maximal effects at different phases of the cell cycle must be chosen. Effective combination of drugs may be obtained by using one agent that arrests cells at one stage, followed by an agent that maximally kills cells during that stage immediately succeeding the block. This cell proliferation arrest may cause a partial synchronization and therefore, with proper drug scheduling, may result in increasing cell kill or even therapeutic synergy, whereas improper scheduling may show no increased cell kill, or even drug antagonism.

v. Schedule dependency : Closely allied to cytokinetic considerations is the phenomenon of schedule dependency which may occur as a result of cytokinetic or biochemical interactions between different drugs. For example, when drugs are administered sequentially, the first drug may produce a delay in cell cycle progression such that when the second drug,

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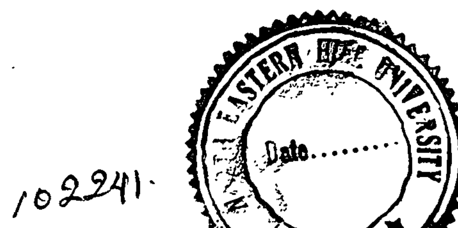


a cell cycle or phase specific agent, is given, the cell may or may not be in a phase of its generative cycle that is sensitive to the action of the second drug. Obviously, the appropriate timing of administration of the second drug is most important. Such a manipulation would produce an improvement in the therapeutic index provided normal host cells have not been affected in precisely the same way. Alternately, the first drug may produce sufficient biochemical imbalance within the cell to attenuate the effects of the second. Schedule dependency has been observed with many drugs and their combinations. It is important to suspect schedule dependent interaction between drugs when agents that possess diverse mechanisms of action and have previously displayed therapeutic activity in treating a disease are found to produce subadditive or antagonistic effects when used in combination.

vi. Miscellaneous considerations : a) Drug doses :- One of the advantages of combining drugs with non-overlapping host toxicity is that full doses of the chemotherapeutic agents can be used simultaneously. If two or more drugs with a similar toxicity spectrum are used, then the doses of each drug must be appropriately attenuated if the drugs are to be used concurrently, or a sufficient time interval must elapse between doses to allow host recovery from the previous drug.

A review of literature indicates that the studies made on the effects of anticancer drugs at cellular level were mostly limited to evaluate their clastogenic potentiality on tumor and on the somatic cells of the tumor bearing host. However, there are reports on germ cell toxicity too. But chronological and systematic studies, particularly on germ cell cytogenetics during the course of therapy and after completion of treatment is rather meagre. A brief review on reported germinal cell toxicity is given for better understanding of the problem.

Germinal Cell Toxicity :

Of the three major classes of genetic damage that is, mutations, structural chromosome aberrations and non-disjunction, only the last two categories can be evaluated in germ cells of experimental animals (Lyon and Cox 1975, Russell 1976). Chromosome analysis in male germ cells as suggested by Adler (1982), is not just one procedure in the field of mutation research but a complex system with which detailed questions about germ cell sensitivity can be answered.

Damage to the testicular germinal epithelium is a potential side effect of cancer chemotherapy and is of particular concern in the cases of male in the reproductive ages. Exposure to drugs often results in azoospermia(Wyrobek et al.1983b).

Even if sperm production recovers, it is uncertain whether the quality and quantity of the spermatozoa are adequate for fertility and whether the spermatozoa are carrying mutation. These questions can be approached by using mouse tumor model as an experimental tool because : i. the development of germ cells of mouse has been studied in detail, ii. the similarity of spermatogenesis in mouse and man, and iii. the genetic heterogenity of these two mammalian species. Moreover, in man, the pattern of loss and recovery of spermatogenesis following cytotoxic treatment is much similar to that of the rodents in many respects. The importance of germ cell studies is further understood by the recent findings in animal models suggesting that offspring may be at risk of cancer or congenital malformations as a result of paternal exposure to mutagenic chemicals (Nomura 1982, Brown 1985, Trasler et al. 1985). The effects of several cytotoxic agents have been evaluated on spermatogonial and other stages of spermatogenesis and spermiogenesis.

Cytotoxic drugs generally cause oligospermia by affecting the spermatogonia (Schilsky et al. 1980) and possibly later stages of spermatogenesis (Neumann et al. 1976, Jackson and Schnieden 1982). Alkylating agents which are known to have a dose related effect may cause testicular atrophy, but Leydig cell function remains unaffected (Graner 1981). MTX produces oligospermia, which improves within a few months of stopping treatment

(Sussman and Leonard 1980). There are reports of azoospermia also after radioiodine (^{131}I) therapy (Handelsman et al.1980). Twenty three different drugs representing the various classes of chemotherapeutic agents have been tested in recent years (Carter et al. 1981, Meistrich et al. 1982, Meistrich 1984); these include: antimetabolites and inhibitors of nucleotide metabolism - hydroxyurea, cytosine arabinoside (ara-c),MTX, 5-FU, 6MP; antibiotics - ADR, daunorubicin,bleomycin and actinomycin D; acridine derivatives - AMSA; alkylating agents- CP, chlorambucil, MC, THIO, nitrogen mustard, busulfan ; other compounds reacting with DNA (including cross-linking agents) - bischlorethylnitrosourea (BCNU), cyclohexychloro-ethylnitrosourea (CCNU), cisplatin, procarbazine; microtubule dissociating agents - vinblastine,VC and corticosteroids - prednisone. Twenty one of these twenty three drugs produce no cytotoxicity to stages later than leptotene spermatocytes,5-FU and cisplatin are the only exceptions (Meistrich et al. 1982). Meistrich (1984) found that differentiated spermatogonia are the most sensitive cell type whereas spermatocytes and spermatids are relatively resistant. However, exceptions are also there. Prednisone produces no detectable cell killing. 5-FU is the drug which has an effect on post-spermatogonial cells at doses which are insuffient to kill spermatogonia. 5-FU affects only pachytene spermatocytes. Zygotene spermatocytes are also sensitive towards high doses of5FU, because the damage to spermatocytes. is expressed as cell killing mainly when the

cells pass through their meiotic divisions. Cisdiamminedichloroplatinum II (CDDP) results in killing of some cells at all stages from zygotene spermatocytes through step 5 spermatids; it also affects the function of the sertoli cells. Several patterns of differential sensitivity of the spermatogonia and spermatids of spermatogenesis have been observed by Meistrich et al.(1982). ACT, CCNU, DNR, 5-FU, 6-MP,MC,and PCB produced some large, round, presumably diploid spermatids. Another type of abnormality observed only after CDDP administration is the formation of binucleate spermatids. BCNU, CCNU, PCB and THIO cause a delay in spermiation. Several drugs viz., PCB and 6-MP result in the production of abnormally elongated spermatids. Testicular sperm head counts obtained after 29th. day of injection indicate that prednisone and 6-MP do not affect spermatogonia while administration of 5-FU and procarbazine kill nearly all the spermatogonia. Sperm counts after 56 days of treatment revealed that CCNU, CDDP, HN_2,6MP, MTX and PRED produce at the highest dose point, upto 25% decline in sperm counts. Seven drugs ACT,BCNU, CHL, DNR, 5-FU, MC and PCB reduced sperm head counts to between 30-60% of controls indicating a significant stem cell killing. THIO produced stem cell toxicity, Meistrich (1984) demonstrated that there are some consistent differences in the stages most sensitive to killing by different classes of chemotherapeutic drugs. Antibiotics preferentially kill spermatogonia at

earlier stages, alkylating agents preferentially kill cells at somewhat later stages, antimetabolites specific for inhibition of DNA synthesis (HU, and ara-C) kill cells at slightly later than most alkylating agents.

The vinca alkaloids are not toxic to stem cells, presumably because these cells are very slowly cycling (Oakberg 1971). Similarly, drugs that affect the availability of DNA precursors (6-MP, MTX, 1-D-arabinofuranosylcytosine and HU) are also non-toxic to stem cells (Meistrich et.al. 1982)except for the drug 5-FU. The explanation for the effectiveness of 5-FU is that its active metabolite has a much longer intracellular life (7-9 days). Van Keulen and de Rooij (1974) demonstrated that A_1 spermatogonia and possibly stem cells were the most sensitive cell types. According to Bucci and Meistrich (1987) , busulfan is a potent killer of spermatogonial stem cells at sublethal doses. Differentiating spermatogonia (types A_1 to A_4) are usually the most sensitive cell types to the effects of other antineoplastic agents including several alkylating agents (Meistrich 1984). MOPP chemotherapy (Mechlorethamine-VC-PCB-Prednisone) is used extensively in treating Hodgkin's disease. A predictable consequence of MOPP is germ cell killing which results in transient or permanent sterility (Devita et al. 1973). In contrast, ABVD chemotherapy (ADR, Bleomycin, VC, decarbazine) is as effective

clinically as MOPP without damaging the germ cells (Bonadonna et al. 1984). The clinical observations with ABVD are somewhat surprising, since atleast one of the agents, ADR has significant cytotoxic and mutagenic action on mouse spermatogonial stem cells (Meistrich et al. 1985). It also produces SCEs in differentiating mouse spermatogonia (Abraham and Franz 1983)., sex linked recessive lethal mutation in Drosophila (Clement et al. 1984) and chromosome damage in mouse spermatocytes derived from treated spermatogonia (Au and Hsu 1980). Goldstein (1987) observed depression in fertilizing frequency in the fifth and the sixth weeks of MOPP post-treatment for the three different drug combinations when procarbazine or VC were the first drugs administered (PMV, PVM, VMP, VPM)but not when mechlorethamine was applied first (MPV, MVP). In all cases the fertilization frequency returned tc control levels by the eighth week of post-treatment. The mutagenic potential of MC was first studied in mammals by Ehling (1971) who found MC induced dominant lethal mutations in mouse. He demonstrated that spermatocytes are the most sensitive stage for the induction of dominant lethal mutations by MC. Neuhauser and Ehling (1973)reported that MC affects both spermatogonia and post-spermatogonial stages. According to Savkovic et al.(1977), MC inhibits mitotic division and mortality of germ cells but have no effect on meiotic stages. Rastogi and Levin (1987), on the other hand, reported that MC results in abnormal sperm

in mice which involved in the formation of banana shaped heads. Increase in the frequency of precocious desynapsis of XY-bivalent in mice upon exposure to MC has been reported by Chakrabarti et al.(1986). Dey et al. (1989) observed a differential effect of MC on precocious desynapsis of XY-bivalent in non-tumor and on S180 tumor bearing male mice. Doxorubicin, on the other hand, causes temporary loss in testicular weight which is regained after a considerable period (Hacker Klom et al. 1986). With treatment of Doxorubicin the loss of spermatogonia and the reduction of 4C cells (primary spermatocytes mainly at pachytene) is followed by a decrease of testicular weight observed corresponds primarily to the loss of spermatocytes and spermatids. Suppression of spermatogenesis and testicular atrophy have been observed in dogs and rabbits subjected to chronic doxorubicin treatment (Bertazzoli et al. 1972)

Two assays are generally carried out to evaluate the survival of stem cells after treatment with anticancer drugs (Wahed et al. 1987a). These assays comprised of the measurement of the levels of the enzymes, LDH-X and the number of sperm heads in testicular homogenates. LDH-X is the X-isozyme of lactate dehydrogenase which in mammals is found only in testis and spermatozoa (Wheat and Goldberg 1975).and LDH enzyme levels provide a finger print in the study of spermatogenesis (Bishop 1968). The study of Wahed et al.(1987a) with 5-FU,

methyl-CCNU, THIO and mitozolamide have shown that THIO and mitozolamide cause the most significant depression in sperm head count and the same result was seen with LDH-X levels. The sperm head count (Searle and Beechey 1974) and LDH-X levels (Erickson et al. 1975, Goldberg and Hawtrey 1970) provide good quantitative markers of the effects of anticancer drugs at germ cell level. Mitozolamide is a new anticancer drug with a broad spectrum activity against murine tumours. This drug, which probably causes cell death by DNA alkylation, is at present undergoing clinical trials. The observation of Wahed et al.(1987b) indicates that mitozolamide has similar effects on the testis to those seen with CP and THIO suggesting a similar mechanism of action. Long term effects have been determined for the approximate 150 chemicals studied with the mouse sperm morphology test, including the forty four agents that were shown to elevate the proportion of morphologically abnormal sperm within the first 5-6 weeks after treatment (Wyrobek et al. 1983a). The results of Meistrich et al.(1985) supported the previous observations showing that all germ cell mutagens tested to-date in mice induced elevated levels of morphologically abnormal sperm.

Pharmacological Aspects of Candidate Drugs

VINCRISTINE : The pharmacology of vincristine (VC) has been studied in a relatively small number of patients. The

disappearance of VC from blood is triphasic with half-lives of 2, 12.5 and 175 min. Over the first 24 hrs. about 10% of the drug is excreted in the urine and 33% in the faeces. Over the 72 hrs. the faecal excretion of VC is nearly 70%. The high rate of faecal excretion of VC is consistent with high biliary excretion and relatively low concentrations of VN excreted in the faeces suggests that the drugs differ in biliary excretion pattern. Following administration of VC, rats excreted magnesium and higher amounts of hydroxyproline in the urine (Araszkiewicz et al. 1987). Increased urinary excretion of Mg and hydroxyproline mav be interpreted as assign of osteolysis. The basis of the profound neuro-toxicity of VC is not certain, but it is presumed that VC retaintion may be substantially higher in nervous tissue. VC is the only commonly used drug which has dose limiting neurological toxicity. There is a peripheral neuropathy, the first sign of which is the loss of ankle jerks and depression of other tendon reflexes. An autonomic neuropathy may develop at the same time causing constipation and occasionally postural hypotension. Cranial nerve palsies have been described following VC therapy, as has recurrent laryngeal nerve palsy and diaphragmatic paralysis due to phrenic nerve damage. VC therapy may be associated with hyponatraemia due to the syndrom of inappropriate antidiuretic hormone (ADH) hypersecretion. Cessation of VC therapy leads to gradual improvement in all side effects. VN has fewer

and less severe neurological toxic effects than does VC, and its dose limiting toxicity is myelosuppression. Polyneuropathy, with sensory disturbances and motor nerve and muscle atrophy is associated with their use (Shelansky and Wisniewski 1969). Ileus and cranial nerve abnormalities also occur rarely (Sandler et al. 1969). These side effects are self-limited and usually respond to cessation of therapy, although residuals are sometimes seen. The vinca alkaloids may cause transient loss of hair, which also regrows during continued therapy. Patients treated with VC develop alopecia alightly more often than do patients who receive VN. VC is free of any significant bone marrow depression (Carey et al. 1963).

In the mouse both VN and VC kill differentiating spermatogonia but not the stem cells (Meistrich et al. 1982). Mauro and Madoc-Jones (1980) demonstrated that VC preferentially kills at late S or G_2. VC was also found to be mutagenic but VN was judged to be nonmutagenic in the in vivo dominant lethal assay (Goldstein 1984, 1987). Cook et al. (1978) observed that spermatogenesis was reinitiated later in the rat treated with VN (Russell et al. 1981).

CYCLOPHOSPHAMIDE : Treatment with CP has been associated w with oligospermia, azoospermia and increased levels of serum follicle stimulating hormone (FSH) in the body (Schilsky et al. 1980, Chapman et al. 1979). Depending on the cumulative dose

and the duration of treatment, spermatogenesis often recovers but this may take years (Buchanan *et al.* 1975, Roeser *et al.* 1978). Being an alkylating agent CP causes severe damage to proliferating cells. The drug was expected to affect the seminiferous epithelium and reduce the number of spermatozoa produced. The number of spermatozoa in the cauda of the epididymis provides a good estimate of spermatozoal reserves (Amann 1981). However, the proliferative phase of spermatogenesis in rat was found unaffected by CP (Trasler *et al.* 1985). There are several qualitative changes in the spermatozoa of the treated males that are not manifested by concomitant quantitative changes. The effects seen are probably not due to a cumulative effect of CP inseminal fluid as i. the drug has a short half life in rodents and man and is eliminated within hours of ending chronic treatment (Graul *et al.* 1967, Bagley *et al.* 1973) and ii. there was no cumulative increase in pre-implantation loss and external malformations with increasing time of paternal drug exposure (Trasler *et al.* 1985). CP has been shown to induce SCEs and chromosome aberrations in male germ cells (Allen & Latt 1976, Goetz *et al.*1980). In addition, clinical studies have indicated that CP can affect the human reproductive system (Schlisky *et al.* 1980). Different underlying mechanisms, both genetic and non-genetic could be postulated to explain the effects, based on

the fact that germ cells are first exposed to CP when they are undergoing different processes : maturation of the spermatozoa, cytoplasmic reorganisation and chromatin condensation (Trasler et al. 1985,1986). Being an immunosuppressive agent, CP alters fertility in humans (Fairly et al. 1972, Buchanan et al. 1975, Fukutani et al. 1981, Watson et al. 1985). CP showed no significant effect on the mean sperm count upto 250 mg per Kg exposure in Syrian hamster (Singh et al. 1987). However, spermatogenesis was significantly affected with time. A minimum dose of 10 mg per Kg body weight reduced the sperm production at week 12, whereas upto 250 mg per Kg showed no effect at one and 4 week. It appeared that the spermatogonium represented by week 12 rather than mature spermatozoa or differentiating spermatocytes was not affected in terms of sperm count by this agent (Singh et al.1987). The initial damage of CP to the epithelium was characterised by vacuolization of the sertoli cells. The primary spermatocytes showed the highest sensitivity to the drug. A high percentage of teratozoospermia was observed at all intervals (Vigil and Bustos-Obregon 1985). The effect of CP was compared with that of a CP-VN combination in rat by Auroux et al. (1986). Twenty days after the end of the treatment of CP-VN combination, a significant decrease in the number of various germ cell types was observed by them. Recovery seemed to have begun in the rats

sacrificed 75 days after treatment. The existence of smaller number of primary spermatocytes suggested a lesion of the differentiating spermatogonia and perhaps the stem spermatogonia. Evidently, such disruptions in the early stages of spermatogenesis are very crucial, because the alterations produced could be perpetuated, while the post-meiotic disturbances affect only a restricted number of sperm generations.

The effect of CP on testis is contradictory. There are reports both in favour (Fairly et al. 1972, Penso et al. 1974, Fukutani et al. 1981), and against (Penn 1979) of the view that CP affects germ cells. Combination chemotherapy with CP for acute leukemia, Hodgkin's disease and other malignancies may also impair Leydig cell function. The alkylating agents in the chemotherapeutic regimens seem to be responsible for the toxic effects on the Leydig cells (Griffin and Wilson 1987). Albanese (1987) reported that CP was effective in inducing heritable, structural chromosome aberrations in oocytes just prior to ovulation and was able to induce heritable chromosome aberrations at the other oocyte stages.

Using absolute weights of reproductive organs, Trasler (1987) found that there was no significant change with drug dose at any time in the weight of pituitaries and testes

in rat. Furthermore, there was no significant change in serum LH, FSH or testosterone concentrations. This would suggest that the pituitary-testicular axis was not markedly affected at any of the time points following the treatments with CP. CP caused no significant change in the weight of the epididymis, ventral prostate or seminal vesicles at low doses (Trasler 1987). However, with the high dose, absolute epididymal weight decreased after three and six weeks of treatment, but not after one or nine weeks . Absolute weights of the seminal vesicles, on the other hand, were only significantly decreased by the high dose of CP after three weeks of treatment. CP has transient effects on both the seminiferous epithelium and spermatozoa reserves in the epididymis of rat. The decrease in testicular sperm numbers could have been due to either a decreased production or an increase in fragility of condensed spermatids and/or spermatozoa. Decrease in sperm production generally correlates well with decrease in testicular weight (Robaire <u>et al</u>. 1979). But a similar trend was not found, so far as,CP treatment is considered. In a study of Trasler (1987), while CP caused upto 60% decrease in testicular sperm numbers, it did not significantly affect testicular weight.

Singh <u>et al</u>.(1987) demonstrated that CP failed to induce

sperm abnormalities in Syrian hamster. Testis weight and sperm count were slightly suppressed at week one and four before returning to normal at week twelve. The study indicated that CP in hamsters did not significantly affect the sperm production as previously reported in other animals. On the other hand, CP was found to induce sperm abnormalities in mice (Pamerantsevs and Ramaya 1980, Wyrobek and Bruce 1975). In the mouse, CP did not induce sperm abnormalities at week one and twelve (Wyrobek and Bruce 1975). However, a slight increase insperm abnormalities from 3% control to 7% in CP treated mice was reported at week 4. But the study on hamster by Singh et al. (1987)showed no effect of this compound on the sperm morphology of Syrian hamster, but spermatogonial population was considerably affected. According to them the sperm morphology assay was negative in establishing the mutagenecity of CP in the hamster germinal tissue within the dose and time tested .

The significant increase in the incidence of congenital malformations in the CP treated mouse specimens (Jenkinson et al. 1987, Knudson et al. 1977) suggested that CP can induce morphologically abnormal offspring in the litters of exposed males. Experimental studies have revealed that when CP is administered to the male rats, the impregnated may sustain a substantial decrease in the number of embryos

(Fritz et al. 1973, Botta et al. 1974, Cooke et al.1978, Adams et al. 1981, 1982) and Fabricant et al. (1983) reported that in the rat, paternal exposure to CP caused post-meiotic cell injuries and behavioural anomalies. Auroux and Dulioust (1985) had shown that CP affected the pre-meiotic phases of spermatogenesis and caused decreased learning capacity in the adult offspring of the treated males. The effects of paternal treatment with CP on progeny outcome of rat was assessed by pre-implantation loss, post-implantation loss and fetalaabnormalities (Trasler 1987). There is a significant dose related increase in pre-implantation loss after six weeks of paternal treatment of CP. Paternal CP treatment induced an increase in abnormal fetuses only after three weeks and significantly only with low dose. The types of malformations seen were principally hydrocephalus, edema and micrognathea. A dose response related increase in post-implantation loss was first seen as early as one week after the initiation of treatment with CP. These observations (Trasler 1987) demonstrated that CP in low daily doses increases post-implantation loss via an effect on spermatozoa during epididymal maturation and suggested that spermatozoa can be modified after entering the epididymis. The exact mechanism of post-implantation loss is not clear. That chromosome aberration and alterations in DNA can result in embryonic loss comes from recent mutagen induced

damage and transgenic experiments (Brewen et al. 1975, Covarrubias et al. 1985). According to Trasler (1987), the higher levels of post-implantation loss was found with the higher doses of CP might result from dose related degrees of DNA damage.

Acrolein, a metabolite of CP is expected to be responsible for teratogenicity of CP. Acrolein hasbeenreported to form adducts with the nucleophile glutathione (Alarcon and MeiOnhofer 1971). CP has beenreported to deplete hepatic glutathione synthesis *in vivo* (Gurtoo et al. 1981). The *in vivo* teratogenicity of acrolein was increased by inhibition of glutathione synthesis with buthionine sulfoximine and decreased by the addition of exogenous glutathione (Slott and Hales 1987a,1987b). Glutathione is present in the male reproductive tract (Li 1975, Grosshans and Calvin 1985, Teaf et al. 1985). How the concentration of this molecule is affected by doses of CP and its role in protecting the seminiferous epithelium from damage due to CP administration is not knwon. The results of NovOtona and Jelinek (1986) concluded that the teratogenicity of CP is associated with its mutagenic activity, provided the latter occurs with mitotic inhibition. Both the cytostatic effect and the large scale degeneration of cells bearing unstable aberrations apparently interfere with morphogenetic functions.

materials & methods

MATERIALS & METHODS

1. Experimental Animal : Random bred male Swiss albino mice, *Mus musculus* of about the same age group(3-mo-old) were housed in the animal house and maintained on standard mice feed and water for at least four weeks before the transplantation of tumor. Random bred mice were chosen because they resemble more closely to genetically heterogenous human population (Rice and O'Brien,1980). The other advantages of using mouse as experimental animal include : i) possess proliferating cells in testis with good yield of different divisional phases.ii)Presence of a satisfactory karyotype,iii) fecund and mature rapidly, iv) sensitive indicator of clastogenic, mutagenic and carcinogenic agents, v) able to convert various procarcinogens/promutagens into active forms,vi) able to withstand experimental stress.

2. Tumor Model : The ascites form of mouse sarcoma 180 (S180) cell line was procured from Tata Cancer Research Centre, Bombay through the courtesy of Chittaranjan Nat. Cancer Research Centre, Calcutta and maintained in the laboratory by weekly intra-peritoneal transplantation into random bred Swiss albino mice.

A close look of literature indicates that the cell line was developed primarily as a 'spontaneous' mammary carcinoma in male Swiss albino mice in the laboratory of Woglom in 1914 (cited by Gay 1965). The solid mammary carcinoma exhibited a rapid and exponential growth rate and metastasized lung and some other connective tissues of the host. Friend (1951) developed this tumor into ascites form by repeated inoculation of the minced sarcoma into intraperitoneal cavity of the mouse. The cell line is now maintained in different laboratories in solid as well as ascites form. The tumor has also been adapted *in vitro* in many laboratories, and maintained by serial *in vitro* passages for successive generations.

2.1. Choice of the Tumor Cell line : S180 tumor cell line was chosen for the following advantages : i) about 100% 'tumor take' in both random bred and inbred mice. ii) uniform transmissibility of the tumor karyptype for successive generations. iii) fixed doubling time as revealed by cell count analysis and BrdU labelling study. iv) tolerance of the host : longer survival with no visible adverse side effects.

The host can survive 17 to 27 days after tumor transplantation. Maximum frequency of dividing tumor cells was obtained on 3-4 day of transplantation with an inoculum size : 1×10^6 cells.

2.2. Transplantation of the Tumor and maintenance of the cell line :

Glass wares and equipments used for the transplantation were sterilised prior to transplantation. Ascites fluid was collected from the tumor-bearing host at log phase of tumor growth and diluted in sterilled saline (0.9%v/w). One part of tumor was suspended into 200 parts of normal saline solution (1:200). From this stock diluted fraction, dead cells were counted simultaneously in a hemocytometer using 0.5% trypan blue as indicator. S180 cells were transplanted to 3-mo-old mice with an inoculum size $1x10^6$ cells per individual .

2.3. Tumor regression analysis :

Tumor regression was analysed by :

i) morphometric study of the tumor volume.
ii)Cell count analysis.
iii) Dead cell frequency using trypan blue as indicator.

3. Chromosome preparation from Seminiferous Tubules :

Meiotic chromosomes were prepared from normal and S180 tumor bearing male mice following the technique of Oud et al.(1979) with little modifications (Chakrabarti et al. 1986). The procedure includes the following steps :

3.1. Collection of Seminiferous Tubules and Hypotonic Exposure :

The dissected testes were placed in 1% tri-sodium citrate solution at room temperature. Tunica albuginea was removed completely. Seminiferous tubules were separated with the help of a fine needle to enhance quick and perfect penetration of hypotonic solution in each tubule. The duration of hypotonic treatment was standardised by repeated trial, and 40 min.hypotonic exposure was found suitable (with a single change) for the present material.

3.2. Fixation : After hypotonic treatment, saline citrate solution was decanted off and replaced slowly by ethanol-acetic acid fixative (3:1 v/v). The material was kept in the fixative at room temperature for 15 min. The fixative was again replaced by fresh fixative and kept at 4°C for 30 min. For a better fixation one change of fixative was given 15 min after keeping at 4°C.

3.3. Dissolution of Seminiferous Tubules: Seminiferous tubules were placed in 30% acetic acid (v/v) for 5 min. followed by gentle flushing by a Pasteur pipette for complete dissolution of tubules. Undissolved materials were removed carefully,and only cloudy suspension was taken for further use, The suspension was centrifuged at

1500 rpm for 10 min; the supernatant was discarded and the deposit was fixed in freshly prepared chilled fixative. The fixative was added very slowly to avoid clumping of the pellet. The fixed material was resuspended, spinned at 1500 rpm and re-fixed afresh. The process was repeated and the material was left to stand at least one hr. at 4°C.

3.4. Spreading of meiotic stages on the slide :

Properly fixed meiocytes were aspirated until a cloudy suspension results. Three drops of cell suspensions were dropped on a clean (grease-free) chilled 50% alcohol-soaked slide and allowed to spread to its maximum extent. The slides were then either dried on a flame or kept in a slanting position for air dry, as the condition required for. The prepared slide was then either stored for further examination or stained immediately.

3.5. Staining : Conventional staining · the cells were stained in phosphate buffer (pH 6.8) Giemsa stain (Gurr, W.Germany) diluted in an appropriate ratio (1:10) for 60 min. The slides were then washed in distilled water to remove the excess stain. Stained slides were observed under 100x oil immersion lens of an Olympus microscope (Tokyo, Japan) using 10x eye pieces.

4. DRUGS :

4.1. Selection of Drugs : Two different drugs used extensively for a 'potential cure' of a variety of human cancer have been selected for the present study. These include : i) Cyclophosphamide (Endoxan) and ii) Vincristine (Oncovin). A brief review on these two drugs is presented :

Cyclophosphamide (CP) and Vincristine (VC) are among the most common anticancer chemotherapeutic drugs used to combat different types of human malignancies all over the world. CP, an alkylating agent is used either singly or in combination with other (physical or chemical) agents to enhance its therapeutic effectiveness, and VC, a spindle poison is used in combination with a variety of drugs. They are extensively used till to date and considered to be the most effective, palliative agents with a therapeutic index significantly higher than that of other agents for the treatment of certain types of cancer. In addition to rapid tumor regression property, easy availability and relatively low cost are major reasons of their extensive use in the developing countries. Among other factors behind the popularity of these drugs, patients' tolerance and compliance with the regimen are also notable. The precise way by which these two agents cause tumor regression is not properly understood, but it is well known that pathways of their action

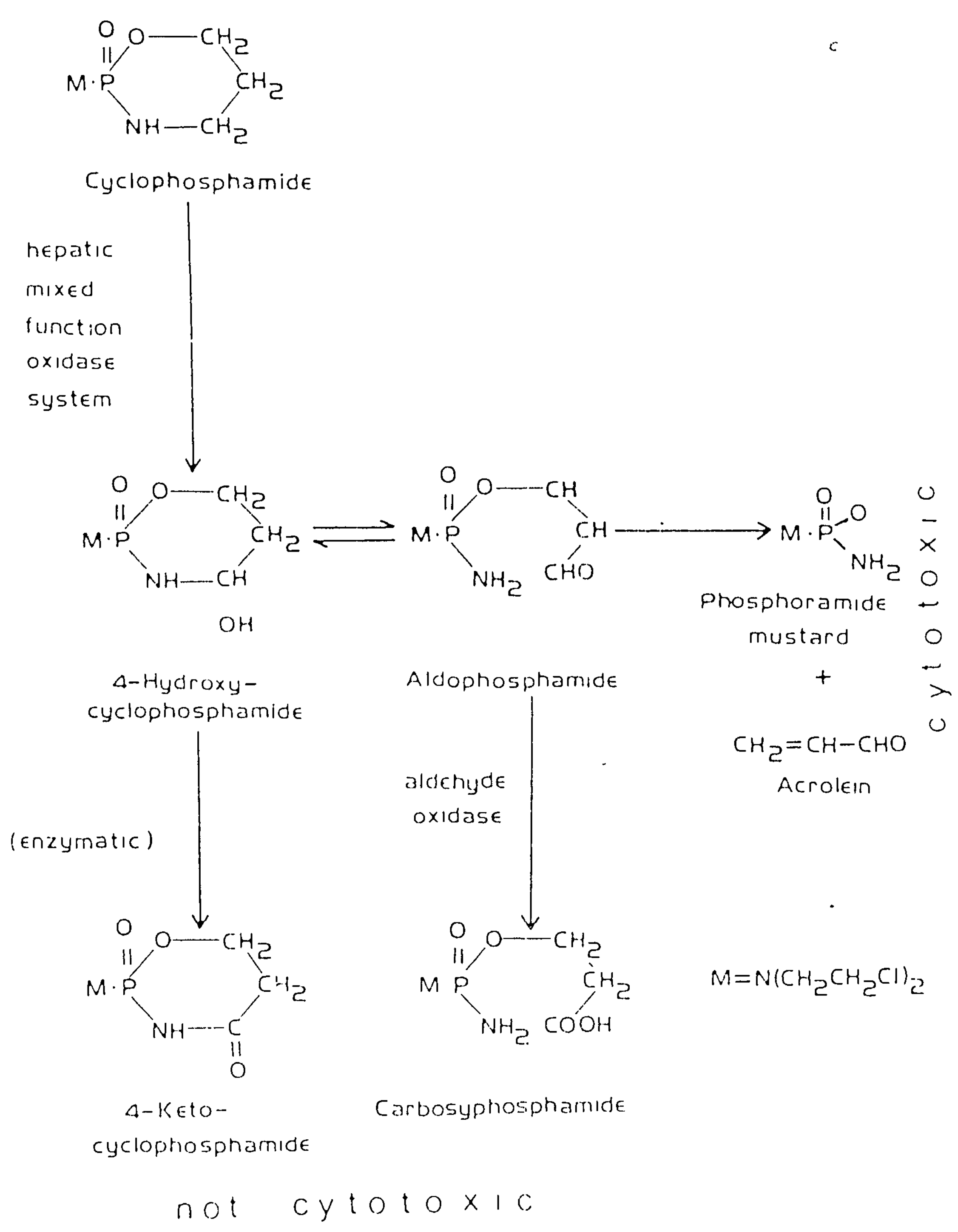
on cell include chromosome damage and division inhibition. Since these two antitumor agents are widely used in our country to curb the increased incidence of cancer death, their effect on meiotic chromosome complements and on certain other germ cells of a murine tumor bearing host has been attempted to assess their cytostatic and cytotoxic potentials at certain selected time points.

4.1.1. Cyclophosphamide (Endoxan) : CP is designated as a latently active form of nitrogen mustard type (Fig.2) which binds covalently to different cell constituents. Of several alkylating agents synthesized and tested for their antitumor activity, CP is now used extensively to a variety of human tumors. Its use as an immunosuppressive agent is also in practice (Friedman et al. 1979). Unmetabolised CP has no activity as an alkylating agent. The toxicity and clinical use of CP has been reviewed by Gershwin et al.(1974).

Fig. 2.

$Cl-CH_2-CH_2$ / $Cl-CH-CH$ — N–P=O — NH – CH / O — CH — CH H_2O

Endoxan


Cyclophosphamide
hepatic mixed function oxidase system
4-Hydroxy-cyclophosphamide
Aldophosphamide
Phosphoramide mustard
+
CH2=CH-CHO
Acrolein
cytotoxic
(enzymatic)
aldehyde oxidase
4-Keto-cyclophosphamide
Carbosyphosphamide
M=N(CH2CH2Cl)2
not cytotoxic

Under physiological condition, CP is positively charged or has high electron density. In spite of a wide variety of negatively charged molecules (nucleophiles) that exist in cells, DNA is the principal target site for CP action. Price (1975) reported that the alkylating agents formed covalent bonds with a number of nucleophilic groups. The nucleophilic groups of proteins and many other molecules can also serve as substrate for clinical attack by alkylating agents. Metabolism of CP occurs through a mixed function of oxigenase system primarily in the liver (Slodek 1971, Cooper and Goldstein 1976), and for this reason CP is apparently inactive in vitro unless activated through'S9'fraction (Natarajan et al. 1976). CP is activated in the liver to 4-hydroxycyclophosphamide (Struck et al. 1971, Colvin et al. 1973). This product, 4-hydroxycyclophosphamide is in equilibrium with its acyclic tautomeric form , aldophosphamide. 4-ketocyclophosphamide and carboxyphosphamide are produced by further enzymatic oxidation. Although these are the major metabolites of the drug, neither compound is significantly toxic either in vivo or in vitro (Struck et al. 1971)[Fig.2].

In addition, some phosphoramide mustard and acrolein are formed from aldophosphamide. There is evidence that phosphoramide mustard is the major cytotoxic principle whereas

acrolein is an irritant that accumulates in the urine and may contribute to the haemorhagic cystisis (Connors et al. 1974). Nornitrogen mustard (bis-/2-chloroethyl7 amine), one of the most abundant metabolites of the cyclophosphamide (Jardine et al. 1978 ,Friedman et al. 1982), is the most abundant product formed within a few hours after the administration of CP in man and experimental animals. It is formed non-enzymatically from many metabolites of CP, including the key alkylating agent (species), phosphoramide mustard. The cleavage of the phosphoramide bond in phosphoramide mustard is rapid. As the adducts are mainly formed at the 7-position of Guanine (Hemminki 1985, Kallama and Hemminki 1986), many secondary reactions such as depurination and imidazole ring opening are expected to ensure (Kallama and Hemminki 1986). Furthermore, the second mustard arm may undergo hydroxylation or cross-linking reactions. These secondary reactions may be important for cytotoxicity and therapeutic effectiveness of CP (Hemminki and Ludlam 1984). Goldin and Wood(1969) suggested that normal tissue detoxifies the circulating species enzymatically to ketocyclophosphamide and carboxyphosphamide. It has been proposed that liver may tend to escape exposure to high concentration of toxic products because of its high activity of aldehyde oxidase (Deitrich 1966), and enzyme that converts

aldophosphamide to the non-cytotoxic compound,carboxy-phosphamide (Cox et al. 1975). Certain forms of tumor becomes resistant to developing higher level of detoxifying enzymes. Classic alkylating agents attach the seven position of guanosine selectively, and this frequently accounts for 90% or more of the total base substitution. Although the 7 position of guanosine is by far the most reactive under most conditions, other sites may be of equal biological importance, viz., 1 position of guanosine, the 1,3 and 7 positions of adenosine and the 3 position of cytosine were also identified as reactive sites.

In addition to its anti-cancer activity, CP is also known to produce both primary and secondary tumor in man. These include : leukemias and urinary bladder carcinoma (Puri and Cambel 1977, Reiner et al. 1977, IARC Suppl. 1982).

4.1.2. **Vincristine (Oncovin)** : The vinca alkaloids played a major role in cancer chemotherapy since 1960s (Jackson and Bender 1978). Investigations on extracts from the periwinkle plant, Vinca rosea Linn. for the possible hypoglycemia activity, led to the isolation of the mitostatic agents : vinblastine and vincristine (Fig.3). They are large dimeric structures composed of an indole nucleus

(Catharanthine) linked to a dihydroindole nucleus (Vindoline). A synthetic derivative of vinblastine, Vindesine (desacetyl vinblastine carboxamide) was introduced into clinical trials in 1970s (Dyke et al. 1979). Recently, vinzolidine , an oral preparation and semisynthetic derivative of vinblastine has been investigated (Budman et al. 1984, Kreis et al. 1986).

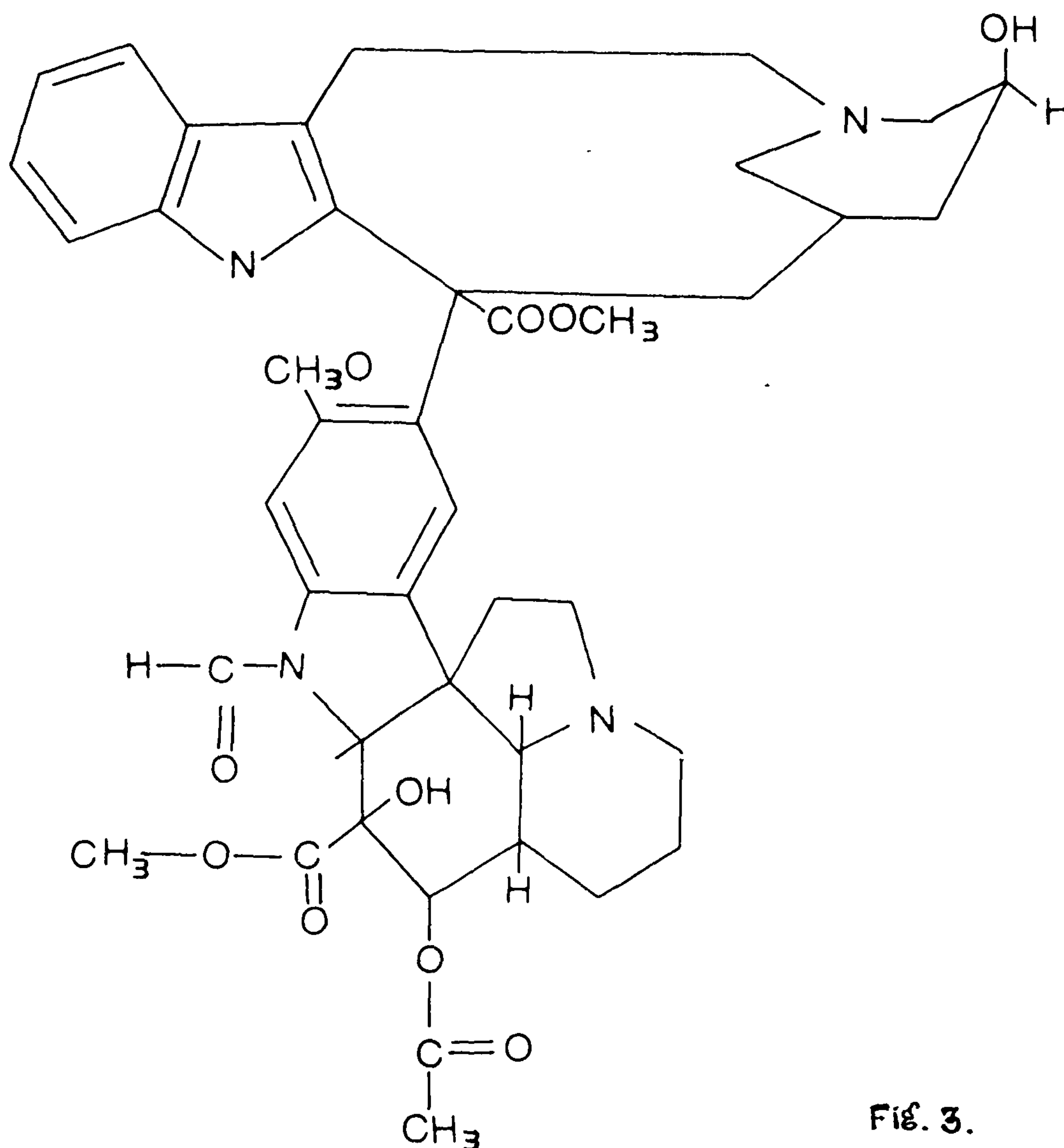
The anti-tumor effect of the vinca alkaloids are several. The major effect appears to be related to the high affinity binding of these agents to the basic protein subunit of microtubules, tubulin which results in disruption of the mitotic spindle apparatus and arrest of cells at metaphase (Jackson and Bender 1978). The neurotoxic effect of VC which is principal dose limiting factor is related to binding and disruption of microtubular structures within neural tissues. The comparative pharmacology of the intravenous bolus injection of the vinca alkaloids has been summerised by Nelson et al. (1980). Using radioimmunoassay, the blood decay was found to be triexponential for VC. The greatest terminal half life in the blood for that of VC was 85$\pm$68.9 hr. The apparent volumes of distribution were extremely high for each agent, indicating extensive tissue binding. There were marked differences in the serum clearance rates, with VC being the lowest

(0.106 ± 0.061 L/Kg/Hr). It has been postulated that the long terminal half-life and low plasma clearance might count for the greater neurotoxicity observed with VC (Nelson et al. 1980).

It is known that principal target for VC action in cell is spindle microtubules which are nothing but an array of tubulin organisation in definite fashion. Recently, Sammak et al. (1987) have presented elegant studies that suggest that spindle microtubules may be in a state of dynamic instability. The dynamic equilibrium depicted in equation is severely displaced by the alkaloids VC and VN. At very high concentrations VC binds to free tubulin molecules resulting in the precipitation of tubulin in the cytoplasm in the crystaline form (Dustin 1984). At low concentrations, these alkaloids inhibit polymerization of tubulin and ultimately prevent the formation of mitotic spindle and thus cell division.

Tubulin exists as a heterodimer comprising of alpha- and beta-forms. At higher concentrations VC may bind to other proteins. Tubulins may be tissue as well as species specific (Gozes and Barnstable 1982) and patterns of tubulin gene expression undergo changes during fetal and neonatal development(Bond et al. 1984, Cummings et al.1984). Evidence

accumulated so far indicates that the modification to either the subunit or the assembled microtubules may affect the action of chemical agents that disrupt microtubular function. According to Donoso *et al.* (1979), and Fellous *et al.* (1985), microtubule associated proteins may modify the action of vinca alkaloids.



Vincristine

Fig. 3.

4.2. Standardisation of therapeutic dose :

In the evaluation of therapeutically induced effect on tumor system, it is needed to define dose response first. The doses used in treatment of human tumor may not necessarily be the therapeutic dose for the murine tumor. Moreover, the therapeutic dose of a particular drug varies not only from one mouse strain/model to another but also on many other factors at the cellular level. These include: tumor type, cell killing, cell renewal, necrosis etc. The tolerance of the host is also an important factor in determining the therapeutic dose of a particular drug for a particular tumor.

4.2.1. Therapeutic dose for single CP therapy:

a. Preparation of stock solution : A stock solution of CP was prepared by dissolving 200 mg. powered cyclophosphamide (Khandelwal Laboratories Pvt.Ltd. under agreement with ASTA-worke A.G. Bielefeld, West Germany)in 10 ml sterilised distilled water just before its injection into tumor bearing host.

b. Therapeutic dose : CP was injected at different doses from a stock soln. on the 4th.day of tumor transplantation when the S180 tumor growth was at log phase (Chakrabarti and Chakrabarti 1978, 1987). In each set a total of 10 male mice of same age group (3-mo-old) were

exposed to single dose of CP and the same dose was repeated for five times for standardisation of the therapeutic dose. Six different doses, o.15ml, o.2o ml, o.25ml,o.3oml, o.35ml,o.40ml. per tumor were tested but not even a single dose was found in which a complete regression of S180 tumor was recorded. A maximum level of regression with least toxic effects was noted at a dose equivalent to 0.25ml per tumor ie. 167mg per Kg body weight. The hosts were (apparently) in good health with minimal visible toxic side effects, like loss of hair which is reversible and new growth of hair was observed while the treatment was in progress. The life span of the tumor bearing host also increased (8 days) upon exposure to single dose of CP. At other doses viz., 0.30ml, 0.35ml per tumor, a remarkable regression of the tumor was recorded but at the same time severe alopecia, oral ulceration, depression in leucocyte count, erythrocytes, platelet and severe lowering of Hb. level were documented. In addition, certain other toxic side effects and high rate of mortality were also encountered. Therefore, these doses were not considered and a therapeutic dose equivalent to 167mg per Kg body weight was chosen for CP single therapy for this particular tumor.

4.2.2. Therapeutic dose for single VC therapy:

a) preparation of stock solution : The stock solution

of VC was prepared by dissolving 1mg potency of VC sulphate (manufactured in India by Biochem Pharmaceuticals PVT Ltd., Bombay) in 10 ml of diluting fluid (benzyl alcohol + sodium chloride) just before injection into s180 tumor bearing mice.

b) Therapeutic dose : VC was injected with different doses from the stock solution on the 4th.day of tumor transplantation when the tumor growth was at log phase. In each set, a total of 10 mice of the same age group (3-mo-old) was used for a single dose, and the dose was repeated several times for standardisation of the therapeutic dose. Six different doses viz., 0.15ml,0.20ml,0.25ml,0.30ml,0.35ml,0.40ml, per tumor were selected initially and day to day observation on the rate and pattern of tumor regression was made by : i. cell count analysis, ii. dead cell frequency,and iii.morphometric study of the tumor size. However, not even a single dose was found suitable for single therapy, because of non-regression at significant level of the tumor. But a maximum regression (temporary) was noted at dose equivalent to 0.30ml per tumor (1mg/Kg body weight) with no apparent loss in general health or death of the tumor bearing host. However, at the other doses, more regression of the tumor was noted with severe weakness of the host with neurotoxicity. Hence this dose was applied in combination with CP for combination therapy which was effective for the present tumor model.

50

4.2.3. Therapeutic dose for Combination Drug Therapy:

Ascites form of sarcoma 180 was treated with a combination of drugs, consisting of VC and CP. The three principles for the use of drugs in combination chemotherapy, as documented by Comis & Carter (1974) was followed:

a. eachddrug was active against tumors, when used alone,

b. the drugs have different mechanisms of action and,

c. the toxic effects of the drugs are not the same so that each drug can be administered at or near to maximum tolerated dose.

In a number of sets,combination of VC followed by CP and CP followed by VC were given on 4th.day of tumor transplantation (log phase of S180 growth) at an interval of 24 hrs. In each set 10 specimens were injected with six different doses of VC/CP viz.,0.10ml, 0.15ml, 0.20ml, 0.25ml, 0.30ml, and 0.35ml per tumor, and the same dose was repeated for five times for standardisation of the therapeutic dose for combination therapy. Two different modes of combinations were tested :

a) Combination I : A maximum level of tumor regression was noted in combination - VC followed by Cp at dose equivalent to 1mg VC per Kg body weight followed by 66.7 mg CP per Kg body weight and it was confirmed by cell count analysis, dead cell frequency and morphometric study of the tumor volume.

in the treated and control series

Index	Materials injected	Route of injection	Dose (per Kg body wgt)	Tissue Fixed or Collected: Tumor regression study	Chr. study	Sperm head assay	Organ weight analysis	Post treatmen time points (in hrs.)
TREATED SERIES:								
i. CP single: therapy	CP	ip.	167mg	Ascites cells	Seminiferous tubules	Epididymis	entire testes	4,24,72,120, 168,288,312, and 336
ii. VC single:	VC	ip.	1mg	do	do	do	do	do
iii. Comb. I :	VC-CP	ip.	1mg VC + 66.66mg CP	do	do	do	do	do
iv. Comb. II :	Vc-CP-VC	ip.	1mg VC + 66.66mg CP + .33mg VC	do	do	do	do	do
CONTROL SERIES:	Sterile Distilled Water	ip.	0.25ml/0.10ml	do	do	do	do	do
	Diluting Fluid	ip.	0.3ml/0.10ml	do	do	do	do	do

b) <u>Combination II</u> : In a separate set, intermittent injection of VC followed by CP followed by VC (VC-CP-VC) at dose equivalent to 1mg VC followed by 66.7mg CP followed by 0.33mg VC per Kg body weight was also found equally effective with no apparent loss in general health or death of the host. At other doses and schedules of VC/CP, remarkable regression of tumor was found but at the same time severe alopecia, GI disturbances , apparent neurotoxicity with high rate of mortality were encountered. Therefore, these doses were not considered as therapeutic dose for the present tumor model.

5. Treatment and Experimental Protocols :

5.1. <u>Treated Series</u> : <u>CP Single</u> - In the CP treated series, each tumor bearing mouse received an ip.injection of freshly prepared CP stock solution @ 167mg oer Kg body weight on the 4th.day of tumor transplantation. The specimens subjected to therapy were sacrificed at two different sets of post-treatment intervals. In the first set,specimens were sacrificed at 4hr,24hr,72hr,120hr, and 168hr of *in vivo* drug exposures to evaluate the early effect (if any) of the drug therapy on germinal cells, and in the second set, drug exposed specimens were kept little longer and sacrificed on 12th., 13th. and 14th.day of therapy to study the relatively late effect of drug therapy at germ cell level (Table 3).

5.2. Treated Series : Combination I :- In the combination I treated series, each tumor bearing mouse received an ip. injection of freshly prepared VC stock soln. @ 1mg. VC per Kg body weight on the 4th.day of tumor transplantation. After an interval of 24hr. the VC treated specimens received another ip.injection of CP stocksoln. @ 66.7mg CP per Kg body weight. The specimens subjected to therapy were sacrificed at different post treatment time points similar to schedule followed in CP single therapy (Table 3).

5.3. Treated Series : Combination II :- In the other treated series, ie., combination II, each tumor bearing host received ip.intermittent injection of VC followed by CP followed by VC @ 1mg. per Kg + 66.7mg + 0.33mg per Kg respectively at an interval of 24 hrs. The initial treatment was started on 4th.day of tumor transplantation, and the treated specimens were sacrificed at the same post-treatment time points observed in other treated series (Table 3).

5.4. **CONTROL SERIES :** A suitable control was made side by side in either cases to evaluate the influence of the solvent(s) if any, on the host. In the control series, tumor bearing mice of identical age group (3-mo-old), maintained in similar laboratory condition were injected

ip. either with sterilised distilled water or with diluting fluid (benzyl alcohol + sodium chloride) in which the drug CP and VC were dissolved respectively. Specimens used as control against CP treated series, received ip. injection of sterilised distilled water only @ 0.25ml per individual on the 4th.day of tumor transplantation when the tumor was at log phase of growth (Table 3).

For control series of Combination I, specimens were injected ip. with diluting fluid followed by sterilised distilled water at dose equivalent to 0.30ml solvent followed by 0.10 ml distilled water (Table 3).

Specimens used as control against Combination II drug treatment series, received intermittent injection (ip) of diluting fluid followed by distilled water followed by diluting fluid @ 0.30ml solvent + 0.10ml distilled water + 0.10ml solvent at an interval of 24 hrs.(Table 3).

6. Collection of Tumor Cells and Metaphase Index Study:

The ascites fluid of S180 tumor from the control and the drug treated specimens was drawn by a syringe and a small drop of it was placed on a clean grease-free slide and smeared carefully. The smear was fixed in Carnoy's fixative for 1hr. The slide was then blotted dry and stained in PMG (Pyronin Y, E.Merck,Darmstad,and Methyl Green,BDH)

6.1. <u>Staining Procedure</u> : According to the technique of Kurnick (1955) as standardised in our laboratory :

i. Cells fixed in Carnoy's fluid were stained for 8 min. in methyl green-pyronin Y.

ii. The slide was then blotted dry.

iii. Immersed in n-butyl alcohol for 5 min. with two changes in each and then dried with the help of filter paper.

iv. Immersed in rectified xylene.

v. mounted in DPX.

In PMG stained smear preparations nuclear material stained greenish colour while cytoplasm took pinkish appearance. Metaphase can be easily detectable by the scattered appearance of condensed chromosomes. Frequency distribution of metaphase stages was recorded in control, CP, Comb.I and Comb.II treated series at different post-treatment intervals, upto'complete regression'of the tumor.

7. Determination of Fertility Potential of S180 tumor bearing mice ;

An evaluation of drugs responsible for damage of testicular cells would provide valuable information to the clinician. The use of drugs with no or lower toxicity to testicular cells may then be considered in the treatment of patients of reproductive age who have reasonable prognosis.

Since spermatogonial stem cell survival appears to provide a measure of the duration and permanence of chemotherapy induced sterility and oligospermia. Following testicular weights, sperm head counts were determined to find out the fertility status of S180 tumor bearing mice in response to combination chemotherapy and also CP single therapy.

7.1. Experimental Protocol :

7.1.1. Dissecting out of the testes and epididymes -

i. the animals were sacrificed by cervical dislocation.

ii. before dissecting out the testes and epididymes, site of incision was cleaned properly by rubbing the site by 90% alcohol.

iii. the region of the testes was opened by an incision with a sharp-edged scissor .

iv. both the testes and epididymes (left and right) including tunica albuginea were removed with the help of a pair of scissors and forceps.

7.1.2. Organ Weights :

Testes from tumor bearing mice of identical age group (3-mo-old) and maintained in the same laboratory condition were dissected out. Both testes with its tunica albuginea were weighed individually in single pan balance.

7.1.3. Method for Sperm head count :

Sperm head counts were made on the both epididymes following the method of Searle and Beechey (1974):

i. The vasa-efferentia between caput epididymis and testis were severed and fat surrounding the caput was carefully removed. It was then cut at its junction with the cauda epididymis and placed in a solid watch glass.

ii. Both epididymes were thoroughly macerated in 0.2ml of 1% soln. of tri-sodium citrate.

iii. The solution was made upto 2ml, mixed well and allowed to settle in a tube for about 1 min.

iv. a drop of suspension was allowed to run into each chamber of an improved Neubaur hemocytometer, after the coverslip had been pressed down to show Newton's rings.

v. Numbers of sperm heads were counted in the four large corner squares (each consisting of 16 smaller squares) and in the large central square. The contenets of two macerated epididymes were diluted with 2ml of hypotonic soln. and the number of sperm heads counted in a volume of $0.03mm^3$ of the diluted suspension. If N is the number of heads found, the expected number in 1 ml mouse seminal fluid is $3.75 \times 10^6 \times N$.

7.1.4. Analysis of Sperm head morphology :

A. Materials : i) 0.85% saline solution, ii) 0.1% glucose solution, iii) fixative (ethanol + ethyl eather 1:1 v/v), iv) Staining solution : 2.5g of aneline blue was mixed in a morter with a little volume of distilled water. The residue of undissolved stain was allowed to settle and the flüid was decanted into a cleaned and dried conical flask. The residue in the morter was treated with additional distilled water and the process was repeated untill all the aneline blue is dissolved into water. 2.5g of powered eosin Y was then added to the 100 ml soln. of aneline blue and mixed well with the help of a pipette. The stain was filtered and 0.1ml phenol was added to it. The pH of the stain was adjusted to 6.8.

B. Method : i) Dissected epididymes were kept in 5cc mixture of equal volume of 0.85% saline and 0.1% glucose solution. (this mixture prevents acrosomal swelling of the spermatozoa). ii) Epididymes were teased and the content was collected into saline-glucose mixture. The mixture was stirred and all debris were removed. iii) After mixing the suspension with the help of a pipette, 3-4 drops of suspension was placed in one end of a properly cleaned grese-free slide. The narrow end of another side ie., spreading was placed at an angle of 45° on the

suspension by the right hand and then moved back slowly to make contact with the suspension. The suspension was spread out quickly along the line of contact of the spreader with the slide. The moment this occurs, the film of suspension was drawn by a rapid, smooth and forward movenent of the spreader. iv) the film was dried in an incubator/hot plate at 50°C for 2 min. v) after this the material was fixed by keeping dried slides in a mixture of equal parts of ethyl ether and absolute ethanol for 3-4 min.

C. Staining : i) after fixation of the material, the slide was dried in an incubator/hot plate at 50°C. ii) during drving, a liberal quantity of staining solution containing aneline blue and eosin Y (pH = 6.8) was poured on to the slide and allowed to stain for 8 min. iii) the stain was washed properly in running water and blotted dry with a Whiteman's filter paper. iv) the stained slides after drying were placed into rectified xylene and again blotted dry and finally mounted under a clean cover glass (22x50mm). in DPX.

The following regions of the spermatozoa were recognisable by the differential staining behaviour under microscope: i. Acrosome cap - blue, ii. Nucleus - carmine, iii. Neck - unstained, iv. Mid-piece - blue, and tail - light blue.

7.1.5. Criteria for Sperm head analysis :

For scoring sperm head abnormalities, the criteria of Meistrich et al. (1985) has been followed in the present study with some additions. Abnormally shaped sperms were distinguishable according to the following categories :

i) sperm with small, eosin dense, round or eliptical bodies at the location of the sperm nucleus, termed 'microheaded' .

ii) Sperm with deviations in the shape of the nucleus termed, 'sperm with deformed head '.

iii) sperm without hook termed 'hookless'.

iv) others :- macrohead, double headed etc.

8. Criteria for scoring aberrations :

After consulting the literatures published by different investigators in this field (Moutcnen 1969, Pathak and Hsu 1977, Hunt 1987, Daniel and Roane 1987, Yuan and Mailhes 1987) the following aberration types were considered in the present study :

Structural abnormalities:

Chromatid breaks - Any form of discontinuity of a part of either of the two chromatids of a chromosome. The broken acentric part may remain in close association with the

parent centric part or may be far apart from its origin.
Fragments of unidentified origin : when the parent body of the broken fragment was not identifiable.
Exchanges : Characterised by the exchange of chromosome segments within or between two or more chromosome bivalent(s): according to the involvement of the number of bivalents in such translocations these are of two different types: a) Translocation trivalent - when one bivalent and one univalent are involved in the process of exchange, i)autosomal trivalent - involvement of autosomes only, ii) sex trivalent - involvement of both autosomal univalent and sex bivalent.
b) Translocation multivalent : when two or more than two bivalents were involved in the exchange process. Translocation multivalents may occur inbetween autosomes (autosomal multivalents) or inbetween autosomes and sex chromosomes (sex multivalents).

Ploidal anomalies :

Two types of ploidal anomalies were considered.
i) Polyploidy - mostly doubling up (tetraploid) of chromosome complements.
ii) Aneuploidy - any deviation from euploid condition at diakinesis-metaphase I stage. This may be of a) hypoploidy (loss of one or more elements) and b) hyperploidy (addition

of one or more bivalents to the complete set)

Pairing anomalies :

Any special behaviour of chromosomes remaining as univalents during diakinesis-metaphase I stage of meiosis. Depending on the involvement of chromosome or chromosomes univalency may be of two types : i) autosomal and ii) sex chromosomal.

9. Statistical Calculation :

The statistical calculation, *viz.*, standard deviation and critical difference (CD) were done following the method described by Panse and Sukhatme (1978). The comparison of means (value) and their standard in between control and treatment schedules at different therapeutic hours was carried out by Student't' test following Bailey (1959).

observations

OBSERVATIONS

1. Tumor regression & Evaluation of Mean Survival Time :

A maximum degree of tumor regression was noted at 120hrs. of post therapeutic single dose CP exposure. The regression time was reduced to a considerable extent in this tumor model when CP was applied in two different combinations with VC. An examination of treated series(Fig.4a,b) revealed that application of Combination I and Combination II caused a maximum degree of tumor regression at 72hrs. of post therapeutic drug exposure. In no cases, however, a complete regression of tumor was documented (Fig.4a,b).

An evaluation of mean survival time (MST) of specimens subjected to single and combination therapy indicated that the application of drugs increased the mean survival time (MST) to a variable extent. A maximum increase in MST was recorded in Combination II treated series

Table 4 : Mean Survival Time (MST) of S180 Tumor bearing Mice subjected to Single and Combination Chemotherapy

Index	No.of Mice*	MST in Day**	Survivors on day 45 / Total Mice Treated
CONTROL : (Dist.Water and Dill.Fluid)	50	22 (17-27)	0/50
TREATED :			
1. CP single :	50	30 (20-40)	0/50
2. VC single :	50	24 (18-30)	0/50
3. Comb. I :	50	36 (24-48)	3/50
4. Comb. II :	50	40 (23-57)	9/50

* each specimen received ip.injection of drug/distilled water on 4th.day of transplantation of tumor;

** figures in parentheses indicate range of survival.

while application of Combination I also extended the life span of tumor bearing specimens to a little extent (Table.4).

2. Meiotic Complements of Normal Male Mouse :

2a. Spermatogonial metaphase -.Spermatogonial metaphase consists of 40 telocentric chromosomes of decreasing length; 19 are autosomal and one (XY) is sex chromosome pair (Plate 1). Spermatogonial chromosomes generally appear as heavily condensed and extremely fragile bodies. Sometimes the chromatids show a spiral structure and the splitting between two chromatids in some cases is not very distinct. The sex chromosomes demonstrate no heteropycnosis in spermatogonial metaphases.

2b. Primary Spermatocyte - The primary spermatocyte nucleus at interphase is a small, deeply stained body with a few relatively large chromocenters (Plate 2). Due to uneven spiralisation of chromatin threads, the nucleus sometimes exhibits a net like appearance. Both the First and the Second meiotic divisions are sub-divided into: Prophase, metaphase, anaphase and telophase. The following stages of First meiotic division were more or less clearly identifiable in the present study :-

Leptotene - Nucleus is small and round with homogenously dispersed chromatin with 'chromomeres' at irregular intervals along their length. No well defined sex chromatin body is visible at this stage (Plate 3a).

Zygotene - Identification of this stage is highly speculative and at the same time difficult to define clearly under the resolution of light microscope. At this stage, homologous chromosomes become aligned and undergo pairing or synapsis. The telomeres of the chromosomes are frequently attached to the nuclear envelope. Pairing is seemed to be point for point and chromomere for chromomere in each homologue. During this stage the X and the Y chromosomes are indistinguishable from the autosomes (Plate 3b).

Pachytene - The pairing of the homologous chromosomes is complete. Early pachytene cells are small with dense chromatin and occasionally show the beginning of the formation of heteropycnotic 'sex-vesicle' (Plate 3c). Mid-pachytene cells are much larger and show a prominent sex vesicle against a pale background of autosomal elements. Cells at late pachytene may easily be confused with those at mid-pachytene and early diplotene. Sex vesicles appear as one or two deeply stained spots presumably the centromeric areas of the sex chromosomes (Plate 3d).

Diplotene - Early diplotene nucleus is rather large and pale stained body. The paired chromosomes show various degree of separation but remain united at certain points by

chiasmata. The number, shape and morphology of bivalents are variable depending on the number and location of chiasmata. At least one chiasma is visible in each bivalent but many bivalents have more thanoone chiasma (Plate 3e,3f).

Diakinesis - The contraction of the chromosomes becomes accentuated. The bivalents are more evenly distributed in the nucleus. During this period, the number of chiasmata diminishes. By the end of diakinesis, in general, the homologues are held together only at their ends due to extreme terminalisation of chiasma. The long X chromosome becomes progressively straight and even in thickness. The Y chromosome becomes progressively shorter. The free end of the longer X chromosome of the XY pair stains differentially. The XY pair at diakinesis apparently shows an end to end association/pairing. In fact there is no clear cut distinction between late diakinesis and metaphase I under the resolution of light microscope.

Diakinesis/metaphase-I - This stage revealed the existence of 20 clearly differentiated bivalents. Of these, 19 are autosomal and 1 sex bivalent. The autosomal bivalents generally exhibit ring or quadriradial configurations (Plate 3g). The size and the shape of ring or quadriradials vary according to the degree of condensation and advancement of cell division stage. The autosomal bivalents are of

66

various size depending on the length of the individual chromosome pair. The XY pair exhibits a rod-like appearance indicating terminal nature of pairing/association between them. In majority of cases, XY bivalent occupies a peripheral position in the diakinesis/metaphase-I stage (Plate 3h). In some cases (about 2.4%) the X and the Y appear as two separate univalents of unequal size without association.

Metaphase II - Second spermatocytes are rather remarkable by the haploid number and by their characteristic spiralised chromatids in addition to the absence of chiasma. Each metaphase II cell displays a total of 20 chromosomes ie., 40 chromatids.

3. Normal Spermatogenesis :

In mouse, the entire spermatogenesis (Fig.5) consists of twelve stages of characteristic cellular association. The least mature germ cell that enters the spermatogenic cycle (Type A spermatogonium) is located closest to the basement membrane. Usually, spermatogonia become progressively smaller in course of their divisions. The most prominent changes in germ cell during the spermatogonial phase occur in the nucleus. In the primary spermatogonia (in fixed preparation) the chromatin of interkinetic nuclei is finely distributed. With each succeeding

generation, the chromatin becomes more condensed in fewer and larger flakes,called crusty.

Type A spermatogonia - Type A spermatogonia appear as large pale staining nuclei with little evidence of chromatin condensation. Intermediate spermatogonia show the development of irregular chromatin masses in an otherwise pale staining nucleus.

Type B spermatogonia - exhibit increasing degree of chromatin condensation, smaller nuclei with many heteropycnotic masses.

Spermatid - Very early spermatids possess spherical nuclei and partially condensed chromatin, representing the complete haploid chromosome set. As the spermatid chromatin is condensed further, the nuclei begin to undergo conspicuous changes in shape. With further condensation of chromatin and the appearance of the characteristic hook-shaped sperm head, the brightly stained chromocentre of late spermatid nuclei disappears and the mature sperm head appears. By day 21, the first mature sperm appears.

Spermatozoa - The mature spermatozoon consists of two main parts : head and tail. The tail is further subdivided into i. the neck, ii. the middle piece , iii. the principal piece and iv. the end piece.

The head is composed mainly of the nucleus made up of condensed chromatin and acrosomal cap. The form of the

head is largely determined by the shape of the condensed nuclear material occupying the greater part of the head. In addition, the so called head cavities are seen. The acrosomal cap covers the anterior four-fifth parts of the head. The neck is a short segment which connects the head with the middle piece (body). The proximal centriole (anterior knob) fits in the depression of the head and is the junction of the head and neck whereas distal centriole (posterior knob) lies between the middle piece or body.

The middle piece extends from the slender connecting piece of the neck to a ring-like structure called, the annulus. The principal piece gradually tapers towards the end-piece which is characterised by the development of a fibrous sheath.

Distal to fibrous sheath of the principal piece is the end piece. The axial filament continues to its caudal extremity being surrounded by a very thin layer of cytoplasm external to which is the cell membrane. End piece of the tail consists of the terminal portion of the axial filament.

4. **Studies on Non-tumor Control :**

The cytogenetic features of normal (untreated) Swiss albino mice have been observed from specimens of 3-mo-old age group. 25 different randomly selected specimens were used for the purpose. An examination of 25 spermatocytic diak-meta-I cells from each specimen revealed no remarkable shift from normal chromosome profile. However, the presence of hypoploidy, hyperploidy, single autosomal univalency , and sex chromosome univalency was noted.But in no cases, translocations (either X-autosome or autosome-autosome) and univalency involving more than one autosomes were encountered in non-tumor control specimens. The frequency of cells with ploidal variations was also not observed to a significant extent.

5. **Studies on S180 Tumor-bearing Control :**

Chronological observation of germ cells on 25 s180 tumor bearing specimens on and from the 4th.day of tumor transplantation revealed considerable deviation from normal cytogenetic profile of spermatocyte so far as the behaviour of autosomal and sex bivalents are taken into consideration. Significant deviation from normal meiotic behaviour was noted in the form of precocious desynapsis

of both autosomal and sex chromosomal bivalents at diak-meta-I level at certain post-transplantation time points ie., at 4,24,and 72 hrs (Table.5). In most of the cases, the desynapsed elements are dispersed far from each other (Plate 4a,b), while in others they are separated but remained closely apposed (Plate 4c,d). An examination of data presented in Table 5 revealed that both sex chromosomal and single autosomal univalency resulted in a higher frequency in S180 tumor bearing mice compared to non-tumor control. A close look of the data indicated that significant increase in single autosomal univalency was recorded from the very beginning ie., soon after tumor transplantation, which continued upto the end of the experiment ie., 14 day of tumor transplantation. A different trend in the precocious desynapsis of sex chromosomal bivalent was recorded in tumor bearing control, while the occurrence of single autosomal univalency started from the beginning of the experiment and maintained a steady peak upto the end. The frequency of sex chromosomal univalency decreased gradually with the increase in transplantated tumor age (Table 5).

Besides the above abnormalities, small fragments of unknown origin was also noted, but in no case to a significant level. In some cells, the X chromosome appeared as highly despiralised, pale stained long thin thread with darkly stained Y attached

Table. 5 : Reference value of Numerical Chromosomal Anomalies of Normal and , S180 Tumor bearing Mice (Served as Control) [Values in Mean ± SD]

Types of Chromosome	Normal Mice	S180 Tumor bearing Mice							
		Post-transplantation Time Points							
		4hr	24hr	72hr	120hr	168hr	288hr	312hr	336hr
Autosomal Univalency									
One Autosome Pair	: 1.87±0.21	9.00±0.73[c]	10.00±0.73[c]	15.00±.40[c]	12.00±0.61[c]	13.00±0.98[c]	12.12±0.96[c]	13.00±0.83[c]	8.12±0.88[c]
>One Autosome Pair	: 0.00±0.00	1.25±0.47[a]	1.87±0.42[b]	2.00±.47[b]	2.00±0.35[b]	1.75±0.59[a]	1.37±0.37[a]	0.87±0.12[c]	0.75±0.25[a]
X/Y Univalency	: 2.39±0.16	8.67±0.51[c]	5.00±0.73[b]	5.87±0.24[c]	4.00±0.71	3.50±0.41[a]	2.75±0.48	1.75±0.58	2.00±0.41

[a] = $p < 0.05$, [b] = $p < 0.01$, [c] = $p < 0.001$ [p values analysed by 't' test]

at the end (Plate 5).

6. Negative Controls :

Negative controls were maintained side by side by exposing S180 tumor bearing specimens (3-mo-old) only to the drug solvents viz., either distilled water (solvent used in CP therapy) or with diluting fluid (Benzyl alcohol + Sodium chloride, used as solvent for VC) and germ cell stages were fixed at appropriate post injection intervals for evaluation of cytotoxicity, if any (Table 3). Since, no significant difference in terms of observed anomalies between S180 control and negative controls were noted, data obtained in S180 control has been presented and pooled for comparison with different treated series.

7. Treated Series :

7.1. Single CP Therapy :- An examination of spermatocytes on and from 4th.day of tumor transplantation revealed that single exposure of CP at therapeutic dose resulted in various forms of chromosomal alterations at different post-treatment intervals.

7.1.i. Ploidal anomalies - Ploidal anomalies in response to CP exposure were recorded mostly in the formof hypoploidy and hyperploidy at diakinesis-meta-I stage (Plate 6a,b).

Hypoploid count varied from 16 to 19. The most common count was 18, and hyperploidy varied from 21 to 22 bivalents, of which the common count was 21. It was indeed difficult to detect which particular bivalent (or group of bivalents) was added or lost. However, a rough estimation indicated that loss/addition was confined to smaller group of bivalents. Sex bivalents were rarely lost.

Quantitative analysis of CP treated series revealed that administration of CP at therapeutic dose resulted a significant increase in aneuploid (hypoploidal) spermatocytes. This was exhibited by an increase in the frequency of aneuploidy on and from 4 hrs of drug exposure (Table 6). An increase in cells with aneuploid chromosome constitution was more pronounced at 24 hrs. of drug exposure. The frequency gradually dropped down at subsequent hrs. of treatment. The frequency of cells with polyploid chromosome constitution, on the other hand, was maximum at 72 hrs. of *in vivo* drug exposure (Fig. 6).

7.1.ii. Structural anomalies - Sex multivalents were recorded during the course of CP single therapy (Fig.7, Table 7) where larger autosomal bivalent was attached to sex bivalent (Plate 7). It was rather difficult to identify which particular autosomal bivalent was involved in the formation of sex multivalent. Autosomal multivalents

Table. 6 : Ploidal Counts Recorded at Different Time Points in Control & Treated Series

INDEX	HOURS OF *in vivo* DRUG / SOLVENT EXPOSURE																$C.D. at P_{0.05}$	
	4		24		72		120		168		288		312		336			
	aneu	poly	aneu	poly	aneu	poly	aneu	poly	aneu	poly	aneu	poly	aneu	poly	aneu	poly	aneu	poly
Control	1.37	3.50	1.50	3.75	1.62	3.75	1.37	3.50	1.37	3.62	1.50	3.25	1.62	3.62	1.87	3.62	0.30	0.28
CP Single	4.37	2.50	8.50	3.50	5.62	3.87	2.25	3.12	1.37	2.87	1.75	2.62	1.37	3.12	1.62	3.62	0.36	0.34
Comb. I	4.87	2.12	8.00	8.87	6.87	6.50	3.12	5.75	1.50	3.25	1.75	3.00	2.00	3.00	1.50	3.00	0.30	0.43
Comb. II	8.00	3.50	7.12	8.87	2.87	9.87	2.00	10.62	2.25	9.62	1.75	6.75	1.37	3.25	1.50	3.37	0.32	0.40
$C.D. at P_{0.05}$	0.40	0.28	0.38	0.41	0.21	0.33	0.42	0.30	0.33	0.39	0.27	0.46	0.35	0.44	0.33	0.40		

Normal O Mice : aneu = 1.53 ± 0.07 ; poly = 3.58 ± 0.07

Table. 7 : Frequency Distribution of **X-autosome Translocations** in Spermatocytes of S180 tumor bearing Mice Subjected to Single & Combination Therapy

Treatment Series	Hours of *in vivo* drug exposure 4	24	72	120	168	288	312	336
CP Single	0.50	0.25	0.37	0.25	0.25	0.50	0.37	5.75*
Comb. I	0.12	0.50	0.50	0.50	0.25	0.50	1.50*	1.37*
Comb. II	0.50	0.50	0.25	0.50	0.37	0.25	1.25*	1.12*
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

* P values analysed by 't' test $p < 0.01$

Table 8 : Frequency Distribution of Autosomal Translocations in Spermatocytic Cells of Control & Treated Specimens

INDEX	HOURS OF *IN VIVO* DRUG/SOLVENT EXPOSURE							
	4	24	72	120	168	288	312	336
CONTROL	0	0	0	0	0	0	0	0
CP Single	0.50	0.25	0.00	10.62*	12.75*	14.62*	15.75*	16.37*
COMB. I	0.00	0.50	0.50	0.25	0.37	2.25*	5.62*	2.25*
COMB. II	0.37	0.00	0.50	0.00	0.50	14.37*	9.75*	8.62*

* $P < 0.001$ (analysed by 't' test)

mostly involving larger autosomes were also documented in CP single therapy series (Fig.8, Table 8). Theye are of two different categories ; multivalents formed by two bivalents or multivalents formed by more than two bivalents (Plate 8a,b).

7.1.iii. Pairing anomalies - Pairing anomalies were recorded in the form of univalents at diak-meta.I. Both autosomes and sex chromosomes had shown the precocious desynapsis which were visualised in the form of univalents (Plate 9a). An examination of the autosomal univalency revealed that more than one autosomes may also be involved in the formation of univalents (Plate 9b). In all the cases the smaller group of autosomal bivalents were mostly involved in univalent formation.

An examination of data of CP treated series (Table 9) indicated that administration of CP caused a drastic increase in the frequency of single autosomal univalency from the very beginning of the experiment ie., from 4hrs.which continued upto 168 hrs. when it reached the maximum level. After this period an abrupt decrease in the frequency was recorded. The effect of CP on autosomes invloving more than one resulting in univalent formation was found maximum at 168 hrs. of in vivo drug exposure(Fig 9).

Data obtained from(Table 10) CP treated series revealed

Table. 9 : Frequency Distribution of Autosomal Univalency at different Time Points in Control & Treated Series.

INDEX	HOURS OF *IN VIVO* DRUG / SOLVENT EXPOSURE																C.D. at $P_{0.05}$	
	4		24		72		120		168		288		312		336			
	one auto	>one auto	one auto	>one auto	one auto	>one auto	one auto	>one auto	one auto	>one auto	one auto	>one auto	one auto	>one auto	one auto	>one auto	one auto	>one auto
CONTROL	9.00	1.25	10.00	1.87	15.00	2.00	12.00	2.00	13.00	1.75	12.12	1.37	9.75	0.87	8.12	0.75	1.10	0.61
CP Single	16.00	1.87	18.00	1.62	27.00	2.00	18.87	2.12	24.87	2 62	12.25	1.25	9.12	0.62	7.75	0.62	1.33	0.63
COMB. I	13.25	2.00	13.75	2.12	15.25	1.62	16.25	2.87	14.50	0.87	12.62	1.37	14.87	2.25	16.12	3.00	1.25	1.01
COMB. II	14.12	1.87	15.75	3.12	16.37	3.12	15.00	2.37	14.00	2.25	13.25	2.12	15.25	2.37	16.25	3.25	1.23	1.14
C.D. at $P_{0.05}$	1.87	0.96	1.66	0.80	1.15	1.12	0.87	0.92	1.44	0.78	1.27	0.62	1.17	0.85	0.91	1.47		

Normal O Mice : one auto. = 1.87±0.21;
>one auto. = 0±0.

Table. 10 : Frequency distribution of Cells with **X-Y Univalency** at different Time-Points in **Control & Treated** Series.

INDEX	HOURS OF *IN VIVO* DRUG / SOLVENT EXPOSURE								**C.D. at $P_{0.05}$**
	4	24	72	120	168	288	312	336	
CONTROL	8.87	6.00	5.87	4.00	3.50	2.75	1.75	2.00	0.73
CP **Single**	8.00	16.00	7.00	2.62	2.00	2.25	2.00	2.50	1.01
COMB. I	10.00	14.50	6.00	4.12	3.00	2.50	2.00	1.75	0.84
COMB. II	10.37	7.00	6.00	5.37	4.87	3.25	1.75	2.25	0.65
C.D. at $P_{0.05}$	0.82	1.39	0.83	0.49	0.78	0.29	1.01	0.82	

X-Y univalency in **Normal male Mice** : 2.39 ± 0.16

that there is a significant alteration in sex chromosome univalency at early hours of therapeutic exposure. The maximum frequency (about 16%) of XY univalency was recorded at 24 hrs of drug administration which abruptly decreased at the subsequent post-treatment intervals (Fig.10).

7.2. Combination I Therapy :- Combination I drug schedule, ie., combination of VC followed by CP at therapeutic dose exerts various forms of alterations in germ cell cytogenetics of S180 tumor bearing mice.

7.2.1. Ploidal anomalies - An examination of spermatocyte cells of tumor bearing mice subjected to VC treatment followed by CP revealed the ploidal anomaly mostly in the form of aneuploidy and polyploidy at diak-meta.I stage of meiosis (Plate 10). Among aneuploidy, hypoploid count varied from 16 to 19 and hyperploid count varied from 21 to 23. The most common count of hypoploidy and hyperploidy were 18 and 21 respectively. The precise detection of the particular bivalent/bivalents involved in the process of hypoploidy/hyperploidy was rather difficult, but an arbitrary estimation indicated that loss/addition of the bivalents was non-random in the sense that it was confined to smaller group of autosomal bivalents. No loss/addition of the sex bivalents was observed in Combination I treated series.

Quantitatively, there was a sharp increase in aneuploid (hypoploid) population from the very beginning of the treatment ie., from 4hrs. of drug exposure. A maximum increase in aneuploid populations of S180 tumor bearing mouse subjected to Comb.I was observed at 24hrs. At 72hrs of treatment, a slight decrease in the frequency was noted followed by a drastic fall at the subsequent intervals upto the end of the experiment (Fig.6). On the other hand, a remarkable increase in polyploidy was documented at 24 hrs. of drug exposure. The frequency gradually dropped down at subsequent hrs. (Table 6) .

7.2.ii. Structural anomalies - Qualitative analysis of Comb. I treated series revealed the existence of sex multivalent where a member of the large autosomal bivalent group was involved in translocation with the XY bivalent. It was rather difficult to detect which particular autosomal bivalent was translocated,

This is evident from Table 7 that Comb.I produced maximum X-autosome translocations at 312 hrs. of drug administration (Fig 7). This is evident from the observation that comb.I also induced autosomal multivalents and larger autosomal bivalents were apparently more prone to the multivalent formation. Data obtained from Comb.I treated series (Table 8) revealed a maximum frequency of autosomal multivalents at 312hrs of drug administration (Fig. 8).

7.2.iii. Pairing anomalies :- Meiocytes of Comb.I treated series displayed univalency in relation to autosomal and sex chromosomal bivalents. One or more than one autosomal bivalents belonging to the smaller chromosome group had exhibited such pairing anomalies (Plate 11).

Besides these abnormalities and structural alterations, certain other forms of aberrations like selective chromosome despiralisation involving either X chromosome or autosomes have also been documented in different treated series (Plate 12a,b).

7.3. Combination II Therapy :- Intermittent dose schedule ie., VC followed by CP followed by VC (VC-CP-VC) caused a number of changes in germ cells of S180 tumor bearing specimens.

7.3.i. Ploidal anomalies - Ploidal anomalies in response to intermittent drug therapy in tumor bearing hosts were recorded mostly in the form of aneuploidy and polyploidy at spermatocytic diak.-meta.I of cell division. There was a wide range of variation in hypoploid and hyperploid count. Hypoploid count varied from 17 to 19 and hyperploid count varied from 21 to 22. The most common count of hypo- and hyperploidy was 18 and 21 respectively. It was rather difficult to detect which particular bivalent or bivalent group was lost in hypoploidy. Similarly, it was much

difficult to detect the proper identity of the extra chromosome or chromosome sets in hyperploidy. However, a rough estimation indicated that the loss/addition was nonrandom and limited among the smaller bivalent group. Sex bivalents were never lost or added to.

Data obtained from the combination II treated series indicated that the drugs used in experiment as intermittent dose caused a drastic increase in aneuploid (hypoploid) population (Table 6) which was more pronounced and maximum at the beginning of the drug therapy ie., at 4hrs. of drug administration, and maintained a steady peak at 24 hrs. A notable depletion in aneuploid population was observed at the subsequent intervals upto the end of the experiment (Fig 6).

Comb.II drug schedule exerts an early effect on polyploidy within 24 hrs. The polyploid population in comb.II treated specimens exhibited a significant increase upto 120hrs. when it reached the maximum level. After this period a reverse trend was noted with the depletion in polyploid frequency which was significant at 312 hrs. when it was almost at par with the control (Fig.6, Table 6).

7.3.ii. Structural anomalies - Structural anomalies in the form of X-autosome translocation was recorded in response

to intermittent drug exposure where there was an exchange between XY and a larger autosomal bivalent. Data obtained from Comb.II treated series (Table 7) revealed that a maximum level of X-autosome translocation was noted at 312hrs. of drug administration (Fig.7). Meiocytes of Comb. II treated series at the stage of diak.-meta-I of cell division revealed the presence of autosomal multivalents configurations where it was found that the larger autosomal bivalents were more prone to involve in this exchange process (Plate 13 a,b).

Quantitatively, autosomal multivalent formation involving two or more bivalents was found to its maximum level at 288 hrs. of drug administration (Table 8) followed by a gradual decrease at the subsequent post-treatment intervals (Fig 8) of therapy.

7.3.iii. Pairing anomalies - An examination of the spermatocytes of S180 tumor bearing host revealed the presence of pairing anomalies of autosomes and XY bivalent in the form of univalents. These anomalies were confined to the smaller groups which are mostly involved in this process. An analysis of data (Table 9) revealed that this drug schedule induced single autosomal univalency to its peak level at 72 hrs of drug exposure, when the frequency of separation was about 16.37% followed by a depletion at the

subsequent hours upto 288 hrs. The frequency of univalency again raised and reached a peak (16.25%) at 336 hrs of therapy (Fig.9). But a different trend was observed when the involvement of more than one autosomal univalency was considered. A maximum frequency was noted as early as 24hrs of drug exposure which maintained its peak at 72 hrs. and afterwards showed a gradual decline upto 288 hrs. The frequency raised and reached at its peak at 336 hrs. when it was about 3.25% (Fig 9; Table 9).

Comb.II treated specimens had shown maximum frequency of univalency in relation to X and Y chromosome from the very beginning of the experiment (ie., from 4 hrs.) which gradually dropped down at the subsequent intervals (Table 10).

8. **Sperm head Assay :**

8.1. S180 Tumor bearing Control :- Chronological studies on sperm head morphology on 25 S180 tumor bearing mice on and from the 4th.day of tumor transplantation revealed the presence of abnormal spermatozoa with deformed head, microhead, hookless and macrohead etc. (Plate 14). But in no cases these abnormalities reached a significant level compared to normal mice.

Table 11 : Frequency distribution of abnormal sperm count in Control & Treated Series
(values in Mean ± SD)

INDEX	HOURS OF *IN VIVO* DRUG / SOLVENT EXPOSURE							
	4	24	72	120	168	288	312	336
CONTROL	4.25±0.20	4.25±0.12	4.62±0.32	4.87±0.14	4.37±0.22	4.75±0.34	4.67±0.20	4.90±0.18
CP Single	4.02±0.09	4.22±0.17	4.45±0.16	4.75±0.22	4.25±0.19	4.90±0.19	4.72±0.19	4.45±0.26
COMB. I	4.10±0.29	4.27±0.09	4.80±0.30	4.62±0.33	4.67±0.12	5.00±0.18	4.85±0.29	5.20±0.27
COMB. II	4.30±0.27	4.52±0.10	4.60±0.27	4.95±0.20	4.72±0.29	4.77±0.12	4.67±0.11	4.80±0.23

Value in Normal Mice = 4.62±0.12

8.2. Single CP Therapy - An examination of mature spermatozoa from epididymis of CP exposed tumor bearing specimens revealed no significant alterations in the frequency of sperm head abnormalities. Table 11 incorporates the data on the incidence of normal and abnormal sperm heads noted after therapeutic exposure and the same is graphically depicted in Fig.11.

8.3. Comb.I Therapy - Like CP single therapy, Comb.I treated series also revealed a maximum frequency of abnormal spermatozoa at late hours of therapeutic exposure. However, the abnormalities observed did never reached to a significant level compared to controls (Fig.11).

8.4. Comb.II Therapy - Like CP and Comb.I treated series, the intermittent treatment showed no significant alterations in sperm head abnormalities in S180 tumor bearing specimens. However, the maximum frequency of spermatozoa with abnormal heads was encountered at 120 hrs of *in vivo* drug exposure (Fig 11).

9. **Epididymal Sperm Count :**

9.1. Single CP Therapy : Spermatozoa count from epididymis of CP treated specimens revealed a gradual depletion from the very beginning of the treatment (Table 12). But a significant depletion was noted only from 288 hrs and onwards(Fig.12)

Table. 12 : Total Count of Sperm at different Time-Points in Control and Treated Series ($\times 10^6$)

INDEX	HOURS OF *IN VIVO* DRUG / SOLVENT EXPOSURE								C.D. at $P_{0.05}$
	4	24	72	120	168	288	312	336	
CONTROL	87.35	85.10	88.10	86.20	86.85	83.85	85.45	84.20	3.89
CP Single	89.75	88.55	86.85	84.55	81.60	75.80	67.85	60.60	5.84
COMB. I	89.00	88.35	87.80	87.00	86.45	85.40	84.35	84.00	5.14
COMB. II	85.85	86.70	88.80	89.00	87.35	84.55	84.75	81.90	5.23
C.D. at $P_{0.05}$	5.96	4.48	4.14	5.25	4.50	3.87	6.17	7.03	

Total Count of Sperm in Normal Mice = 85.89 ± 0.82

9.2. Comb. I Therapy - Data obtained from Comb.I treated series (Table 12) indicated that unlike single CP therapy, there was no significant alteration in total sperm count.

9.3. Comb.II Therapy - Like comb.I drug schedule, Comb. II therapy was also unable to induce any significant effect on total epididymal sperm count of S180 tumor bearing specimens (Fig 12).

10. **Testis Weight Assay :**

Table 13 incorporates the data on the incidence of testis weight observed in normal, tumor bearing control and in different treated series, and the same is depicted in Fig.13.

10.1. Single CP Therapy - The measurement of testis weight in CP treated series revealed a decrease in testis weight which was more pronounced and significant (compared to control and normal) on and from 12th.day of CP administration upto the end of the experiment (Table 13).

10.2. Comb.I Therapy - Data displayed in table 13 indicated that administration of this drug schedule decreased the testis weight to a considerable extent, but not to a significant level. A maximum decrease in testicular weight was noted at 336 hrs of last administration of the drug (Fig 13).

Table. 13 : Testis Weight at different Time-Points in Control & Treated Series(g)

INDEX	HOURS OF *IN VIVO* DRUG/SOLVENT EXPOSURE								C.D.at $P_{0.05}$
	4	24	72	120	168	288	312	336	
CONTROL	0.103	0.108	0.103	0.104	0.104	0.104	0.101	0.105	0.004
CP Single	0.103	0.101	0.102	0.105	0.100	0.095	0.085	0.074	0.004
COMB. I	0.102	0.104	0.103	0.102	0.106	0.101	0.099	0.097	0.004
COMB. II	0.103	0.104	0.101	0.103	0.102	0.099	0.099	0.098	0.004
C.D.at $P_{0.05}$	0.004	0.003	0.004	0.003	0.004	0.004	0.006	0.004	

Testis Weight in Nommal Mice = 0.104 ± 0.93

10.3. Comb. II Therapy - Measurement of testis weight (Table 13) in response to intermittent drug exposure like Comb.I series revealed no significant change (Fig.13).

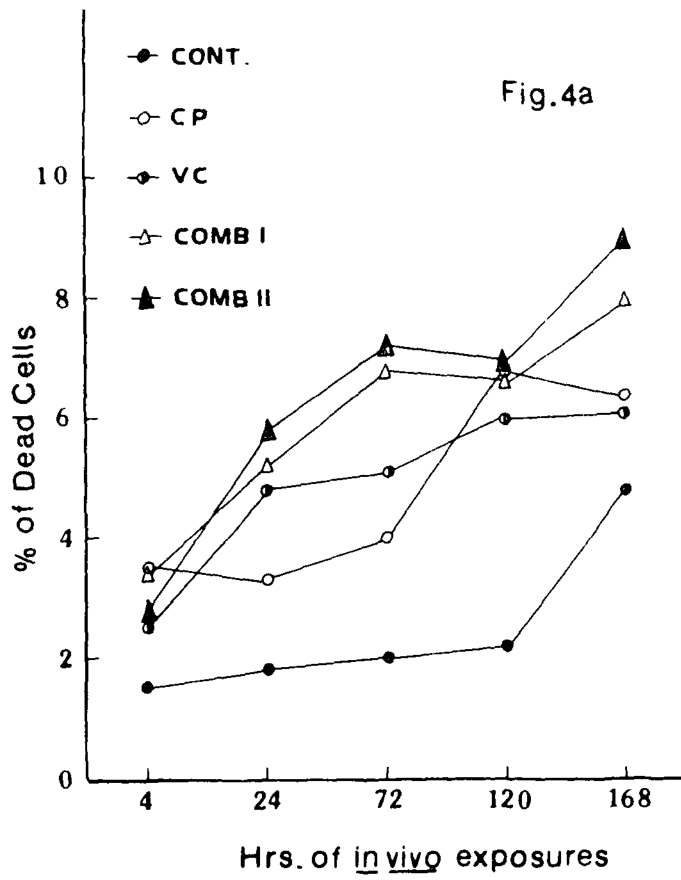
figures

Fig. 4a. ——

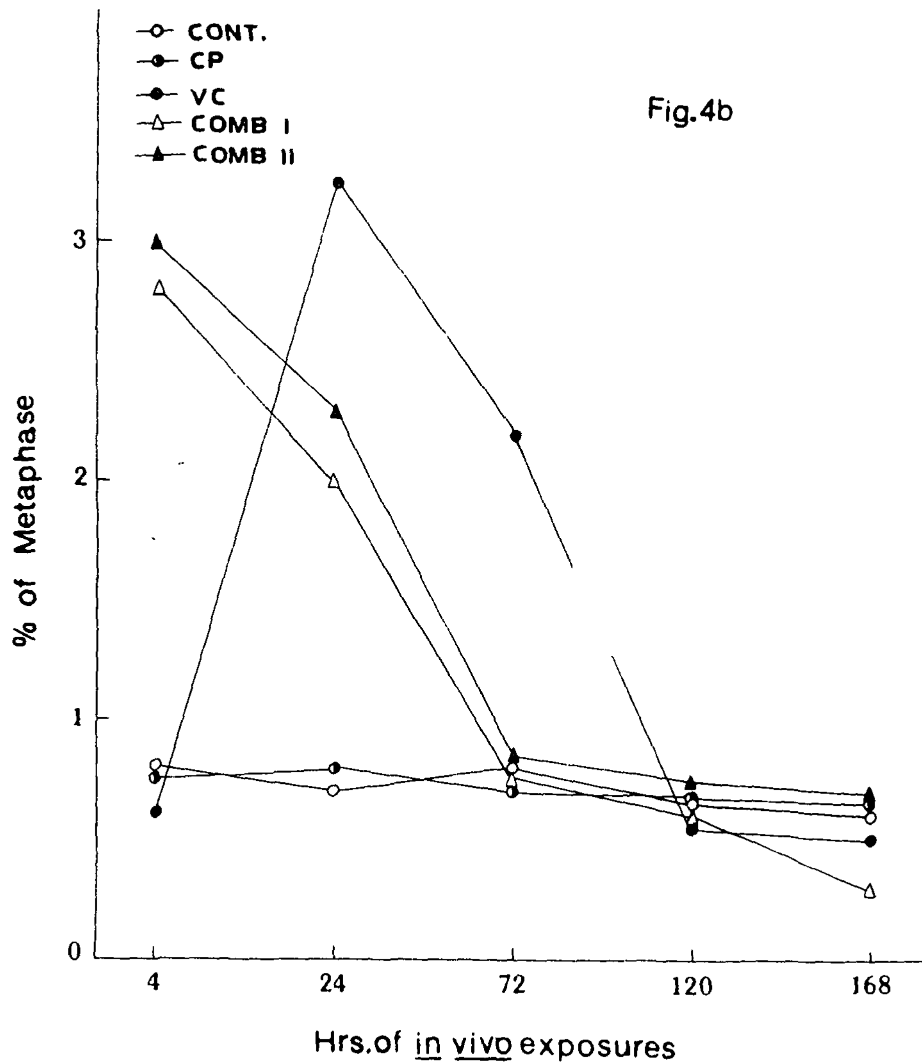
Frequency distribution of dead cells at different therapeutic schedules.

Fig. 4b.——

Distribution frequency of MI in S180 ascites tumor upon exposure to different therapeutic schedules.



CONT.
CP
VC
COMB I
COMB II
Fig.4a
% of Dead Cells
0
2
4
6
8
10
4
24
72
120
168
Hrs. of in vivo exposures



CONT.
CP
VC
COMB I
COMB II
Fig.4b
% of Metaphase
0
1
2
3
4
24
72
120
168
Hrs.of in vivo exposures

Fig.5

Fig. 5. Sequence and Kinetics of Spermatogenesis in Mouse

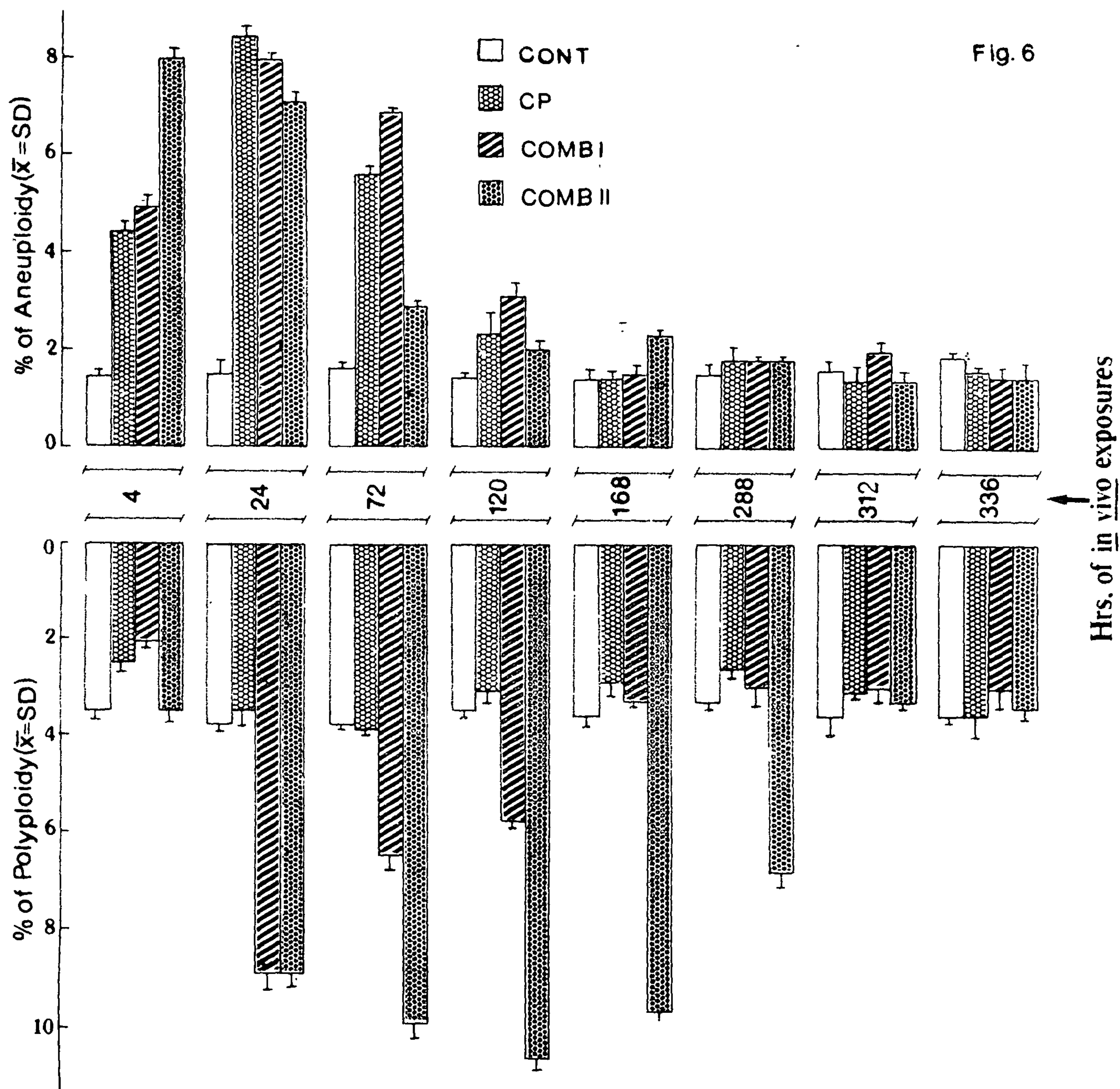


Fig. 6. Frequency Distribution of Aneuploidy and Polyploidy in primary spermatocytes of S180 Tumor bearing mice upon in vivo exposure to CP and CP-VC drug combinations.

Fig.7

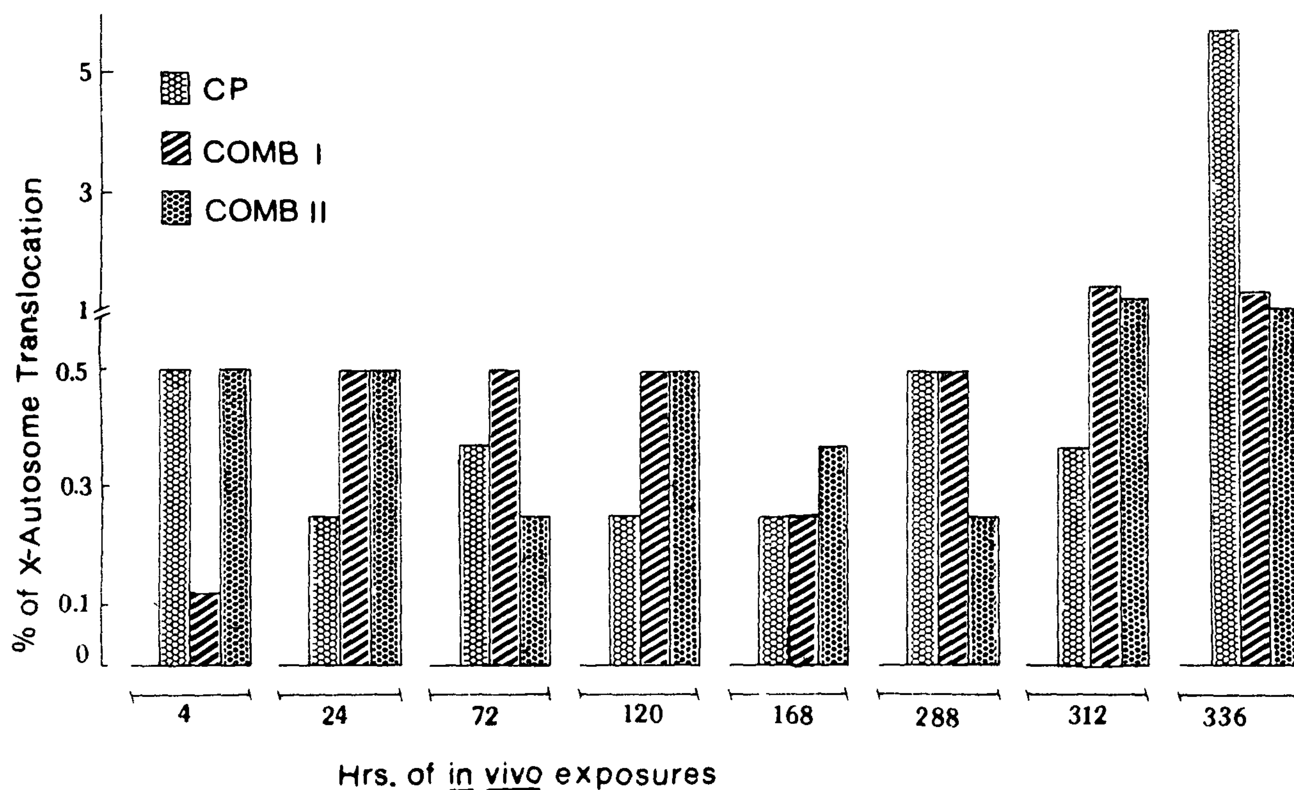


Fig. 7 : Frequency Distribution of X-autosome translocations in the Spermatocytic Cells of S180 Tumor bearing mice at different post treatment time points after administration of CP and CP-VC drug Combinations.

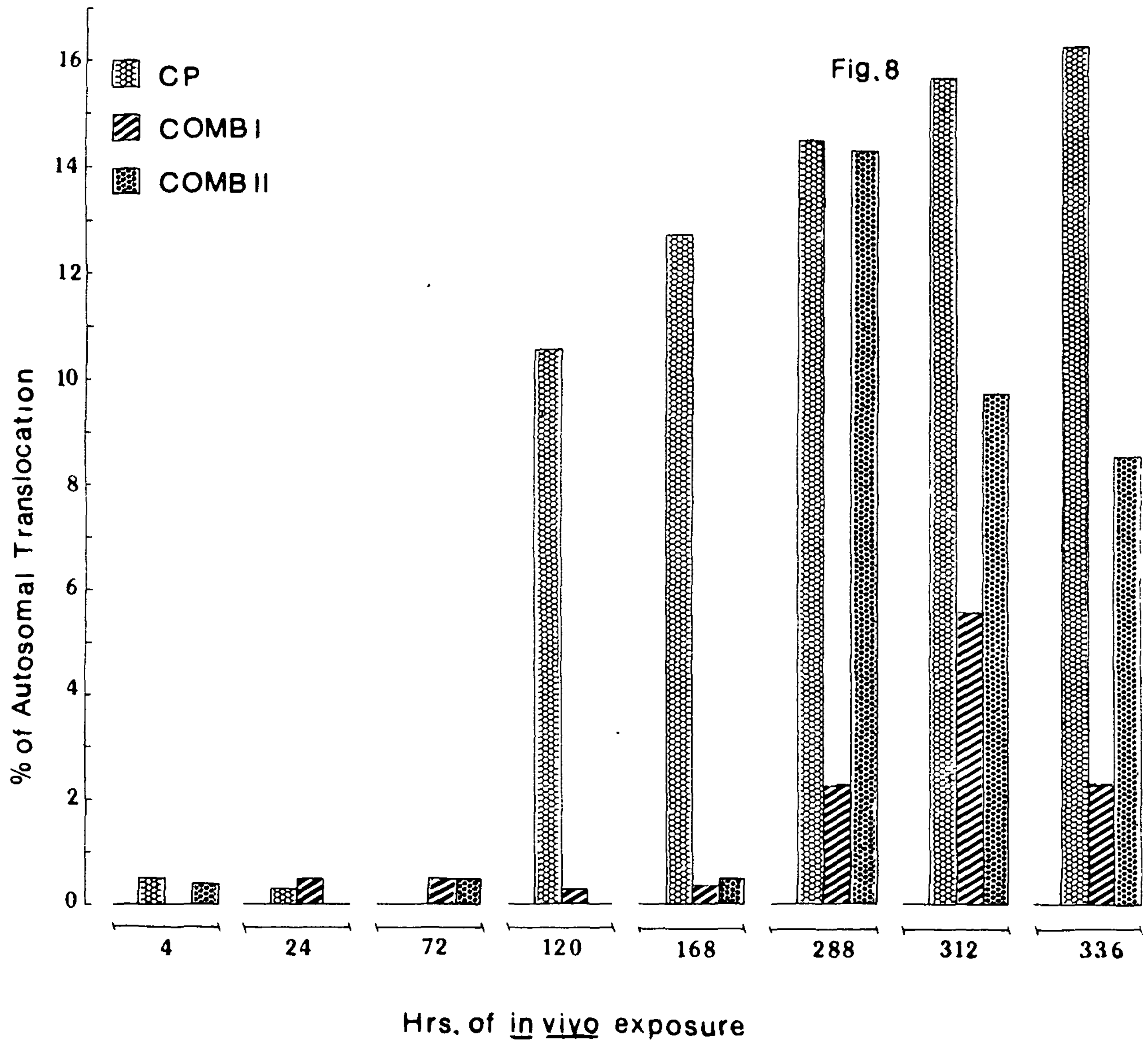


Fig. 8 : Frequency Distribution of Autosomal Translocations in primary spermatocytes of S180 Tumor bearing mice at different Post Treatment Time Points after administration of CP and CP-VC drug Combinations.

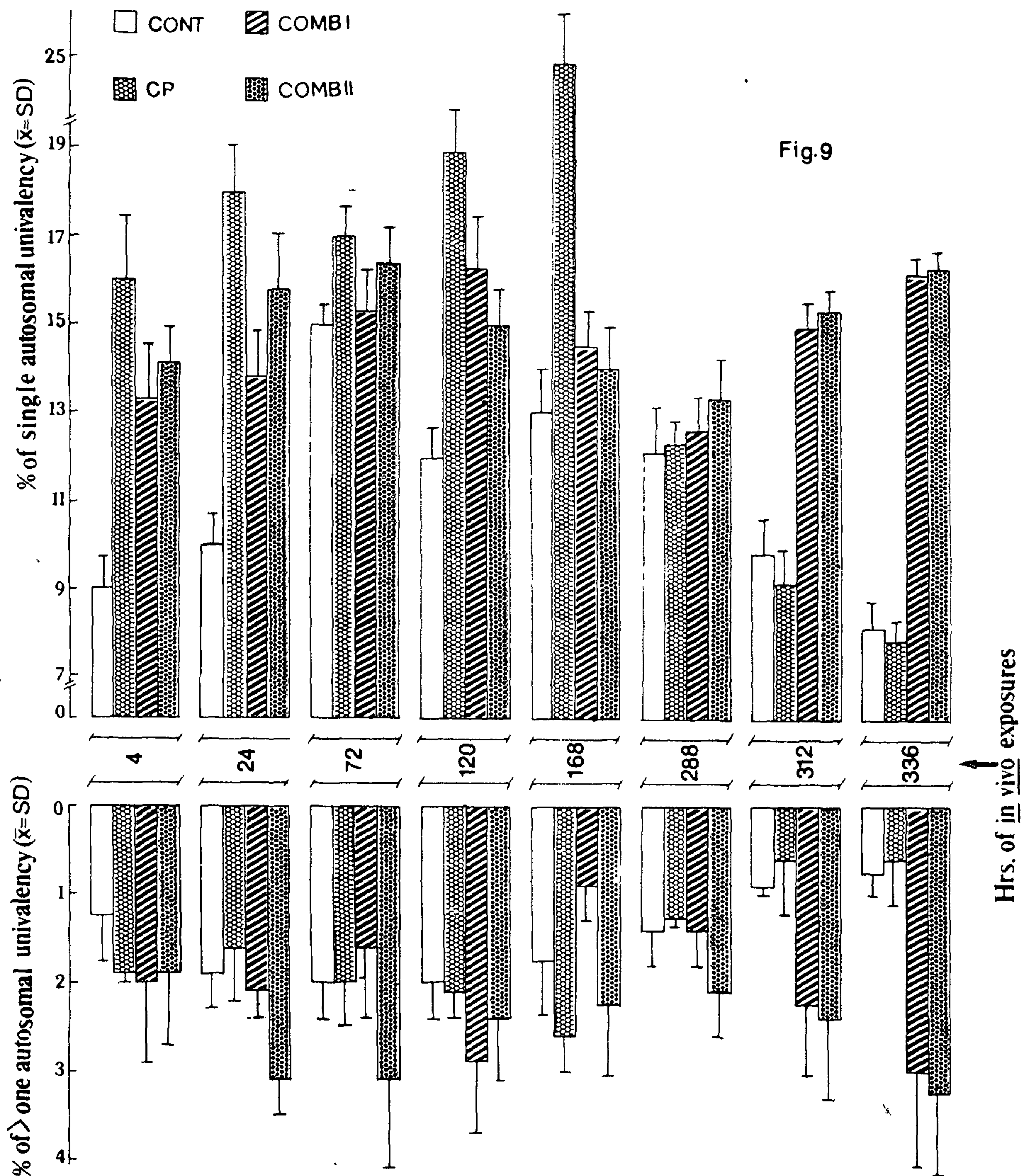


Fig. 9 : Frequency Distribution of Autosomal Univalency at Different post therapeutic time points in (Control) and (S180 Tumor bearing Mice).

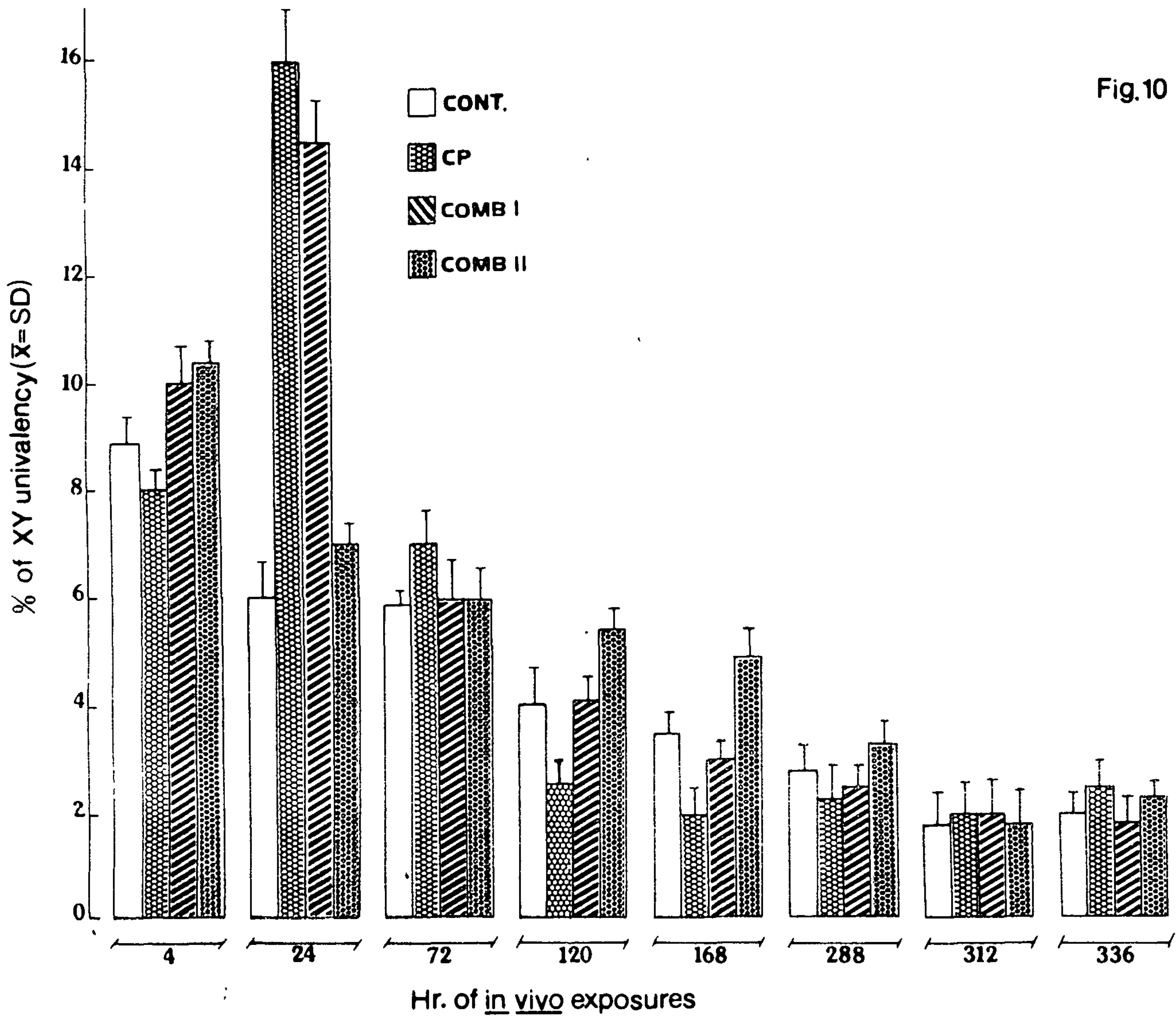


Fig. 10 : Frequency Distribution of XY Univalency in Spermatocytic cells of S180 tumor bearing mice after exposure to Single and Combination Drug schedules.

Fig.11

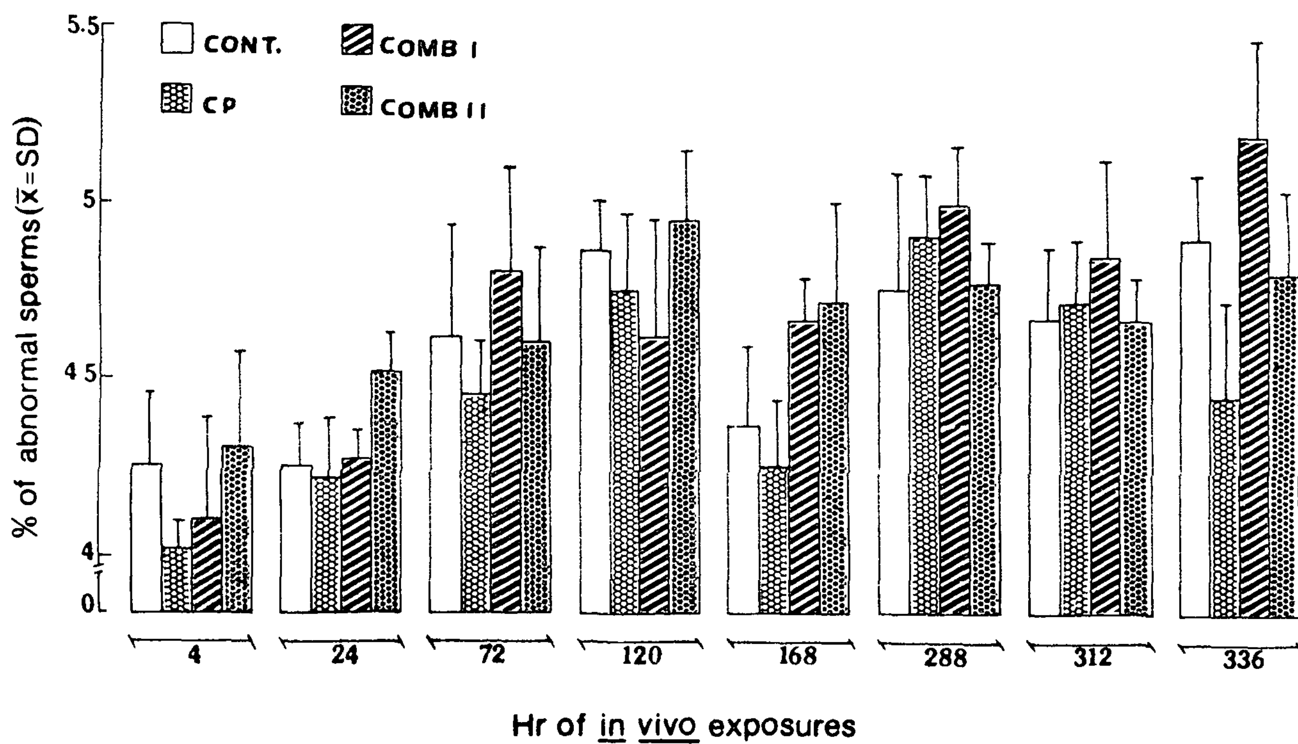


Fig. 11 : Frequency Distribution of Abnormal Sperm in Control and different Treated Series.

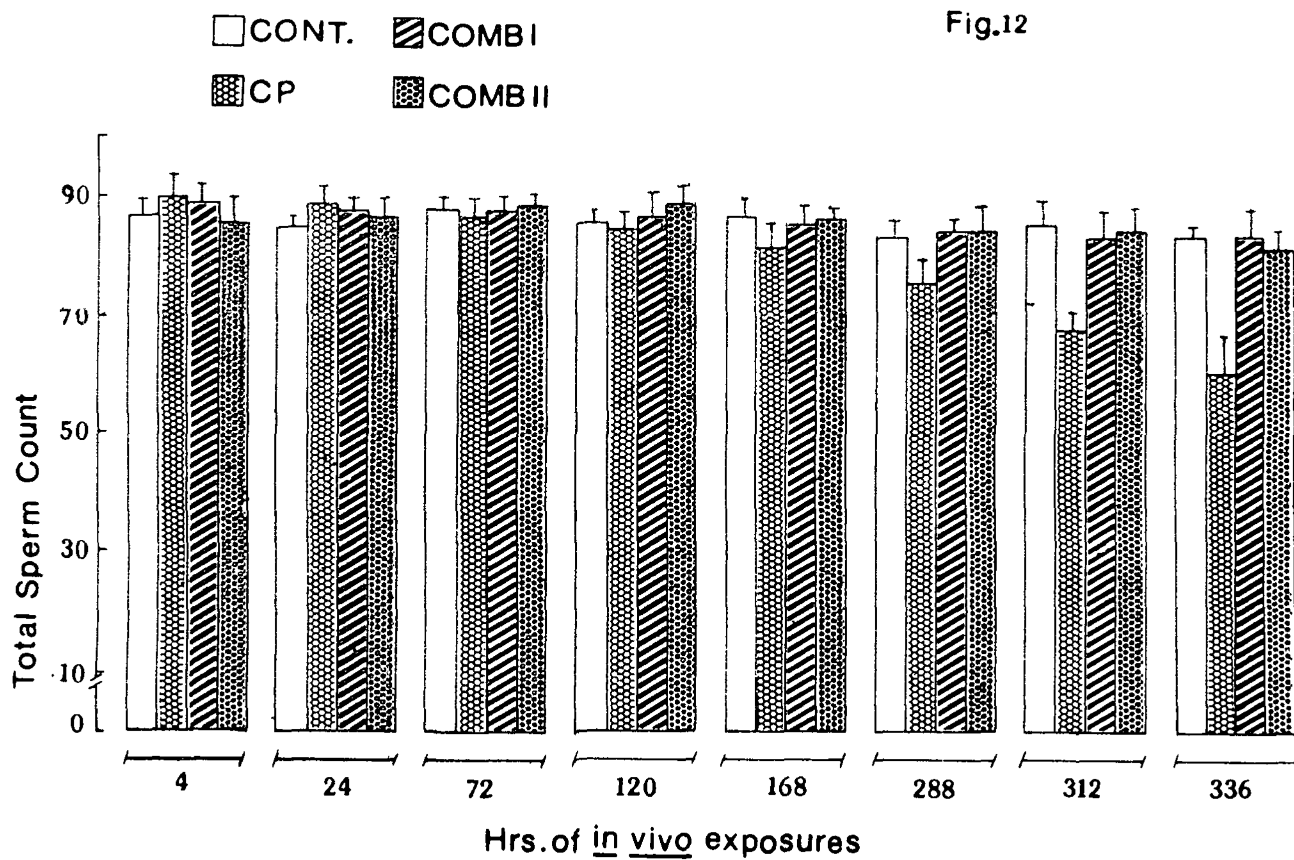


Fig. 12 : Frequency Distribution of Total Sperm Count at different post treatment time points in the epididymis of S180 Tumor bearing mice.

Fig.13

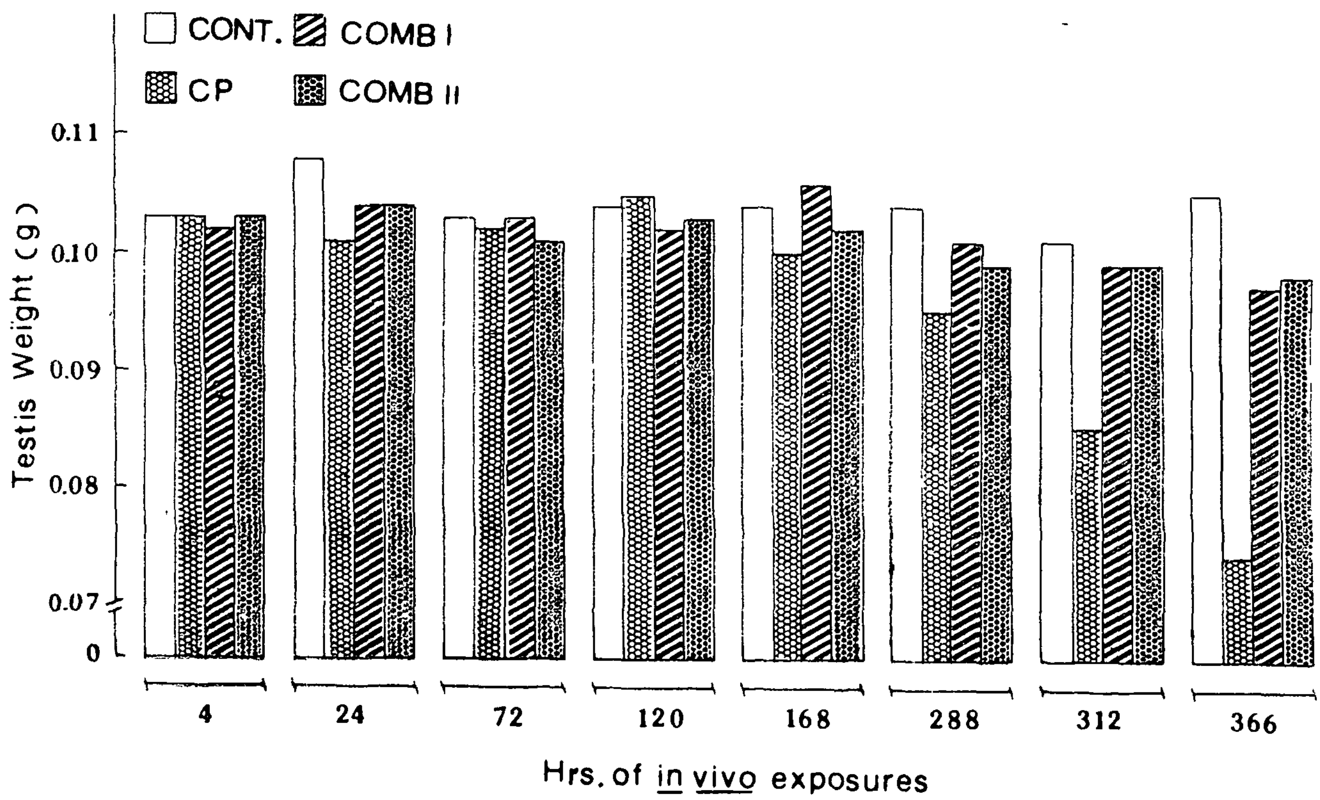


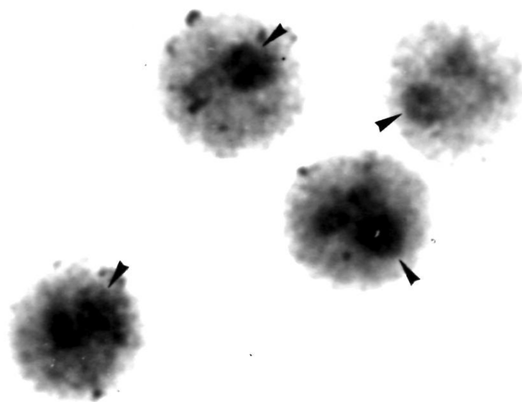
Fig. 13 : Histogram showing Distribution Frequency of Testis Weight in S180 Tumor bearing mice at different post therapeutic time points.

Plates

Plate 1. Spermatogonial metaphase of Normal Mouse showing 40 telocentric chromosomes of decreasing length.

Plate 2. Primary Spermatocyte nuclei with dark stained chromocenters (arrowed).

1



2

Plate 3. Stages of First Meiotic Division :

Leptotene

Zygotene

Early Pachytene

Late Pachytene (Sex Vesicle)

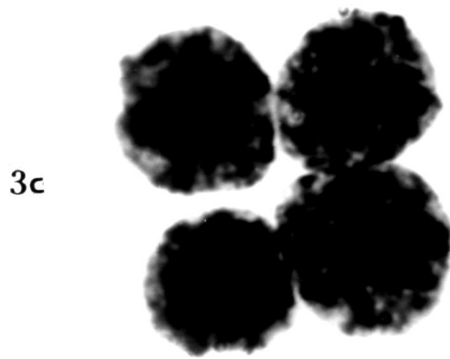
Early Diplotene
(X-Y Bivalent arrowed)

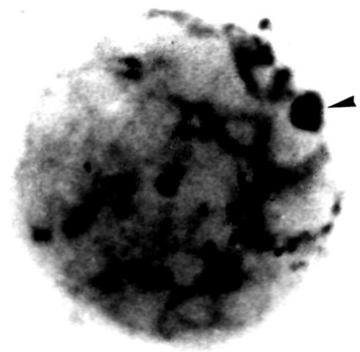
Late Diplotene
(X-Y Showing positive heteropycnosis)

3a

3b

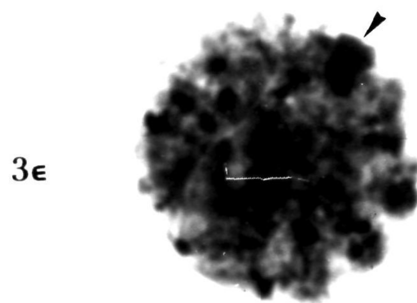
3c

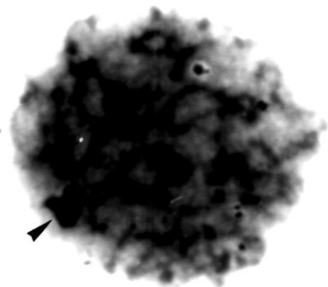




3d

3ε





3f

Plate 3g. Diakinesis-Metaphase I stage of Normal mouse showing 20 bivalents. Arrow head points terminally paired XY bivalent.

Plate 3h. XY bivalent showing a peripheral disposition (arrowed) in a Diak-Meta.I plate

3g

3h

Plate 4.

Diak-Meta.I spread from testis of S180 tumor bearing specimens showing various pairing anomalies :

a. widely separated X and Y chromosomes (arrowed),

b. widely separated autosomal bivalent (arrowed),

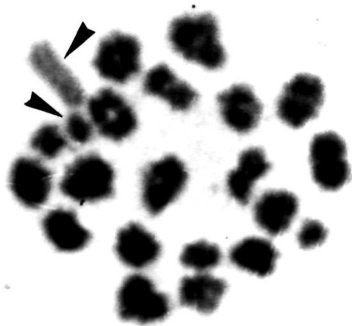
c. desynapsed X and Y bivalent disposed in a close proximity (arrowed),

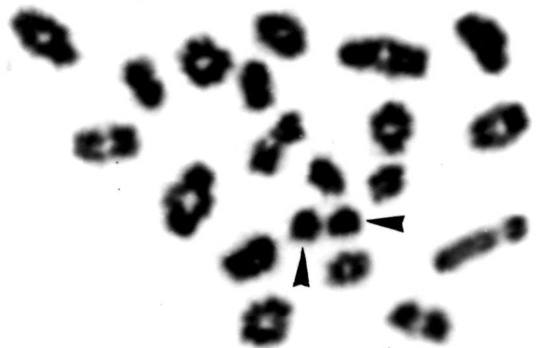
d. desynapsed autosomal bivalent disposed in a close proximity (arrowed).

4a

4b

4c





4d

Plate 5.

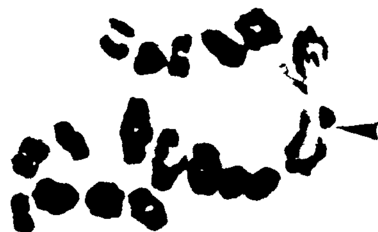
Diak.-Meta.I stage showing despiralised X chromosome with darkly stained Y attached at the end.

Plate 6a

Hypoploid Diak. - Meta.I from CP-treated specimen with 18 bivalents.

Plate 6b

Hyperploid Diak. Meta.I from CP treated tumor bearing mouse showing 21 bivalents



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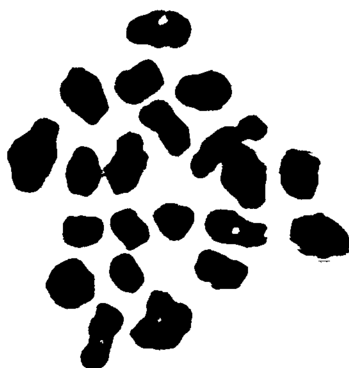
6a



6b

Plate 7.

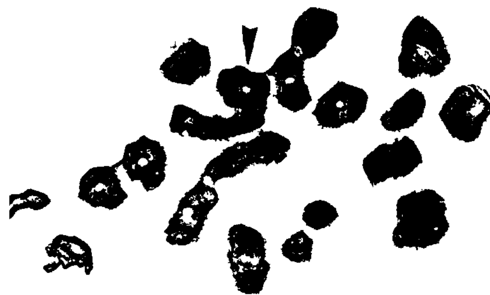
Diakinesis-Metaphase I spread from CP exposed S180 tumor bearing mouse showing X-autosome multivalent (Arrowed)

Plate 8a

Autosomal multivalents involving two autosome pairs observed in CP-exposed tumor bearing mouse (arrowed).

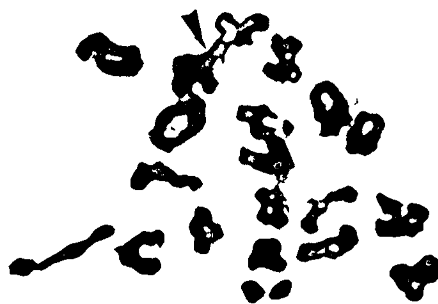
Plate 8b

Autosomal multivalent formed by more than 2 autosomal bivalents in spermatocytic cell of CP-exposed S180 tumor bearing mouse(arrowed).



7

8a



8b

Plate 9a ——

Diakinesis-Metaphase I showing desynapsed autosomes and sex chromosomes in CP treated S180 tumor bearing mice (arrowed).

Plate 9b ——

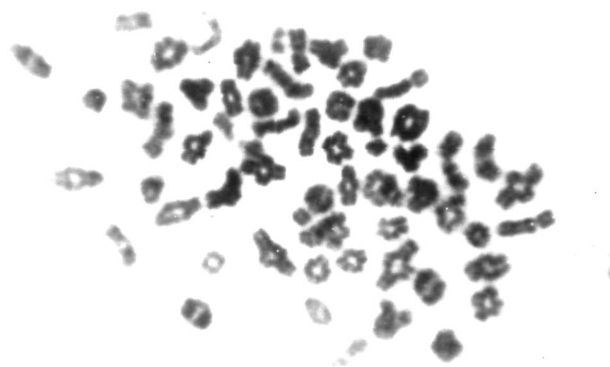
Diakinesis-Metaphase I spread from CP exposed tumor bearing mouse showing more than one autosomal bivalents involved in univalency (arrowed).

9a

9b

Plate 10. Polyploid Diakinesis-Metaphase I stage from a Combination I treated mouse .

Plate 11. Diakinesis-Metaphase I spread showing single autosomal univalency in a tumor bearing mouse treated with Combination I.



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11

Plate 12a. Diakinesis-Metaphase I stage from a Combination I treated tumor bearing mouse showing despiralised X (arrowed).

Plate 12b. Despiralised autosomes produced as a result of Combination I treatment in S180 tumor bearing mouse.

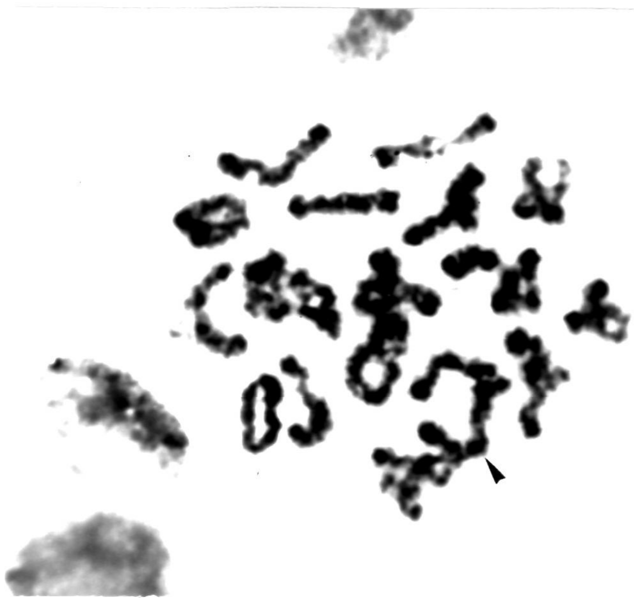
12a

12b

Plate 13a. b.

Diakinesis-Metaphase I spreads from S180 tumor bearing mice showing autosomal multivalents induced by Combination II drug schedule (arrowed).

13a



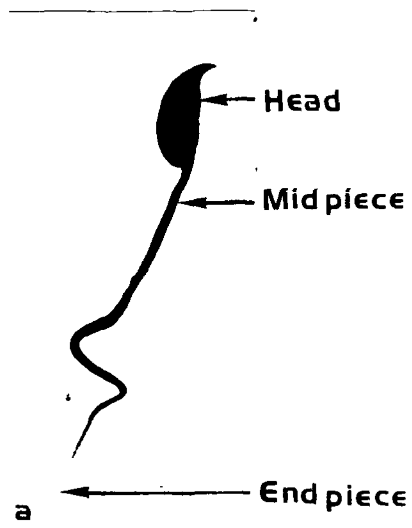
13b

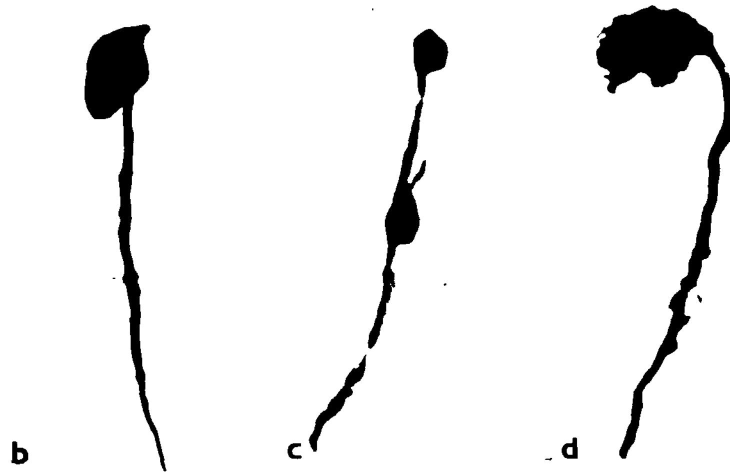
Plate 14. Normal and various forms of abnormal sperm head from epididymis of S180 tumor bearing mice subjected to chemotherapy:

Normal

Deformed Microhead Macrohead

Hookless Banana Head Other abnormalit





14

discussions

DISCUSSIONS

S180 mouse tumor model was chosen for the present study to investigate the effect of two most common antineoplastic drugs on germinal cells at some selected post treatment time points in response to therapeutic stress. The cytostatic agents selected are two popular anticancer drugs used to combat certain types of human malignancies. The use of these two agents is increasing day by day, particularly in the countries belonging to the Third World. Each drug has different mode of action : Vincristine is mitostatic alkaloid while, cyclophosphamide is an alkylating agent. Extensive reviews on the pharmacokinetics and therapeutics of CP and VC are now available. Individual cytotoxic effects of the drugs on somatic cells of man and other mammalian specimens, both *in vivo* and *in vitro* have also been published. But adequate attention has not been paid on the genotoxic potential of the drugs and their combinations on different germ cell stages of tumor-bearing animal models in response to therapeutic stress. Moreover,

reports available in the literature on the effects of CP on meiocytes and on fertility potential of man and laboratory animals are rather conflicting (Fairly et al. 1972, Penn 1979, Singh et al. 1987). Since one of the pathways of cyclophosphamide action is interference with the synthesis of cellular DNA at S-phase, most of the studies made so far in this regard were designed to evaluate the effects either after 19th.day or 35th. day of the last treatment of the drugs. During this period, as per time sequence established by Oakberg(1956,1957), the target spermatogonial stemcells (which are in an active phase of DNA synthesis) would enter spermatocytic or spermatozoa phase, accordingly. In the present project, attempt has been made to assess the effect of CP single therapy and its combination with VC on germ cells of S180 tumor bearing mice at certain chosen time points during and after potential regression of tumor.

The rate of tumor regression in response to single and combination therapy was assessed by following the protocol described earlier, and the influence of therapy on mean survival time (MST) of tumor bearing specimens was estimated. Data were compared with untreated (and negative) S180 tumor bearing control as well as non-tumor mice. A close look of the data presented in Fig.4

reveals that a maximum regression (potential cure) of tumor was achieved on day-4 for CP single therapy and, day-3 for different combinations viz.,Comb.I & Comb.II. A maximum increase of +8 days inMST for CP single therapy , +14 days for Comb.I and +18 days for Comb.II series was also documented in the present experiment.

It is evident from the data that short termexposure of CP single as well as in different combinations with VC at single therapeutic dose produced various types of cytotoxic effects on the germinal cells of tumorbearing mice. Major types of abnormalities recorded at spermatocyte level were in the formoof: chromosome univalency, ploidal anomaly and exchange configurations at bivalent level. In addition, effect on chromosome condensation was also documented but not to a significant level. The effect at the spermatozoa level, though, not conspicuous, includes alterations in sperm head morphology. Decreased sperm counts in the epididymis and loss of testis weight to a significant extent have also beenrecorded at certain time points in response to therapeutic stress.

One of the major types of abnormalities observed in the spermatocytic cells upon in vivo exposure of the drug and drug combinations was sex chromosomal and autosomal

univalency, referred to as 'behavioural aberrants' by Lin et al.(1971)- a condition where in autosomes or sex chromosomes remain as univalent during diakinesis-metaphase I. In normal condition, the X and Y chromosomes of the mouse are expected to be associated in a terminal bivalent pairing/association (long arm-long arm) in primary spermatocytes at the diak-meta.I stage of meiosis. The presence of synaptonemal complex has been identified between the terminal region of the X & the Y chromosomes in early pachytene cells (Solari 1970, Moses et al. 1976). Any deviation from this may result in the formation of univalency. Univalency may occur as a consequence of asynapsis or desynapsis. Asynaptic condition is depicted by the complete absence of bivalents in diak.-meta.I of meiosis, whereas desynapsis results in the simultaneous occurrence of univalents and bivalents (Schleirmacher 1970, Golubovskaya 1979). Occurrence of univalents along with bivalents in high proportion at diak.-meta.I in the present study can be attributed to desynapsis.

An analysis of the data plotted in figure 10 reveals that about 2.5% cells show precocious desynapsis of XY bivalent in normal mice. Reports on the 'spontaneous' occurrence of premature desynapsis of XY bivalent are

not very uncommon. Lin et al.(1971) documented sex chromosome univalency to the extent of about 6.17% in 3-4 month old inbred Swiss mice. A range of 3.8% to 8.5% desynapsis frequency has been suggested for C57BI mice by Leonard & Leonard(1975). Chandley and Speed(1987) also recorded about 6% precocious separation of XY in normal mouse spermatocytes. Earlier, Evans et al.(1980) reported 5-10% separation index for normal mouse. It is also evident from published literature that the frequency of XY desynapsis may reach 70% to 90% in mouse with sex reversal (Sxr) and with other form of abnormalities (Winsor et al. 1978, Chandley and Fletcher 1980). Lyon et al.(1981) considered pairing failure as possible reason behind sex chromosome univalency in mouse. But this was later criticised by several other workers on various grounds (see Chandley and Speed 1987). DeBoer et al.(1986) assumed that genetic diversity of the Swiss random bred mouse leads to variants in the extent and adjustment of meiotic pairing and furthermore, that pairing is related to spermatocyte survival. Gollapudi et al.(1981) advocated the possibility of genetic control of the pairing mechanism and univalency resulting in a genetic or chromosomal factor inherited from high frequency parents.

In the present study, a low frequency of 2.5% spermato-

-cytes in normal random bred Swiss mice with precociously desynapsed X and Y chromosomes is in close agreement with the findings of Murthy and Subramanyam (1985), Das and Nayak (1988). However, the frequency of such cells in tumor bearing control specimens with 4-day old tumor was as high as about 9%, and in all subsequent post-transplantation intervals the desynapsis frequency remain within the range (Fig.10). In fact the frequency peak decreased gradually with an increase in post-transplantation interval. Thus the frequency of about 9% is sex chromosome univalency in control specimens again is in close agreement with the earlier reports available in the literature. A comparison of data reveals that there is a significant difference in the frequency of sex chromosome univalency between control and different treated series. Single exposure to CP at therapeutic dose caused an increase in the frequency at certain post treatment time points <u>viz.</u>, 24 and 120 hrs. Interestingly, significant difference in this regard was noted only at 24 hrs. in case of combination I series, while a similar result was obtained at 120 hrs. in combination II series. In all the cases the difference between control and treated series was at 5% level. Again, when the data of single CP treated series and Comb.II treated series (VC-CP-VC) were compared, a different trend was noted. In all post treatment timepoints,

beginning from 4 hrs. upto 288 hrs, the frequency was significantly high in combination II series (Table 10). There are reports on the influence of alkylating agents and other chemicals on the induction of precocious desynapsis of X and Y chromosomes of mouse (Schleirmacher 1970, Chakrabarti et al. 1986, Dey et al.1989). But so far as the present author is aware, there is no short-term exposure study on the effect of CP and CP-VC combinations on the phenomenon of precocious desynapsis during therapy at spermatocytic level. Thus the present study clearly points out that application of these drugs at therapeutic dose influences precocious desynapsis of sex bivalent in spermatocytic cells of tumor bearing mice and the effect is more pronounced if the drugs are applied in a combination : VC followed by CP,followed by VC ie,in Comb.II in the present experiment, while an alternative combination ie, prior application of VC followed by CP was not that effective so far as the precocious desynapsis of XY bivalent is concerned.

A similar trend in relation to precocious desynapsis of autosomal bivalents was also documented in the present experiment. Like sex chromosome univalency, single autosomal univalency was also observed almost consistently at a significant level from the very beginning of the

experiment upto the end of the experiment. The result is more consistent compared to sex chromosomal univalency. A difference in the level of significance was documented both in single CP as well as in combination therapy. Like sex chromosome univalency, the level of significance in case of single autosomal univalency was also noted at 5% level (Table 9). A similar trend was not documented when data of control and more than one autosomal univalency were compared. In fact no significance was seen in univalency related to more than one autosome pair. Again, when the differences between the series, CP single and different Combinations were compared, it was noted that the difference is significantly higher in two combination series mostly during late hours of therapeutic exposure. This indicates that both the combinations seem to be more effective in inducing single autosomal univalency in comparison oto CP single therapy.

The mechanism by which CP and CP-VC combinations affects XY and autosomal pairing and causes precocious desynapsis, is difficult to ascertain. Several possibilities may be discussed. CP is known to be clastogenic and its clastogenic effect may be responsible for inducing desynapsis of XY bivalent which are only terminally associated/paired

during prophase of first meiotic division. Since, in the present investigation no structural abnormality in the form of simple break or deletion in relation to sex chromosomes or autosomes noted, it is difficult to ascertain that a breakage-separation was in operation.

During meiosis, the X and Y chromosomes of mouse like other autosomal bivalents remain associated from pachytene onwards to metaphase I. The precise orientation of these bivalents is contradictory. It has long been assumed that there is a homologous pairing segment of variable length of the X and the Y pair, and an obligatory chiasma keeps the two chromosomes together(Kollar and Darlington 1934, Solari 1974, Chandley and Speed 1987). According to the other school, the synapsis between the X and Y in mouse,is non-homologous. Crossing over never occurs as normal event in this pair, and orientation of X and Y is mediated only by an achiasmatic telomeric association (Ashley 1985, 1987). With this knowledge in mind, one can assume that CP acts on cementing portion of synaptonemal complex that keeps the X and the Y chromosomes together for a short segment throughout meiotic prophase I and thereby causes precocious disjunction of the bivalent. Such possibility is not very unlikely, bacause it is known that, in addition

to its effect on chromosomal DNA, CP can also form co-valent bonds with nucleophilic groups in a variety of molecules including protein. Moreover, report on extensive synaptinemal complex damage in the spermatocytes of mouse, Chinese hamster and American hamster within 72 hrs. of CP administration to a variety of doses have been published recently (Allen et al. 1987). The effect, according to Allen et al.(1987) is dose dependent. So far as the desynapsis of autosomal bivalents is concerned, it has been noted in the present study that the phenomenon is somewhat non-random and restricted mostly among the smaller autosomal bivalents. Regarding the selective separation of XY bivalent and smaller autosomal bivalents in different treated series, it may be suggested that these chromosomal elements remain associated only for a short segment and become the primary target for cytotoxic insult offered by the drug which compels them to separate precociously compared to their counterparts long before the onset of Anaphase I. However, although, there are published reports on the selective action of CP on synaptinemal complex, there is no such report known to the present author on the interference of VC with the formation of synaptinemal complex. Therefore, the reason behind a significant increase in sex chromosome and autosomal univalency in

response to combination therapy (compared to CP single therapy) remains unexplained. The condensation effect of VC on chromosomes may aid or enhance theprocess of premature desynapsis of bivalents.

The other most significant effect of CP single and combination therapy include ploidal variation. The occurrence of aneuploidy with hypohaploid chromosome count was repeatedly observed at significant level in response to in vivo drug exposure in almost all post treatment time points upto 120 hrs. (Fig.6,Table 6).The aneuplo frequency in mouse is known to be variable. According to Lin et al.(1971), the frequency varies from 2.7% to 3.2%, while according to Leotard et al. (1987) it is strain dependent, and wide spreading (between 4.6% and 34.8%). But there is no report on their frequency in t tumor bearing mice. In the present study, the frequency of such aneuploid cells was within 2% range which is in close agreement with observations of Lin et al.(1971). A comparison of data presented in Fig.6 reveals that there are significant differences at 5% level between control and different drug exposed series upto 120 hrs. (for Comb.II- 168 hrs). Significant difference was also documented at certain time points between CP single therapy and Combination chemotherapy. A variety of

chemicals are known to produce aneuploidy during spermatogenesis (Hansmann 1983). Non-disjunction and selective or random chromosome elimination are often attributed to the aneuploid condition in mitotic and in meiotic cells. Chemicals that induce non-disjunction include among others, MTX, MMS, EMS, bleomycin, natulum, CP, Cd, trenimon, carbendazim, colchicine, VC. Thus both CP as well as VC are known to be potent inducer of non-disjunction. But the way by which these two drugs either singly or in combination produce higher frequency of aneuploid cells within 4 hrs. in the present experiment is difficult to explain. The possibility of non-disjunction is unlikely because the time period is insufficient for the last division of potentially affected spermatogonial stem cells to reach spermatocytic phase followed by drug treatment. The only possibility lies in the selective elimination of individual bivalents at certain phase of meiotic prophase I. The observed frequency of aneuploidy, thus in the present experiment, seems to be highly irregular and at the same time difficult to explain.

The influence of the drug and drug combinations on the frequency of polyploid cells at diak.-meta.I stage was very striking and found to be significant at various

post treatment time points, both at early as well as late hrs. of therapeutic exposure (Table 6). The frequency of polyploid cells in normal and non-tumor specimens was 3.5% (3.58±0.07) which is in close agreement with the results obtained by Leonard (1973) who demonstrated that spontaneous ploidal range varies from 3-6% in normal mouse. However, Kar and Das (1987) observed 5.34% polyploidy in random bred Swiss albino mice. No significant difference in the frequency of polyploidy between normal and S180 tumor bearing control was found. This indicates that the transplantation of tumor had no visible influence on the phenomenon of polyploidisation in this mouse tumor model.

The precise significance of ploidal anomaly has been discussed in length by various investigators. Cell fusion and spindle disruption followed by endoreduplication are often attributed to induction of polyploidy in somatic as well as in germinal cells. VC is a potent spindle disrupting agent and at the same time, an active spindle inhibitor. But the effect on spindle protein of dividing spermatogonial cells can not be manifested until the completion of stipulated period when the

affected cells enter into spermatocytic phase. In the present experiment, high incidence of polyploidy was documented from 24 hrs. of drug exposure. The incidence was very high in combination groups, particularly in Combination II treated specimens. The trend continued upto 288 hrs. after which it was almost at par with that of the control. In contrast, the effect of CP single therapy on the incidence of polyploidy was not significant at any time point. It is thus evident that not the CP but the administration of VC is responsible for higher induction of polyploidy observed in two different Combination treated series. Moreover, an examination of data presented in table 6 reveals that application of Comb.II therapeutic schedule seems to be more effective in inducing polyploidy at diak-meta.I cells of meiotic prophase I. However, the mechanism of the induction of polyploid cells within 24 hrs. of drug exposure remains inconclusive. Only possibility which can explain the phenomenon is the fusion of contiguous cells as reported earlier by Dym and Fawcett (1971) and also by Beatty _et al._ (1975). Reported occurrence of polyploidy within 24 hrs. and at late hrs. ranging from first week to second week time period in response to other non-anticancer drugs ais known in normal mouse. (Reddy and Subramanyam 1985,Murthy and Subramanyam 1985).

One of the most interesting aspects of the present study is the occurrence of chromosomal structural changes in the form of various exchange configurations at spermatocytic level. The exchanges recorded were mainly of two kinds : sex chromosome-autosome translocations and autosome-autosome translocation. It may be mentioned that not even a single incidence of translocation was encountered in control or in normal non-tumor specimens. An examination of data (Table 7,8; Fig.7,8) reveals that the effect is significant at 1% level at certain post treatment time points. The significance is pronounced in case of autosome-autosome translocation particularly when the difference between CP single therapy and control was taken into consideration. An analysis of data indicated that administration of CP at single therapeutic dose produce exchanges involving both autosomes and sex chromosomes from 120 hrs onwards. The effect was also visible, ofcourse, to a lesser extent, in Comb.I and Comb.II treated series. However, unlike numerical chromosome anomalies, the occurrence of structural chromosome abnormalities has not been documented at early hrs. of therapeutic exposure, and the involvement of sex chromosomes in the production of translocation has been documented only at one particular time point (ie., 336 hr) to a significant extent in case of CP single therapy.

It is generally argued that, in most cases, DNA damage produced by drugs and chemicals will not result in the formation of aberrations and exchanges *unless* the cell is in S-phase between the time of treatment and sampling. The appearance of various exchange configurations in the form of autosomal and sex chromosomal multivalents to a significant extent within 5 days of in vivo drug exposure and their continued presence upto the end of the present experiment ie., 14 day, can be explained in a different way. It is clear from the observation that spermatocytic cells examined on the fifth and the following days of initial treatment of CP single and CP-VC combinations, are not the direct descendants of those spermatogonial stem cells that were in S-phase during the time of last drug treatment. Because, a five day time period is not adequate for a spermatogonial stemcell to enter spermatocytic phase in the process of spermatogenesis. It is,therefore, obvious that translocations noted in these cells were not mediated through the interference of the drugs with the spermatogonial S-phase. Some other mechanisms of drug action on germinal cells must be there which resulted in DNA damage and misrepair manifested into visible chromosome structural changes within a relatively short time period in the present experiment.

The extent of DNA synthesis of mouse germ cells have been studied by various investigators. According to Monesi (1962), DNA synthesis in the mouse primary spermatocytes lasts,, on an average, 14 hrs. and is not observed at late phases of spermatogenesis. In contrast, Ghosal and Mukherjee (1971) estimated the duration of leptotene to be 2-3days , with DNA synthesis lasting for 29 hrs. It is also known that diakinesis-metaphase I occurs in different strains of mouse on an average of 10 to 12 days after preleptotene (Oakberg 1956, Dietrich and DeBoer 1983). Therefore, any chromosomal DNA damaging effect of a drug or a chemical which is mediated through the interference of spermatogonial S phase would require this minimum period to be visualised at diak.-meta.I. The first appearance of translocations in spermatocytic cells in the present experiment was made on day 5 of CP single exposure. Kofman Alfaro and Chandley (1971) obtained unscheduled DNA Synthesis (UDS) in vitro at all stages of gametogenesis of mouse, except for advanced spermatids and spermatozoa. This UDS is used for the repair of DNA damage at cellular level during the process of spermatogenesis. A maximum synthesis of UDS was recorded during zygotene and pachytene. DNA synthesis during zygotene is related to homologous pairing while DNA synthesis during pachytene is used to repair

nicks and other lesions in DNA chain. CP is known to interfere with the synthesis of DNA during S phase. Reports are also available which indicate that CP and certain other mutagens viz., MMS, PCB, EMS etc. can induce UDS in meiotic and post-meiotic stages upto about midspermatids and sperm cells (Sega 1974,1982, Schmid et al. 1978, Burgin et al. 1979). It is thus proposed that the drug interferes with the UDS and the repair synthesis of DNA during zygotene-pachytene complex and thereby causing misrepair of DNA strands which results in the production of various exchange configurations documented at diak-meta.I of meiosis in CP treated series. The explanation also fits well with the established time sequences of meiosis. In mouse, zygotene lasts for approximately 2 days, pachytene for 5 days and diplotene approximately 3 days; then comes diakinesis-metaphase I (Dietrich and DeBoer 1983). Obviously, cells affected at the pachytene would enter diak.-meta.I within 4 to 9 days depending on whether the cells were in early, mid or late pachytene stage during the last exposure of the drug. Any effect at this stage will be visible at diak.-meta.I stage from 5 day onwards. The reported damage of synaptinemal

complex within 3 days of in vivo CP administration at different doses in mouse spermatocytic pachytene stage by Allen et al. (1987) also strengthen the view that CP can produce cytotoxic effect within a short time in mouse spermatocyte.

The other effects of CP and its combinations were documented at spermatozoa level. In the present study single exposure of CP at therapeutic dose produced no sperm head abnormalities to a significant level, but its effect on testes weight and sperm count were significant at certain time points. So far as the CP single therapy is concerned, the effect is seemed to be of delayed nature. Significant difference between control and single CP therapy was noted on and from 12 day onwards. But the way by which CP produces reduction in sperm count is not clearly understandable from the present experimental set up. Earlier Trasler(1987) demonstrated that CP at low daily doses increases post-implantation loss via an effect on spermatozoa during epididymal maturation and suggested that the spermatozoa can be modified after entry in the epididymis. It may, therefore, be assumed that CP at therapeutic dose, in the present experiment, affected the epididymal spermatozoa

102241

and caused reduction in total sperm count. The loss of testis weight to a significant level upon CP exposure may also be explained in the same line.

So in the retrospect, it can be said that CP singly and in combinations with VC produced germ cell cytotoxicity both at meiotic and post meiotic stages within a short time period in S180 tumor bearing mice. The significant types of effects include : Precocious desynapsis of sex chromosome and autosomal bivalents leading to univalency at diak-meta.I stage of meiosis which has far reaching clinical consequences. The gonosomal univalency in male, is often associated with abortion of primary spermatocytes, sterility and meiotic break down (Beechey 1973, Chandley 1981). Cytogenetic studies on F_1 progeny of parents subjected to CP chemotherapy along with dominant lethal assay may add further information on chemotherapeutic risk at genetical level.

The other significant effect of CP and CP-VC combinations in this tumor model is the increase of cells with aneuploid and polyploid chromosome counts . The precise clinical significance of ploidal anomaly has been discussed in length by various investigators.

Unlike numerical chromosome anomalies and univalency, the occurrence of structural abnormalities in response to drug therapy was not significant at early hours of therapeutic exposure but the appearance of exchange configurations in the form of autosomal and sex chromosomal multivalents in higher frequency was well documented at late hours. So far as the present author is aware, there is no report on the induction of chromosomal structural changes and translocations in spermatocytic cells by short term therapeutic exposure of CP. The probability of recovering a translocation is very low after clinical treatment. Since the time period required to visualised structural chromosome changes mediated through the interference with S phase DNA synthesis of spermatogonial cells, is not sufficient in the present experiment, the cells displayed translocations were certainly not spermatogonial stem cells at the time of treatment. It has been postulated that either the drug interferes with repair synthesis of DNA during pachytene or with UDS at different stages of spermatogenesis resulting in the induction of various exchange configurations observed at diak-meta.I stage.

104

The other significance of therapeutic exposure of CP documented in the present study is its effect on sperm count and testis weight which also has clinical importance. It has been repeatedly demonstrated by different investigators that depletion in sperm count and the loss of testis weight are related to male sterility in man and laboratory animals.

Thus the present study on S180 tumor bearing mice in association with the results published by other investigators in this field show the potential risk of CP and CP-VC combinations to human populations and may represent a useful contribution in further studies of cancer patients exposed to single and combination drug therapy.

summary

S U M M A R Y

The genotoxic effects of two widely used antineoplastic drugs, both singly and in combinations, on meiotic and post-meiotic stages of a tumor bearing mouse model have been assessed at certain chosen time points.

The tumor model : Transplantable murine tumor, mouse sarcoma 180 (S180) adapted to out bred strain of Swiss albino mice was chosen for the present experiment for the following advantages : i. about 100% tumor take in inbred and outbred strain of mice, ii. uniform transmissibility of the tumor for successive generations, iii. prolonged survival of the 'host' with transplanted tumor, iv. fixed doubling time of tumor cells. v. tolerance of the mice, vi. easy to maintain in the laboratory. The ascites form of the tumor was adapted to out bred strain of Swiss albino mice because of their close resemblance with genetically heterogenous human populations.

Selection of drugs : Two effective and commonly used anticancer drugs of diverse mode of action viz., Cyclophosphamide (CP) and Vincristine (VC) were chosen for single and combination chemotherapy.CP is an alkylating

agent while VC is a mitostatic alkaloid. These two drugs are now extensively used in different parts of the globe particularly in the countries of the Third World for a potential cure of various forms of human tumors.

Treatment schedule : The effects of the drug and drug combinations were evaluated after exposing tumor bearing specimens of identical age group to the therapeutic dose determined by repeated trials after 'tumor take'. Tumor regression upon drug exposure was assessed by: morphometric study of tumor volume, cell count and dead cell frequency, and metaphase index study. A maximum regression of ascites tumor (Potential cure) for CP single therapy series was noted on day 4, at a dose equivalent to 167 mg CP per Kg body weight. A maximum regression in Combination I (VC followed by CP) and Combination II (VC followed by CP followed by VC) was recorded on 3rd day of last drug administration @ 1 mg VC + 66.7 mg CP (for Comb.I) and 1 mg VC + 66.7 mg CP + 0.33 mg VC (for Comb. II)respectively. Mean Survival Time (MST) was also estimated and compared with that of the normal and control.

Meiotic chromosome complements of tumor bearing mice : Characterization of meiotic chromosome complements of mice with transplanted tumor was done by conventional Giemsa staining. The diakinesis-metaphase I stage of spermatocytes revealed the existence of 20 bivalents of which 19 were autosomal and the rest was XY. The autosomal bivalents were mostly ring or quadriradial in appearance while the sex bivalent was rod-like with the X and the Y chromosomes disposed in a long arm-long arm pairing or association. Spontaneous ploidal variation in

terms of aneuploidy and polyploidy was noted but never to a significant extent compared to normal mice. No structural chromosome abnormality at diakinesis-metaphase I stage was encountered in tumor bearing mice.

The effect of the drug and drug combinations was assessed both at spermatocytic as well as spermatozoa level at certain chosen time points : 4hr, 24hr, 72hr, 120hr, 168hr,288hr,312hr and 336hr of _in vivo_ drug exposure. A parallel control was maintained with S180 tumor bearing mice (of the same age group) exposed _in vivo_ to drug solvents ie., either sterile distilled water or diluting fluid as the case may be, for identical time points selected for treatment series.

The major cytotoxic effects of the drug and drug combinations include : Chromosome univalency, Ploidal anomaly, and chromosome structural changes involving both autosomes and sex chromosomes.

Sex chromosome univalency,referred to as precocious desynapsis of XY bivalent in the text,was induced to a less significant extent compared to autosomal univalency in different treatment series. The application of drug combinations (both the schedule) was found to be more effective in comparison to the single administration of CP so far as the phenomenon of chromosome univalency was considered.

Ploidal variations were recorded in the form of aneuploidy (both hypo- and hyperploidy) and polyploidy (mostly tetraploidy) at diakinesis-metaphase.I stage. The

incidence of aneuploidy in a higher frequency was documented from the very beginning of the experiment while the frequency of cells with polyploid chromosome counts to a significant extent was recorded from 1 day onwards in the present experiment. The incidence of polyploidy was significantly higher at all post treatment time points in Combination II treated series compared to CP single therapy. The precise mechanism which caused significantly higher incidence of polyploid cells within a short time period remains speculative. The possibility of cell fusion in response to drug administration has been assumed.

Structural chromosome abnormalities, mostly in the form of autosomal multivalents in diakinesis-metaphase I cells were observed predominantly in tumor bearing specimens subjected to CP single therapy. The effect was recorded to a significant extent from day 5 onwards and continued upto the end of the present experiment. In contrast, the occurrence of sex-autosome multivalents in response to drug exposure was documented to a much lesser extent in different treated series (significant at 1% level only at one time point in CP treated series). Interestingly , the induction of chromosome structural abnormalities in the form of autosomal multivalents although documented to a significant extent at several post treatment time points in Comb.II treated specimens, its occurrence was not equally frequent in specimens exposed _in vivo_ to Comb.I drug schedule.

The occurrence of chromosome structural changes within five days of the last exposure of the drug or drug combinations has been explained according to the following :- since a five day time period is not sufficient for spermatogonial stem cells (affected at S phase of the cell cycle) to enter diakinesis stage, it is proposed that the drug/drugs either affected repair synthesis of DNA at pachytene or else interfered with Unscheduled DNA synthesis (UDS) at any later stages of meiosis which was finally manifested into structural chromosome changes recorded at diakinesis-metaphase I stage in the present experiment. This explanation also fits well with the time sequence established for mouse spermatogenesis by different laboratories.

The effect of short term exposure of CP and CP-VC combinations on sperm head morphology and epididymalsperm count has also been assessed. It was revealed that the drug or drug combinations although not effective in inducing structural alterations of epididymal sperm head within the time points examined, caused significant depletion in total sperm count and loss of testis weight from day 12 onwards. It has been suggested that CP alone at therapeutic dose can affect epididymal sperm pool of S180 tumor bearing mouse which in turn results in reduction of organ weight.

The clinical and cytogenetic s'gnificance of CP single & CP-VC combination induced germ cell cytotoxicity has been discussed. The present study on S180 tumor bearing mice, in association with the results published by other investigators in this field point, the potential risk of CP involved drug combinations to human patients and represent useful contribution in further studies of cancer patients subjected to single and combination drug therapy.

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LIST OF RESEARCH PUBLICATIONS.

Dey D, Das SB and Chakrabarti S. 1986 : Premature disjunction of XY bivalent in response to mitomycin C. MOUSE NEWSL. 75 : 21.

Das SB, Dey D, Chakrabarti A. and Chakrabarti S. 1986 : Selective endoreduplication of robertsonian marker chromosome in a murine tumor cells (S180). CELL & CHR.RES. 9 : 28.

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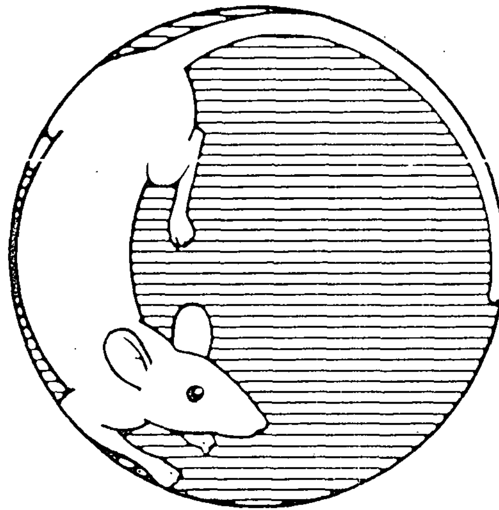

JULY 1986

No. 75

BURDWAN UNIVERSITY

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Received : 18 May 1986

Research News

Premature Disjunction of XY bivalent in response to Mitomycin Treatment

Out bred strain of male Swiss albino mouse, *Mus musculus* when subjected to mitomycin C treatment at therapeutic dose (0.3ml per 25 g from a stock soln.prepared by dissolving 2 mg potency mitomycin C in 5ml sterile distilled water) for 24,48,and 72 hr showed premature disjunction of XY bivalent in diplotene/early diakinesis. The data obtained showed a statistically significant difference ($p < 0.05$) with control (distilled water injected) and normal. The effect is not dose dependent but a maximum frequency of cells with separated X and Y chromosome was noted at 24 hr (38%). A similar effect of this widely used antitumour agent has not been documented earlier.

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SELECTIVE ENDOREDUPLICATION OF ROBERTSONIAN MARKER CHROMOSOME IN A MURINE TUMOUR CELL (S 180)

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ABSTRACT

The appearance of a new, stable and transmissible biarmed marker chromosome has been documented in ascities form of mouse sarcoma 180 cell line maintained *in vivo* in outbred strain of Swiss albino mice. Morphometric analysis and banding study revealed that the new marker is identical with the original biarmed marker already present in the cell line, and has been formed by selective endoreduplication of the existing marker.

Robertsonian translocation—formation of one biarmed chromosome by fusion between two acrocentric or telocentric chromosomes has been the subject of investigation since the time of its discovery by Robertson (1), particularly after the introduction of the term by Matthey (2). Its role in tumour karyotype evolution is a matter of controversy (3, 4). Almost all murine tumour cell lines, particularly the ascites forms possess one or more rb-marker chromosomes (5). But their exact significance in tumour adaptation has not been clearly established.

Mouse sarcoma 180 (S 180) is one of the oldest known murine tumour cell lines, originated as a 'spontaneous' breast carcinoma in a male mouse (6). Friend (7) developed the ascites form by repeated intraperitoneal transplantation of the minced solid tumour. The first report on the chromosome complements of S 180 showed a modal number of 86 with no identifiable marker chromosome (8). Bianchi et. al. (9) reported as many as 10 marker chromosomes in this cell line ('S 180A) maintained *in vitro* (10), Chakrabarti and Roychoudhuri (11) studied the chromosome complements of this cell line maintained *in vivo* in close inbred strain of Swiss mice and found the existence of one biarmed marker chromosome. Ghosh and Chaudhuri (12) confirmed the existence of one biarmed marker chromosome in this cell line and by the help of

improved techniques they analysed all the marker chromosomes present in this cell line. In the present paper we report the occurrence of a new rb-marker chromosome in this cell line now maintained in outbred strain of Swiss albino mice.

Materials and Methods :

The ascites form of mouse sarcoma 180 is maintained by serial intraperitoneal transplation in outbred strain of Swiss albino mouse with an inoculum size of 1 × 10^6 cells per 3 month-old individuals. On the fifth day of tumour transplantation, chromosomes were prepared from the ascites fluid after 1.30 hr of colchicine exposure (10). Slides were prepared by air drying and stainied in Giemsa stain diluted in phosphate buffer (1:20) at a pH 6.8. C—band was performed by slight modification (13) of the the technique of Sumner (14) and for G—band the technique of Seabright (15) was followed.

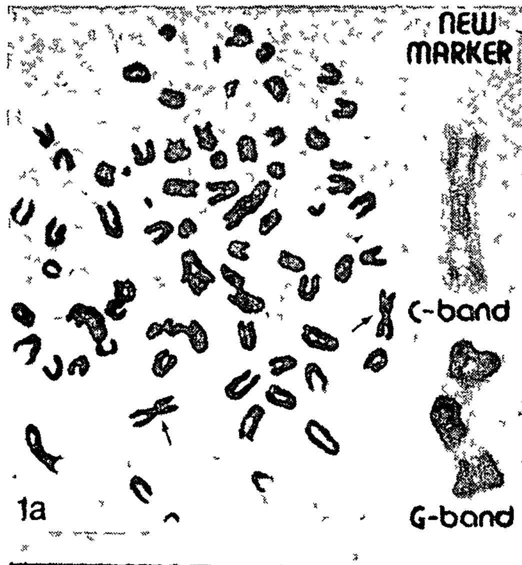
Observations and Discussions :

More than 40 successive tumour cell generations were studied and the chromosomal findings from 50 metaphases at each cell generation revealed that about 45% metaphase population possess a new biarmed marker chromosome along with the three marker chromosomes already present in the cell line (10 13, 16 (Fig. 1a). About 35% of the remaining metaphases on the other hand displayed only one such marker chromosome (Fig. 1b). Rest of the metaphase population showed an irregular distribution of marker chromosome, hence not taken into account for present purpose.

C—band analysis of the new marker revealed the existence of two closely situated dark spots in the middle region of early metaphase chromosome while condensed late metaphase chromosomes showed a single extended C—band in the centromere region. The C—band profile of this new marker is exactly identical with that of the original rb-biarmed marker chromosome of the cell line (10).

G—band study also indicated an exactly identical band profile in the two biarmed marker chromosomes. It thus appeared that this new marker like the old one is also a product of rb-fusion between two nonhomologous chromosomes, viz., t(16; 14) (Fig. 1a). The identification of the chromosomes involved in rb-fusion has been made by following Cowell (17).

The occurrence of one or more biarmed maker chromosomes in mouse ascites tumour cell lines has been reported by different investigators (5, 9-12). In all known cases the biarmed makers were formed by fusion between nonhomologous members of the karyotype. However, recently Spira (18) has documented the existence of the t (2; 17) rb homozygous marker chromosomes in mouse T-cell leukemia. But the possible mechanism of the formation of such marker in two copies has not been mentioned. The present findings on the occurrence of Robertsonian marker chromosomes viz., t (16;14) in two copies in S180 cell line will add further cytological data in the field of murine tumour



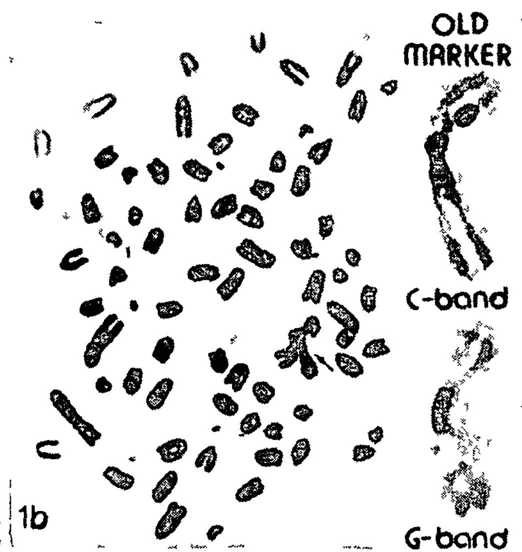


Fig 1a. Metaphase spread from S180 tumour showing two biarmed marker chromosomes.

Fig 1b Metaphase spread from S 180 tumour showing only one biarmed marker chromosome.

chromosome evolution Since the new marker resemble the old one in band pattern and there occurred no reduction in modal number of the cell line, we suggest that this new marker has originated by selective endoreduplication of the old one. Spontaneous occurrence of endoreduplication of S180 chromosomes (part or whole) has been reported earlier by Chakrabarti and Roychoudhury (11). The presence of the new marker chromsome in about 45% metaphases and its perpetuation through successive cell generations indicate that like many other mouse ascites tumours (5, 19) the karyotype of S180 still is on the way of reconstruction and the presence of a new Robertsonian marker may be needed for the adaptation of this tumour strain in a new strain of murine host

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Precocious Disjunction of XY-Bivalent in Mouse Germinal Cells Exposed *in vivo* to Mitomycin C

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Anticancer-antibiotic mitomycin C was found for the first time to induce precocious disjunction of sex bivalent in mouse spermatocyte The effect is nonrandom because a similar result has not been documented in any of the autosomal bivalents

Mitomycin C (MC), an anticancer-antibiotic, is now widely used for the regression of various forms of human cancers[1-3] Being a polyfunctional alkylating agent, MC acts on chromosomal DNA and impairs DNA replication by cross-linking base pairs, particularly the G-C moiety of the DNA duplex[4] After reduction MC can form covalent bonds with nucleophilic groups in a variety of molecules including RNA and protein[5] Various cytological effects of MC have been reported[6-8] Its role in the production of differential C-band in mouse tumour chromosomes *in vivo* has also been discussed in recent years[9] But very little is known about the effect of MC on the gonial cells of mammals[10] During the course of our investigations on the effect of MC on somatic and germinal cells of S180 tumour bearing mouse (a response to therapeutic stress), we noted an interesting and previously unknown effect of MC on the sex bivalent of mouse

Materials and Methods

The 16 week old out-bred strain of Swiss albino mice were subjected to MC treatment @ 0 3 ml per 30 g body weight (therapeutic dose for S180 tumour model[9]), from a stock solution prepared by dissolving 2 mg potency MC (Kyowa Hakka Kogyo Co Ltd, Tokyo) in 5 ml sterile distilled water Gonial chromosomes were prepared from testis after 24 hr of *in vivo* drug exposure by little modification of the technique suggested[11] One modification was that the 'acetic acid treatment' step for seminiferous tubules had been deleted A parallel control was of specimens injected with sterile distilled water only The meiotic chromosomes of both treated and control specimens were stained in Giemsa stain diluted in phosphate buffer at a *p*H 6 8

Results

Normal mouse spermatocytic diakinesis/metaphase I contains 19 autosomal bivalents and an XY sex bivalent (Fig 1) The bivalent condition is clearly detectable from diplotene to metaphase I An analysis of 200 diakinesis/metaphase I cells from four treated specimens revealed a number of cells (about 38%) where the Y chromosome showed precocious dissociation from the XY pair and in most of the cases remained displaced, far from the X (Fig 2) A similar phenomenon has not been seen in any of the autosomal bivalents The XY in control specimens, on the other hand, maintained the characteristic bivalent condition throughout the meiotic prophase I In addition to precocious disjunction, the XY bivalent in some cells showed certain other abnormalities which have neither been recorded in autosomal bivalents of the same (MC treated) specimen nor in the germinal cells of control specimens These include unequal decondensation and/or despiralisation and uneven condensation of X chromosome alone or both –X and Y (Table 1) Similar abnormalities including precocious disjunction of XY were also recorded at other post-treatment

Table 1—Frequency Distribution of Chromosome Abnormalities in XY Bivalent after MC-treatment

Hr of mitomycin exposure	Precocious disjunction of XY (%)	Despiralization of XY bivalent (%)	Unequal condensation of XY bivalent (%)	Unequal condensation of X chromosome only (%)
24 hr*	38 (–)	8 (2)**	4 (–)	5 (–)

*Out of 200 diakinesis/metaphase I studied from 4 specimens
**Figures in parentheses indicate control value

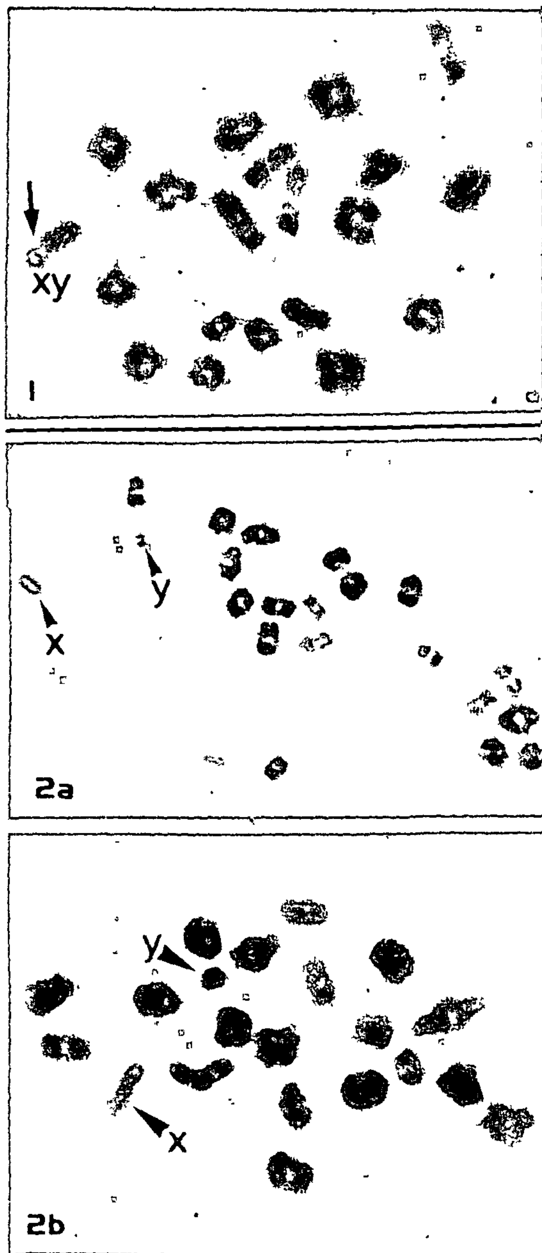

Fig. 1—Diakinesis spread from a normal male mouse showing 19 autosomal and one XY sex bivalents, arrow points out the position of XY bivalent Fig 2—Diakinesis spreads from mitomycin C treated male mouse showing precocious disjunction of XY (arrow)

intervals but in a very low frequency. In no case, however, the control or the normal specimens exhibited identical abnormalities.

Discussion

Report on the occurrence of spermatocytic chromosome abnormalities due to chemical exposure in mammals is very limited[10]. The mechanism by which MC affects the sex bivalent and causes precocious disjunction of XY is difficult to ascertain. One of the possibilities includes its clastogenic potentiality on heterochromatin. The Y chromosome of mouse and other mammals is almost exclusively heterochromatic. During meiosis in mouse the X and Y chromosomes like other autosomal bivalents remain associated from pachytene to metaphase I. The precise orientation of X and Y during meiotic prophase I of mouse and other mammals is controversial. Two contradictory schools exist. It has long been assumed that there is a homologous pairing segment of variable lengths in the XY pair of all mammals and an obligatory chiasma is formed in the region which keeps the two chromosomes together from pachytene to metaphase I[12,13]. In mouse, synapsis from the distal ends of the acrocentric X and Y has been reported to involve up to 90% of the Y and 30-35% of the X[14]. According to the other school, the synapsis between X and Y in mouse and man is nonhomologous[15]. Crossing-over does not occur as a normal event in this pair, and orientation of XY is mediated only by an achiasmatic telomeric association[15,16]. It may be assumed that MC acts on the cementing protein that keeps XY chromosomes together throughout meiotic prophase I and thereby causes precocious disjunction of XY bivalent during diakinesis. Such a possibility is, however, not very unlikely, because it is known that, in addition to its effect on chromosomal DNA, MC can also form covalent bonds with nucleophilic groups in a variety of molecules including RNA and protein[5].

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M[illegible]TIC CHROMOSOME ANALYSIS OF S 180 TUMOR BEARING MICE IN RESPONSE TO SINGLE DRUG THERAPY

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SUMMARY

Sarcoma 180 [illegible] mice were exposed *in vivo* to therapeutic dose of Cyclophosphamide [illegible] Specimens subjected [illegible] 24, 72, 120 and 168 h/s after the drug [illegible] chromosome display at [illegible] were [illegible] frequent than the sex [illegible] the induction [illegible] the [illegible] to drug therapy.

INTRODUCTION

[illegible] been [illegible] [illegible] through [illegible] DNA synthesis. Uptil now very little has been [illegible] particularly during the course of tumor [illegible] been [illegible] of a tumor bearing mouse.

MATERIAL AND METHODS

Ascites form of mouse sarcoma 180 [illegible] were [illegible] Swiss albino mice with [illegible] the [illegible] when the tumor [illegible] dose of Cyclophosphamide [illegible] was injected. The therapeutic dose [illegible]

[illegible] was standardised by repeated [illegible] control was maintained [illegible] sol [illegible] tende list [illegible] In [illegible] minal [illegible] fluid [illegible] at 24, 72 [illegible] regression was studied by [illegible] analysis of [illegible] (*ii*) dead cell count using [illegible] metaphase index study from PMG stained smear preparation. Gonadal chromosomes were prepared from testis by little modification of the technique of Ona *et al.* (1979). The meiotic chromosomes were stained in Giemsa stain diluted in phosphate buffer at pH 6.8.

OBSERVATIONS

'Potential cure' of S 180 tumor was noted at 120 hrs of CP exposure at therapeutic dose and regression spectrum was determined and confirmed by metaphase index frequency study.

Meiotic diakinesis/metaphase I of normal mouse spermatocyte contains 19 autosomal and an XY sex bivalent. The autosomal bivalent condition is clearly detectable from diplotene to Metaphase I from conventional Giemsa stained preparation. The XY bivalent shows [illegible]

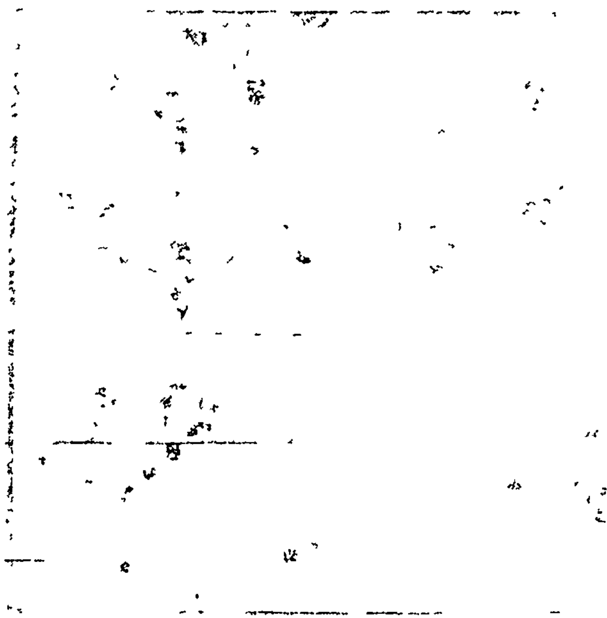

FIG. 1 (*a*) Normal meiotic diakinesis/Metaphase I

FIG. 1 (*b*) Arrow indicates autosomal [illegible]

FIG. 1 (*c*) A cell carrying two [illegible]

FIG. 1 (*d*) Sex chromosomal univalent

go pairing throughout the meiotic prophase I. Large number of brightly stained meiotic cells were screened for locating well spread stages of diakinesis, Metaphase I with a view to scoring changes both structural and numerical and any special behaviour of autosomes and sex chromosomes. Among the abnormalities in drug exposed specimens the univalent behaviour of certain homologues ranked first. This univalency (Fig 1) seemed to be random in nature. The autosomal univalents involving the smallest pair were more frequent than the sex chromosomal univalents. The sex chromosomal univalency was recorded maximum in 24 hrs. treatment series (10%) and autosomal univalency was maximum in 72 hrs. (26%) of drug exposure. The smallest autosomal univalents were frequently involved in the process. The frequency of autosomal and XY univalency is given in Table 1.

TABLE 1. Frequency distribution of chromosome abnormalities in Autosomal and XY *bivalent after Cyclophosphamide treatment*

Hrs. of CP Exposure		*No. of XY Univalents*	*No of Autosomal Univalents*	*Total % of Univalents*
4	Control	7	12	19
	Treated	7	18	25
24	Control	6	8	14
	Treated	10	16	26
72	Control	6	12	18
	Treated	9	26	35
120	Control	0	10	10
	Treated	5	24	29
168	Control	5	10	15
	Treated	7	25	32

DISCUSSION

Antibiotics (Subramanyam and Reddy, 1975), tranquilisers (Kamada, *et al.*, 1971, Subramanyam and Murthy, 1975) hallucinogens (Cohen and Mukherjee, 1968 Fernandez *et al.*, 1973) oral contraceptives (Carr, 1967), adeiphane (Jameela and Subramanyam, 1979), Sulpha drugs (Sharma 1971), Paracetamol (Laxminarayana *et al.*, 1980) and many other commonly used drugs are known to cause meiotic chromosome aberrations in mice. The reported occurrence of univalency in response to above drug exposure is not very uncommon. Dissociation of XY bivalents in

diakinesis/metaphase-I spermatocytes was reported by exposure to various physical agents specially temperature (Waldbieser and Chrisman, 1986; Garriot and Chrisman, 1980). Univalency has also been reported by single treatment of Mitomycin C (Chakrabarti *et al.*, 1986). In the present experiment we noted a gradual increase in autosomal univalency with the increase in treatment hr. and in decrease in tumor population. The situation is, however, different in case of sex chromosome univalency. There is controversy regarding the genetic consequence of univalency. In our experiment we noted that the smallest autosomal pairs are prone to univalency. This may be due to the fact that the binding force in the form of chiasmata is not too many in number in these univalents and are not strong enough to keep the chromosomes together as bivalents, hence appear to desynapse precociously either spontaneously or under the influence of various agents. The presence of univalents in controls in a low frequency is not surprising as they are reported to occur spontaneously in many untreated materials (Lin *et al.*, 1971; Beechey, 1973; Purnell, 1973). According to Biddle *et al.* (1985), sex chromosomal univalency is genetically controlled. The presence of univalents reflects asynapsis or early separation due to a faulty chiasma formation and may be considered to indicate non-disjunction inducing potential (Pacchierotti *et al.*, 1983). According to Brewen and Preston (1978) univalents may result from minor structural rearrangements such as deletion. Another alkylating agent, Ethylene Oxide increases the frequency of cells with autosomal and XY univalents and according to Ribeiro (1987), the occurrence of univalents is due to the possibility that this substance induces non-disjunction. Many cytogenetic studies on subfertile mice and men have revealed prematured separation of the X and Y chromosomes and a low incidence of subsequent meiotic stages in the testis (Beechey, 1973; Chandley, 1973; Chandley *et al.*, 1976; Burgyoni, 1979). These researchers have concluded that X-Y dissociation leads to death of the affected spermatocytes, thus lowering fertility. The way by which CP at therapeutic dose produces autosomal as well as sex chromosomal univalency in the present experiment is not clear, but it is evident from the comparative data that the drug can affect meiocytes even at early hrs. of *in vivo* exposure long before the spermatogonia enter meiosis and can produce numerical chromosome abnormality of the host. The significance of this precocious separation in response to drug exposure is far reaching.

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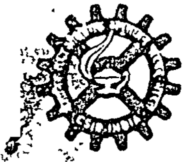

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Title Page

PRECOCIOUS DESYNAPSIS OF XY-BIVALENT IN MOUSE GERMINAL CELLS : INFLUENCE OF MITOMYCIN C ON TUMOUR BEARING AND NORMAL MICE

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Key words : Sex bivalent, precocious desynapsis, S180 tumour model, anticancer drug, chemotherapy, sterility.

Abrr.used : MC = Mitomycin C ; SC = synaptinomal complex

SUMMARY :

A comparative study on the cytotoxic potential of anticancer antibiotic mitomycin C (MC) has been made on tumour bearing and normal mice considering precocious desynapsis of sex bivalent (in male germinal cells) as parameter. The study indicates a strikingly differential effect of the drug on the phenomenon in two different types of mice. The administration of MC at therapeutic dose although enhances the frequency of precocious desynapsis of XY-bivalent in normal mouse to a significant extent (compared to control), the same drug at the same dose fails to produce a similar effect in tumour bearing specimens. Discussions have been made on : i)the probable cause of this differential effect,ii)the mechanism of MC action on precocious desynapsis of sex bivalent and,iii) the possible significance of the findings in relation to cancer chemotherapy.

Cont. 3

INTRODUCTION : Cytogenetic assays on the genotoxic potential of drugs and chemicals are of continuous importance. Hsu (16) advocated that an increase in chromosome abnormalities in somatic cells may enhance the chance of developing neoplasia while in germinal cells any such abnormalities may lead to a high frequency of spontaneous abortion,birth defects and heritable chromosome rearrangements.

Of various therapeutic modalities adopted in recent years for a 'potential cure' of different human malignancies,chemotherapy has become much popular for some obvious reasons. Although one of the pathways of action of most antitumour drugs is chromosome damage, cancer therapeutic agents have not,as a rule,been selected by studying their effects on somatic and germinal cells of the host. Mitomycin C, an anticancer antibiotic is extensively used to treat various kinds of human malignancies (6,18,22). Its use as single therapeutic agent in the regression of breast carcinoma and leukemias, particularly in the treatment of CML is fast increasing (8). The drug is also effective against sarcomas when applied in appropriate combination with other antitumour agents of diverse nature. Being a polyfunctional alkylating agent,MC impairs DNA replication by cross-linking base pairs particularly the G-C moiety of the DNA duplex (27). Upon metabolic activation,the drug transformed into very reactive alkylating agent and after reduction it can form covalent bonds with nucleophilic groups in a variety of molecules including RNA and protein (30). Various cytotoxic effects of MC have been reported. But most of these reports are on somatic cells of man and other mammals(21,25). The

Cont..4

production of chromosome structural changes in somatic and germinal cells of mouse upon exposure to MC has been reported by Adler (1). MC induced premature disjunction of sex bivalent in mouse germ cells has been published from this laboratory (9). More recently, Allen et.al.(2) have recorded an extesive SC damage in mouse spermatocytes upon exposure to MC. In the present paper we report on the influence of MC on precocious desynapsis of XY-bivalent in germinal cells of the normal(ie.,non-tumour bearing)and tumour bearing male mice.

MATERIALS AND METHODS :

The Animal Model : Out bred strain of Swiss albino mouse, *Mus musculus* of about the same age group constituted the animal model for the present investigation. Out bred mice were chosen because of their close resemblance with genetically heterogenous human population (23). Two month old male mice,procured from an animal supplier of Calcutta, were housed in the animal house and maintained on standard mice feed and water for atleast four weeks before exposure to MC.

The Tumour Model : Out bred strain of male mice bearing the ascites form of sarcoma 180 (s180) constituted the other experimental model for the present investigation. The s180 tumour cell line,originally procured from Chittaranjan National Cancer Research Centre,Calcutta, is maintained in this laboratory for last 12 years by serial intraperitoneal transplantation into 3-mo-old Swiss albino mouse.

The drug and the treatment schedule: Stock solution of MC was prepared by dissolving 2 mg potency MC (manufactured in India by Biochem Pharmaceutical Industries under licence: Kyowa Hokko Kogyo Co.Ltd., Japan). Both the(non-tumour bearing)normal and s180 tumour bearing male mice of the same age group(3 mo-old) received intraperitoneal

CONT. 5

injection of MC at therapeutic dose(4mg.per Kg.body weight). The therapeutic dose for this s180 tumour model was established by repeated trials on tumour bearing mice at Log phase of tumour growth. A maximum regression (as determined by morphometric analysis of tumour volume, dead cell frequency and mitotic index) was noted after 120 hr of in vivo drug exposure. In either cases drug exposed specimens were sacrificed by spinal dislocation at five different post-treatment intervals: 48, 72,96,120 and 168 hrs. Concurrent controls with two specimens per time point exposed to sterile distilled water was also examined. In the two treated series six specimens were sacrificed at each time point and in each case.

Meiotic chromosomes from seminiferous tubules of the treated and the control specimens were prepared by following the technique of Oud et.al. (24). In brief: seminiferous tubules were collected after removing the tunica albuginea of testes in 1% tri-sodium citrate solution. After 30 minutes of hypotonic exposure seminiferous tubules were kept in ethanol: acetic acid fixative (3:1 v/v)for 30 min.at 8^{0}C. After primary fixation tubules were dissolved in 30% glacial acetic acid, centrifuged at 1,500 rpm for final fixation in ethanol:acetic acid (3:1) fixative. After one day preservation slides were prepared by following air dry technique. The meiotic chromosomes of both control and treated specimens were stained in Giemsa stain diluted in phosphate buffer (1:10 v/v) at a pH 6.8.

RESULTS : Normal mouse spermatocytic diakinesis/metaphase-I contains 19 autosomal bivalents and one XY sex bivalent (Fig.1). The bivalent condition is clearly detectable under microscope from diplotene onwards to metaphase I. The X and the Y chromosomes show an end-to-end

CONT: 6

association/pairing and the autosomal bivalents,depending on the position of chiasmata,exibit ring,rod or quadriradial configurations (Fig.1).

An analysis of 400 well spread diakinesis/metaphase-I stages from control specimens revealed the existence of about 11% spermatocytes in which X and Y chromosomes showed precocious desynapsis with no typical end-to-end association (Fig.2). The normal value,established by analysing 100 diakinesis/metaphase-I stages from 6 male individuals also resembled the control value (hence they were plotted together in Fig.3).

An examination of the treated specimens at 5 different post-treatment intervals,on the other hand, revealed a significant shift in the frequency of XY separation from the control and the normal value. The histograph presented in figure.3 depicts the frequency distribution of diak./meta-I stages with precociously desynapsed XY bivalent in normal/control and the different treated specimens. It is evident from the histograph that the administration of MC at therapeutic dose influenced the frequency of precocious desynapsis of sex bivalent to a considerable extent.The frequency of separation was significantly higher at each time point in drug exposed non-tumour bearing specimens. A maximum frequency of about 20% cells with separated XY bivalent was noted at 96 hr of drug exposure while a minimum frequency of 13% was documented at 72 hr.of MC exposure. A steady peak was maintained at other time points upto the end (ie.,168 hr) of the experiment. A different and to some extent a contrasting situation was documented in the other experimental series. The frequency of precocious separation of XY bivalent in drug exposed s180 tumour bearing mouse was either almost at par or else lower than the control value (Fig.3).

CONT. 7

A maximum depletion of separation frequency was documented at 96 hr of post-treatment interval when the frequency of precociously desynapsed XY bivalents dropped down to about 3%. The frequency at other time points although showed an increase,never (except for 48 hr) reached the control/normal value.

DISCUSSIONS : Premature desynapsis of the XY bivalent with respect to autosomes has been repeatedly noted by various investigators. Chandley and Speed (11) documented about 6% precocious desynapsis of sex bivalent in normal mouse. Earlier Evans et.al.(14) also reported 5-10% separation of sex bivalent in normal mouse. Lyon et.al. (20) proposed pairing failure as possible cause behind XY univalency in diak./meta-I in mouse and other mammals. But this has been criticised by other workers on various grounds (see Chandley and Speed,1987). Ashley (3) imposed adaptive significance on the phenomenon and suggested that precocious separation of XY bivalent may serve to prevent genetic exchange along an extensive portions of the non-homologous synapsed region. In the present study we also recorded about 11% cells with precocious XY desynapsis in control/normal mice which showed a significant increase (as high as 20%) after MC administration in non-tumour bearing mouse at all post treatment time points. This clearly points out that MC has definitive influence on precocious separation of XY bivalent in male mouse. It may be cited in this connection that one of the main pharmacological sanctuary sites for the cytotoxic drugs in the body is testicle (8). Hence it is not very unlikely that the drug,MC upon entry into the body of the host will transported to the testes and produce various cytotoxic effects including premature desynapsis of XY bivalent in proliferating germ cells.

CONT. 8

A different result was obtained in the other experimental series where the meiocytes of S180 tumour bearing mice were exposed identically to the same therapeutic dose of the drug. There was a steady fall in the frequency of XY separation at all post treatment intervals in this series except for 48 hr when the frequency was almost at par (12%) with that of the control/normal. Interestingly, a maximum decrease was noted at 96 hr of treatment in tumour bearing mouse when the frequency was only 3% against 20% recorded in non-tumour bearing specimens in one hand, and 11% in control/normal on the other. This is difficult to explain. It is true that upon entering into the blood stream, MC in tumour bearing specimens rushed to the target tissue and the bulk of the drug was utilised at the tumour site leaving very little or no drug to be available for testes or other tissue or organs . This, however, is only a partial explanation of the situation because, at all post treatment time points (except for 48 hr) the frequency of separation was lower than the control. One possibility may include an increase in intracellular level of protective agent like glutathione in testes and other organs of tumour bearing mice.

The precise mechanism by which MC affects XY pairing (of mouse) and causes precocious desynapsis is difficult to ascertain. Several possibilities may be discussed. The clastogenic potential of MC on chromosomal heterochromatin is well documented. The Y chromosome of mouse and other mammals is almost exclusively heterochromatic and MC is known to have preferential effect on chromosomal heterochromatin (12,15,17). It may, therefore, be assumed that MC acted on Y heterochromatin and thereby caused breakage which in turn results in sex chromosome univalency in treated specimens. Since in our present preparation no structural abnormality

CONT . 9

in the form of break or deletion was noted in the X and the Y chromosomes it is difficult to conclude that a breakage-separation event was in operation.

During meiosis, the X and the Y chromosome of mouse,like other autosomal bivalents remain associated from pachytene onwards to meta.I. The precise orientation of X and Y chromosome during meiotic prophase-I of mouse is controversial. Two contradictory schools exist. It has long been assumed that there is a homologous pairing segment of variable lengths of the X and Y pair,and an obligatory chiasma is formed in the region which keeps the two chromosomes together from pachytene to metaphase-I (11,19,26). According to the other school the synapsis between X and Y in mouse is nonhomologous (4). Crossing over never occurs as normal event in this pair and orientation of X and Y is mediated only by an achiasmatic telomeric association (3). It may be assumed that MC acts on the cementing portion of SC that keeps the X and Y chromosome together for a short segment throughout meiotic prophase-I and thereby causes precocious disjunction of X and Y bivalent. Such a possibility is not very unlikely; because it is known that, in addition to its effect on chromosomal DNA, MC can form covalent bonds with nucleophilic groups in a variety of molecules including RNA and protein (30). Moreover, a report on the extensive SC damage in mouse spermatocytic cells upon MC administration to a variety of doses has been published (2). The study of Allen et al.(2) indicated that among mouse, chinese hamster and American hamster, the synaptonemal complex of mouse is most sensitive to MC action. Regarding the selective separation of XY bivalent it may be suggested that these two chromosomes remain associated only for a short segment by terminal association (see Ashley, 1985) and become the primary target for any

cytotoxic insult offered by the chemical or physical agent which compells them to separate precociously long before the onset of anaphase-I when other autosomal bivalents remained synapsed throughout the length.

It is thus evident that MC, which is now widely used to treat various forms of human malignancies, has influence on precocious separation of sex bivalent in normal and nontumour bearing mouse. There are report which suggests that premature XY dissociation of mouse and man leads to the death of affected spermatocytes thus lowering fertility (5, 7,10,29). Reduction in testis weight in specimens with higher degree of XY separation has also been documented by Chandley and Speed (11). Further study is thus needed to establish this previously unknown cytotoxic potential of MC and to impose any restriction to its frequent use in treating various human cancer patients.

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5 On the Stability and Significance of a Robertsonian Marker Chromosome in S 180 Mouse Tumour.

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Cytogenetic characterization of Sarcoma 180--a long-reared murine tumour has been done for several successive *in vivo* cell generations by the application of modern methologies including G—,C—,Q—, and N—banding. Sister choromatid differentiation (SCD) and SCE studies were also made by *in vivo* BrdU-chasing technique. The formation and perpetuation of a stable biarmed marker chromosome have been traced and its resistance to clastogenic effect of several anticancer drugs has been discussed.

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7. Premature Disjunction of XY Bivalent in Mouse Germinal Cells in Response to Chemotherapeutic Stress.

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The synapsis between the X and the Y in mouse is nonhomologous, and in normal meiosis X and Y chromosomes, like other autosomal bivalents remain associated from pachytene onwards to metaphase I. The precise orientation of XY bivalent is not clearly known. In the present study we noted premature disjunction of XY bivalent in tumour-bearing mouse in response to chemotherapeutic stress offered by a number of antitumour drugs. In all drug treated cases a significant number of cells showed premature separation of sex bivalent either in diakinesis or in early metaphase I. The effect is unusual and nonrandom in nature. A similar kind of effect has not been documented in any of the autosomal bivalents. Discussions are made on the significance of premature disjunction of sex bivalent in relation to cancer therapy.

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302 MEIOTIC CHROMOSOME ANALYSIS OF S180 TUMOUR BEARING MICE IN RESPONSE TO DRUG THERAPY

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Sarcoma 180 tumour bearing mice were exposed *in vivo* to therapeutic dose of Cyclophosphamide, an anticancer alkylating agent. Specimens subjected to therapy were sacrificed at 4, 24, 72, 120, 168h after the drug exposure and the testes were processed for meiotic chromosome display at diakinesis/metaphase I complex Mitoclastic effects of the drug were visualized by the presence of univalents at a significant level when compared with that of the control. The autosomal univalents were more frequent than sex chromosomal ones Discussions have been made on the induction and the possible consequence of univalency due to drug therapy.