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Molecular cytogenetic analysis of chemically induced aneuploidy in germinal and somatic cells of mice: studies with topoisomerase II inhibitors

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To my Family

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CURRICULUM VITAE

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LIST OF PUBLICATIONS:

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CONTENTS

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMCA	7-amino-4-methylcoumarin-3-acetic acid
ATP	Adenosine triphosphate
BACs	Bacteria artificial chromosomes
bp	Base pair
CI-1	Hamster cell line derived from embryonic lung cells
CREST	Calcinosis, Raynaud's phenomenon, Eosophageal dismobility, Sclerodactyl and Telangiectasia
d	Day (s)
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytidine Triphosphate
dGTP	Deoxyguanosine Triphosphate
dH_20	Distilled water
DNA	Deoxyribonucleic acid
Fig.	Figure
g	Gram
µg	Microgram
GSF	Forschungszentrum für Umwelt und Gesundheit
h	Hour (s)
ISIS3	*In situ* imaging system
kb	Kilobase pairs (kilobase unit of length of 1000 basepairs)
kg	Kilogram
L	Liter
µL	Microliter
LBNL	Lawrence Berkeley National Laboratory
LD50	Dose that kill 50% of animals
LINL	Lawrence Livermore National Laboratory
M	Molar mass
MER	Merbarone
mM	Milimole
nM	Nanomole
µM	Micromole

M-FISH	Multiplex-Fluoroscence *in situ* hybridization
mg	Milligram
min	Minute
ml	Millileter
N	Normal solution
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolution per minute
SD	Standred diviation
SKY	Spectral karyotyping
VP-16	Etoposide
UV	Ultraviolet
χ^2	Chi-square -Test
YACs	Yeast artificial chromosomes

A. INTRODUCTION

1 General Overview

Topoisomerase (topo) II is an essential enzyme, which relieves torsional stress in DNA during transcription, replication and cell division by causing transient cleavage of DNA, strand passing and religation (Wang, 1996). The inhibition of topo II is a very powerful principle of cancer chemotherapy and topo II inhibiting drugs are the backbone of most chemotherapeutic strategies. As well as inducing cell death because of both 'mitotic catastrophe' and the induction of apoptosis (Lock and Stribinskiene, 1996), topo-II-targeted drugs can induce mutations. Such events may have important consequences in cancer chemotherapy. Firstly, germ cell mutations may be transmitted to future generations. Secondly, mutations may lead to the development of secondary tumours from cells that were not originally neoplastic. Thirdly, mutations may lead to drug resistance, limiting further therapy.

In this introductory chapter, a general overview of the mutations, regulatory evaluation of aneugens, mechanisms, incidence and aetiology of aneuploidy are presented. The processes of mitosis, meiosis, erythropoieses, spermatogenesis and oogenesis will be discussed briefly. This is followed by a description of classical methods for the detection of chemically induced aneuploidy. Finally, the topoisomerases/topo II inhibitors [etoposide (VP-16) and merbarone (MER)] and the thesis aim are presented in the last section of this introduction.

2 Mutations

Mutations are heritable changes in the genome of a cell or an organism. These changes may be expressed, for example, as a change in the structure of a protein, which alters or abolishes its enzymic properties. Mutations occur spontaneously or may be induced by physical and chemical agents. In general, mutations are detrimental because in most cases they lead to defects in gene function. There are three different levels at which mutation takes place, namely at the DNA sequence level (gene mutations), at the chromatin structure level (structural chromosome aberrations) and at the chromosome number level (numerical chromosome aberrations).

2.1 Gene Mutations

Gene mutations are changes in base pair sequence within a gene. Such mutations were formerly also called point mutations. Gene mutations can occur by base substitutions (one base is substituted by another), or by deletion or addition of one or more bases from one or more codons. Additions or deletions change the reading frame of the DNA and are known as frameshift mutations (Drake et al., 1983; Hartl et al., 1988). Gene mutations, which lead to a codon change, which specifies the insertion of the "wrong" amino acid into a polypeptide, are called missense mutations. A missense mutation may lead to the production of a defective protein if it occurs at a critical site in a polypeptide (e.g., at the active centre of an enzyme, or at the site at which a ploypeptide should fold). In general, missense mutations are expressed as decreases in function (such as partial growth, or increased temperature sensitivity) rather than by total loss, and can often be identified by this leaky property. Gene mutations, which convert a codon normally specifying an amino acid to one, which is a terminator codon (a "full stop" in the genetic code) causes polypeptide synthesis to stop prematurely, with the production of defective proteins containing short polypeptide fragments. Mutations of this type are nonsense mutations. Because gene mutation are caused by very subtle DNA changes they are usually detectable only by the effects of their expression in the mutant cell or individual, that is by a change in phenotype (e.g. a change in an easily determined biochemical function).

2.2 Structural Chromosome Aberrations

Chromosome aberrations are changes in chromosome structure or number. Structural chromosome aberrations involve gross alteration of the genetic material and are detected by light microscopy in appropriately prepared cells during cell division, when DNA is condensed by chromosomal proteins to form chromosomes (stainable element). Cytologically detected damage includes chromosome breakage and various chromosomal rearrangements that can result from broken chromosomes. Aberrations may involve breakage before DNA replication so that both chromatids of a chromosome are affected (chromosome-type aberrations) or breakage after DNA replication so that just one of the two chromatids of a replicated chromosome is affected (chromatid-type aberrations). Some aberrations are stable, in that they can be transmitted through repeated cell divisions and therefore persist in the cell population. Major classes of chromosomal rearrangements that can be transmitted in populations of cells or organisms are deletions, duplications, inversions, and balanced translocations. Many of these

rearrangements can be detected with staining techniques (e.g. trypsin-giemsa banding) that reveal banding patterns on chromosomes but cannot be detected with routine cytogenetic analysis of unbanded chromosomes. Each chromosome in a karyotype has a banding pattern, which is characteristic for the individual chromosome of the given species. In recent years, molecular cytogenetic staining procedure with fluorescence-labelled DNA probes (chromosome painting such as SKY and M-FISH, comparative genomic hybridization: CGH) opened a new area of chromosome diagnostics (Jentsch et al., 2000; Klein et al., 1999).

Besides stable aberrations, chromosome breaks give rise to acentric fragments (i.e. broken chromatin pieces with no centromere), dicentric chromosomes, ring chromosomes, and various other asymmetrical rearrangements that are unstable, in that they usually bring about the death of the cell through loss of vital genetic material (Carrano and Natarajan, 1988). Although many of the aberrations routinely scored in cytogenetic analysis are unstable, they provide direct evidence of chromosome breakage and are representative of aberrations as a whole. Easily scored aberrations include chromatid breaks, chromatid exchanges, acentric fragments, dicentric chromosomes, ring chromosomes, and some reciprocal translocations (Carrano and Natarajan, 1988).

2.3 Numerical Chromosome Aberrations

These are changes in the number of chromosomes in the genome. When mutations change the number of whole chromosome sets present, polyploid cells result. When mutations change parts of chromosome sets, aneuploid cells result. Polyploidy such as triploidy (3*n*) and tetraploidy (4*n*) are common in the plant kingdom and are even represented in the animal kingdom, e.g. in liver cells (Vinogradov et al., 2001). An odd number of chromosome sets makes an organism sterile because there is not a partner for each chromosome at meiosis, whereas even numbers of sets can produce standard segregation ratios. Allopolyploid plants are obtained by crossing two related species and then doubling the progeny chromosomes through the use of colchicine (COL) or through somatic cell fusion. These techniques have important applications in crop breeding, because allopolyploids combine the useful features of the two parental species into one type. Polyploidy can result in an organism of larger dimensions; this discovery has permitted important advances in horticulture and in crop breeding.

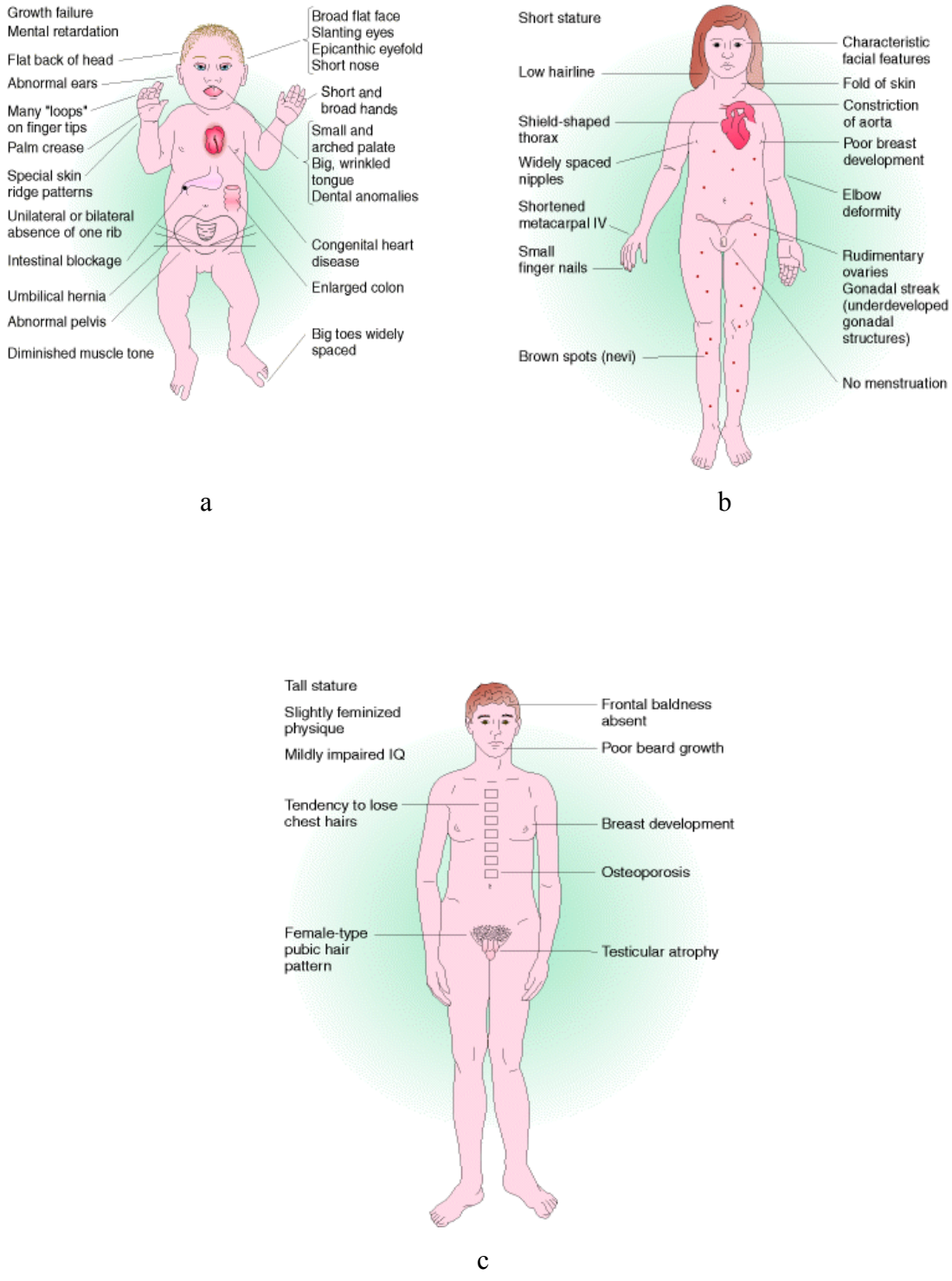


Fig. 1. Characteristics of Down syndrome in an infant: (a), Turner syndrome (b) and Klinefelter syndrome (c). Adapted from Vogel and Motulsky, 1982.

Aneuploids also are important in the engineering of specific crop genotypes, although aneuploidy per se usually results in an unbalanced genotype with an abnormal phenotype. Examples of aneuploids include monosomics (2*n*-1) and trisomics (2*n*+1). Aneuploid conditions are well studied in humans. Down syndrome (trisomy 21), Turner syndrome (XO) and Klinefelter syndrome (XXY) are well-documented examples (Fig. 1). The spontaneous level of aneuploidy in humans is quite high and produces a major proportion of genetically based ill health in human populations.

3 International Regulations for Mutagenicity Testing

The possible role of chemicals in the induction of both cancer-related genetic changes and genetic changes implicated in human birth defects has stimulated the development of a wide range of testing methods capable of detecting and assessing the genotoxic potential of chemicals (reviewed by Phillips and Venitt, 1995). The recognition of the genotoxic potential of a range of chemicals and the availability of validated test methods has led to the development of regulatory requirements in many countries which require the assessment of chemicals for which human exposure may occur (Carere et al., 1995). Thus, selected batteries of test methods are required in many countries to assess the ability of chemicals to induce gene mutations and structural chromosome changes. However, until recently, testing strategies have not included a specific requirement to determine the potential of chemicals to induce aneuploidy, i.e. aneugenic activity (Aardema et al., 1998). The need for an assessment of chemically induced numerical chromosome changes is explicit or implicit in some current regulatory protocols and testing schemes, and has been incorporated in two proposed regulatory guidelines. This assessment is either requested in the initial test battery for new chemicals or in supplementary assays for specific substances. A summary of those regulatory guidelines and testing schemes that address the assessment of numerical chromosome changes is given in Table 1 [for review of the international guidelines (Aardema et al., 1998)].

The determination of the DNA reactivity of newly developed chemicals has been a regulatory requirement within the European Union for the past 20 years. Recent reassessments of the strategies for the testing of chemicals within the European Union has resulted in the increasing recognition of aneuploidy as an important end-point to consider when evaluating potential genotoxicity to both somatic and germ cells. The UK Department of Health's Advisory Committee on the Mutagenicity of Chemicals (COM) has recently recommended a requirement for the measurement of aneugenic potential in its revised guidelines for the testing of chemicals

(Committee on the Mutagenicity of Chemicals, 2000) and it is expected that similar requirements will be introduced throughout Europe. The development of appropriate methods for the detection and assessment of aneugenic chemicals has been a major thrust of a number of research programmes of the European Union (Parry and Sors, 1993). These projects have progressed since that time to develop and to apply a range of methodologies capable of detecting and assessing the significance of aneugenic activity to both somatic and germ cells by the use of both *in vitro* and *in vivo* tests.

Table 1. Overview of regulatory guidelines and testing schemes for assessing numerical chromosome changes. Adapted from Aardema et al., 1998.

	Current	Future
OECD	Report polyploidy and endoreduplication when seen. In vivo micronucleus test can detect whole chromosome loss	None
ICH	In vivo micronucleus tests have the potential to detect some aneuploidy inducers; there may be aneuploidy inducers that act preferentially during meiosis, but there is no conclusive evidence for these chemicals	Same
CEC	For chemicals consider aneuploidy-induction or spindle inhibition at 100 or 1000 t/y (500 or 5000 t cum)	Harmonise with OECD and ICH
Japan	Routine assessment of polyploidy in in vitro cytogenetic assay	Harmonise with OECD and ICH
Canada	In vitro chromosome assay should include some chromosome counts to gain information on potential aneuploidy induction	Harmonise with OECD (and ICH)
US-EPA	None	Harmonise with OECD
US-FDA	None	Harmonise with OECD and ICH

OECD = Organization for Economic Co-operation and Economic Development, ICH = International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for human Use, CEC = Commission of the European Communities, US-FDA = US Food and Drug Administration, US-EPA = US Environmental Protection Agency.

4 Aneuploidy

4.1 Terms and Definitions

The normal species-specific diploid genome is euploid, and contains a complete set of chromosomes from each parent, e.g. *2n* = 46 for humans, *2n* = 40 for mice. Aneuploid and ploypoid cells have chromosome numbers that differ from the normal number for the species. In aneuploidy, the deviation in chromosome number involves one or a few chromosomes, whereas in ploypoidy, the alteration involves complete sets of chromosomes. For example, in humans, where the normal diploid (*2n*) chromosome number is 46, cells with 45 or 47 chromosomes would be described as aneuploid, whereas cells with 69 chromosomes would be described as ploypoid, in this case triploid (*3n*). Aneuploid nomenclature is based on the number of copies of the specific chromosome in the aneuploid state. For example, the aneuploid condition 2*n*-1 is called monosomic (meaning "one chromosome") because only one copy of a specific chromosome is present instead of the usual two found in its diploid progenitor. For autosomes in diploid organisms, the aneuploid 2*n*+1 is called trisomic, 2*n*-1 is monosomic, and 2*n*-2 (where the -2 represents homologs) is nullisomic. In haploids, *n*+1 is disomic. Special symbolism has to be used to describe sex-chromosome aneuploids, because we are dealing with two different chromosomes (X and Y) and the homogametic and heterogametic sexes have different sex-chromosome compositions even in euploid individuals. The symbolism merely lists the copies of each sex chromosome, such as XXY, XYY, XXX, or XO (the "O" stands for absence of a sex chromosome and is included to show that the symbol is not a typographical error).

4.2 Cell Division and Distribution of Chromosomes

4.2.1 Cell Cycle, Progression and Checkpoints

The cell cycle of proliferating somatic cells proceeds through different phases (Fig. 2): a relatively long interphase consisting of the Gap1-phase (G_1), DNA-Synthesis-phase (S) and Gap2-phase (G_2), and a relatively short mitotic phase (M). During the G_1-phase a major part of the RNA and protein synthesis takes place, mainly in preparation for the S-phase. The G_1-nucleus is diploid; the information on each chromosome is present in singular form. In S-phase the DNA and other components of the chromosomes are duplicated, resulting in a G_2-phase

during which there is intense biochemical activity and spindle protein assembly in preparation for mitosis and cell division. Cell differentiation is generally associated with a loss of proliferative capacity (e.g. in neurons or liver cells); these cells are in a resting state, called G_0. Some G_0-cells, such as lymphocytes or liver cells, can be induced by a mitogen or growth factor to enter the cell cycle and divide.

The cell cycle is driven by the sequential activation of key proteins by phosphorylation. Proteins that are called cyclins play an important role (Fig. 2). They activate a series of cyclin-dependent kinases (CDKs), the enzymes that carry out the phosphorylations. Cyclins D1, D2, and D3 activate CDK4 and CDK6 and drive G_0 and G_1 cells through G_1. The cyclin E/CDK2 complex then drives the cells through early S, cyclin A/CDK2 through mid-S, cyclin A/CDK1 (also called cdc2, cell division control protein 2) through late S-early G_2, and cyclin B/CDK1 to the G_2/M transition (Roussel, 1998).

At key transitions during eukaryotic cell cycle progression, signaling pathways monitor the successful completion of upstream events prior to proceeding to the next phase. These regulatory pathways are commonly referred to as cell cycle checkpoints (Fig. 2). Cells can arrest at cell cycle checkpoints temporarily to allow for, (i) cellular damage to be repaired, (ii) the dissipation of an exogenous cellular stress signal, or (iii) availability of essential growth factors, hormones, or nutrients. Checkpoint signaling may also result in activation of pathways leading to programmed cell death if cellular damage cannot be properly repaired. Defects in cell cycle checkpoints can result in gene mutations, chromosome damage, and aneuploidy, all of which can contribute to tumorigenesis.

Cells are arrested in G_1 and G_2 if their DNA has been damaged and progression is delayed until DNA repair has occurred or cell death ensues. DNA damage triggers the sequential activation of proteins first, the mutated in ataxia-telangiectasia (ATM) protein (Kastan et al., 1992), then the p53 tumour suppressor protein, and then the GADD45 protein, which binds to the proliferating cell nuclear antigen and blocks DNA replication (Levine, 1997). DNA topo II is required for chromosome condensation and segregation in eukaryotes (Downes et al., 1994; Uemura et al., 1987). Although these are mitotic processes, their successful completion depends partly on topo II activity during DNA replication and in G_2-phase. Chromosome replication creates two identical sister DNA molecules that are knotted together (catenated). Topo II removes the catenations; in higher eukaryotes, the majority must be resolved before entry into mitosis to allow accurate chromosome condensation (Giménez-Abián et al., 1995). A G_2 checkpoint ensures that DNA catenations have been sufficiently resolved before mammalian cells enter mitosis (Downes et al., 1994). There is also a spindle-

assembly checkpoint. Cells arrest in G_2 and delay their exit from mitosis in response to spindle microtubule disruption by poisons such as COL or nocodazole.

A series of CDK inhibitors are involved in normal and genotoxic damage-induced checkpoint regulation of the cell cycle. The *INK4A* gene product, p16, and two other proteins, p15 and p18, specifically inhibit the cyclin D-dependent Kinases CDK4 and CDK6 and can lead to G_1 arrest. The *KIP1* gene product, p27, as well as p21 and the *KIP2* gene product, p57, inhibit all cyclin/CDK kinases and can arrest the cycle at various points. The amount of the p27 protein is high in G_0 cells but is reduced by mitogens. The tumor- suppressor protein, p53, stimulates production of p21 and thus bocks cell proliferation (Levine, 1997). Mutation of the p53 gene is extremely important in carcinogenesis because of its key role both in DNA damage checkpoints and at spindle assembly checkpoints.

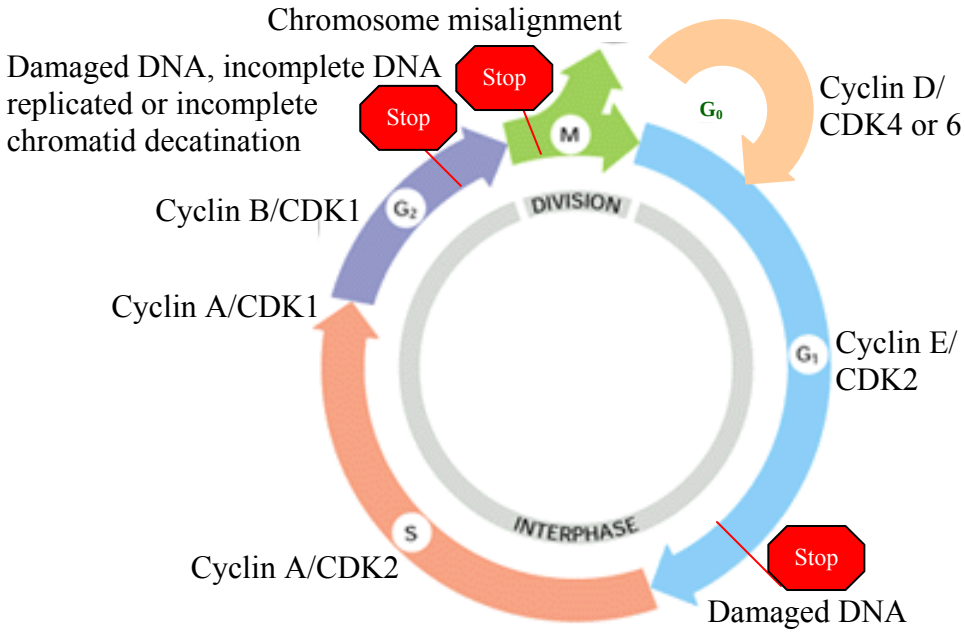


Fig. 2. The cell cycle involves sequential activation of cyclin-dependent kinases (CDKs) by binding to cyclins. The cycle can be blocked at G_1 and G_2 DNA checkpoints or at a spindle assembly checkpoint. G_1 (first "gap" phase), S (synthesis phase = DNA replication), G_2 (second "gap" phase), M (mitotic phase) and G_0 (zero phase). The period in between two M phases is called interphase (= G_1 + S + G_2). Adapted and edited from Alberts et al., 1994.

4.2.2 Mitosis

The process of cell division (M phase) consists of nuclear division (mitosis) followed by cytoplasmic division (cytokinesis). During the prophase of mitosis the chromosomes progressively condense in the nucleus. In the cytoplasm, the centrosomes duplicate, split, and subsequently migrate to opposite sides of the cell to form the spindle poles. The microtubules grow from the centrosomes by polymerization of tubulin molecules. At prometaphase, the nuclear membrane breaks down and the spindle microtubules can enter the nuclear area. Part of the microtubules function to stabilize the spindle (polar microtubules), the other part to capture the kinetochores (kinetochores microtubules), which are tri-laminar protein structures on either side of the centromeres of the chromosomes (Sullivan et al., 1996). The chromosomes shuffle back and forth until they eventually align on the metaphase plate in the centre of the spindle (Rieder and Salmon, 1998). At this stage, the cell has reached metaphase.

In metaphase, all the chromosomes are properly lined up in the metaphase plat halfway between the spindle poles, and the formation of the bi-polar spindle is completed. Most cells remain only briefly at metaphase before proceeding to anaphase. The transition from metaphase to anaphase is triggered by breakage of the link between sister chromatids, which then separate and move to opposite poles of the spindle. Mitosis ends with telophase, during which nuclei re-form and the chromosomes decondense. Cytokinesis usually begins during late anaphase and is almost complete by the end of telophase. At cytokinesis the formation of the nuclear envelope is completed and the contractile ring forms a cleavage furrow at the midbody, the region of microtubule overlap. The cytoplasm is divided at the site of the cleavage furrow, resulting in two daughter cells. Thus each daughter cell receives an identical set of chromosomes (Fig. 3).

4.2.3 Meiosis

Meiosis consists of two successive cell divisions (Fig. 3). The first meiotic division (meiosis I; MMI) reduces the number of chromosomes from diploid (*2n*) to haploid (*n*) chromosome sets and is called reduction division. The second meiotic division (meiosis II; MMII) distributes the chromatids evenly to the two daughter cells. In preparation of the first meiotic division, homologous chromosomes of germinal cells condense and pair up. This pairing of homologous chromosomes after DNA replication is not only a key event for the fidelity of meiotic chromosome segregation, but also allows recombination between

chromosomes of paternal and maternal origin. This critical pairing of homologous chromosomes takes place during an extended prophase of meiosis I, which is divided into five stages (leptotene, zygotene, pachytene, diplotene, and diakinesis) on the basis of chromosome morphology. The initial association of homologous chromosomes is thought to be mediated by base pairing between complementary DNA strands during the leptotene stage, before the chromatin becomes highly condensed. The close association of homologous chromosomes (synapsis) begins during the zygotene stage. During this stage, a zipperlike protein structure, called the synaptonemal complex, forms along the length of the paired chromosomes (Heyting, 1996). This complex keeps the homologous chromosomes closely associated and aligned with one another through the pachytene stage, which can persist for several days. Recombination between homologous chromosomes is completed during their association at the pachytene stage (Alberts et al., 1994), leaving the chromosomes linked at the sites of crossing over (chiasmata). The synaptonemal complex disappears at the diplotene stage and the homologous chromosomes separate along their length.

Importantly, however, they remain associated at the chiasmata, which is critical for their correct alignment at metaphase. At this stage, each chromosome pair (called a bivalent) consists of four chromatids with clearly evident chiasmata. There is a general agreement that this meiotic recombination between the homologues chromosomes plays an important role in the fidelity of chromosome segregation in meiosis, since these chiasmata stabilize the bivalent and prevent premature chromosome segregation (Eichenlaub-Ritter, 1994; reviewed by Ferguson et al., 1996a). Diakinesis, the final stage of prophase I, represents the transition to metaphase, during which the chromosomes become fully condensed.

At metaphase I, the bivalent chromosomes align on the spindle. The kinetochores of sister chromatids are adjacent to each other and oriented in the same direction, while the kinetochores of homologous chromosomes are pointed toward opposite spindle poles. Consequently, microtubules from the same pole of the spindle attach to sister chromatids, while microtubules from opposite poles attach to homologous chromosomes which allows their separation. Anaphase I is initiated by disruption of the chiasmata at which homologous chromosomes are joined. The homologous chromosomes then separate, while sister chromatids remain associated at their centromeres. At completion of meiosis I, each daughter cell has therefore acquired one member of each homologous pair, consisting of two sister chromatids. Meiosis II initiates immediately after cytokinesis, usually before the chromosomes have fully decondensed. In contrast to meiosis I, meiosis II resembles a normal mitosis.

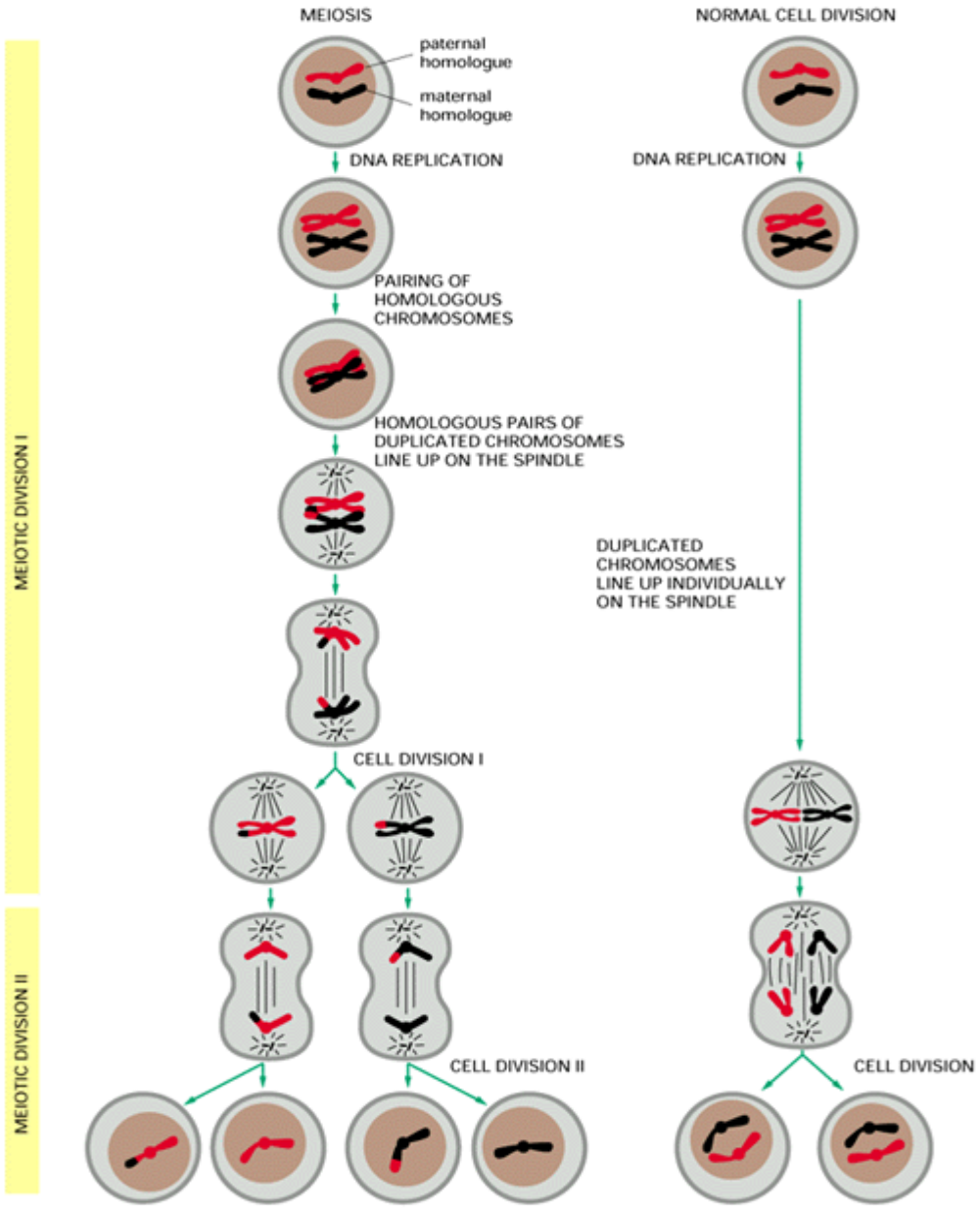


Fig. 3. Diagram of meiosis and mitosis (normal cell division) with one pair of homologous chromosomes. Adapted from Alberts et al., 1994.

At metaphase II, the chromosomes align on the spindle with microtubules from opposite poles of the spindle attached to the kinetochores of sister chromatids. The link between the centromeres of sister chromatids is broken at anaphase II, and sister chromatids segregate to opposite poles. Cytokinesis then follows, giving rises to haploid daughter cells (Fig. 3). At fertilization, the diploid state is restored by fusion of sperm and egg to form a zygote. The genetic constitution of the zygote will be different from that of either parent because of the recombinational events, which occurred during meiosis. In mammals, the sex of the new

individual, which develops from the zygote, is determined by whether it receives two X-chromosomes (female) or an X and a Y (male).

4.3 Mechanisms of Aneuploidy Induction

There are two classical processes that give rise to aneuploidy. The first is non-disjunction of chromosomes at anaphase (Fig. 4), so that one daughter cell gains a chromosome (e.g. trisomic *2n*+1 during mitosis or disomic *n*+1 during meiosis) and the other loses a chromosome (e.g. monosomic *2n*-1 during mitosis or nullisomic *n*-1 during meiosis). The other process is chromosome loss during cell division so that one daughter cell becomes monosomic and the other one remains normal. In the latter case the lost chromosome may form a micronucleus that can be detected cytogenetically (Fig. 6). Alternatively, it may be randomly re-incorporated into either one of the daughter nuclei so that either one daughter cell becomes trisomic and its partner monosomic or both daughter cells become diploid and the aneugenic event is effectively erased. In addition to the two classical processes (chromosome loss and non-disjunction), other pathways could lead to aneuploidy: non-conjunction (when homologous chromosomes fail to establish a paired state), defective centromere division (wrongful separation of sister chromatids at first meiotic division) or extra-replication of chromosome (error of chromosome replication at some time during meiosis, so that an extra copy of a chromosome is generated).

Aneuploidy may arise either spontaneously or by chemical induction during cell division in germinal and somatic cells. Chemicals inducing aneuploidy are called aneugens. Possible targets of these aneugens are molecules

- involved in chromatid attachment and separation (chromosome condensation, crossing-over, kinetochores, chromatid glue proteins),
- that are part of essential DNA-containing structures (centromeres, telomeres),
- involved in cell cycle control (cyclins, CDK's, p53), APC (anaphase promoting complex),
- that are part of the spindle apparatus [tubuline, microtubule associated proteins (MAPs), centrioles],
- that are indirectly involved in the cell cycle (calmodulin, cellular or nuclear membrane).

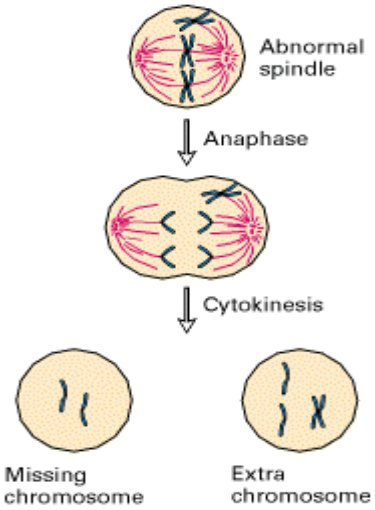


Fig. 4. Nondisjunction occurs when chromosomes segregate in anaphase before the kinetochore of each sister chromatid has attached to microtubules (red lines) from the opposite spindle poles. As a result, one daughter cell contains two copies of one chromosome *(2n+I)*, while the other daughter cell lacks that chromosome *(2n-I)*. Adapted from Murray and Hunt, 1993.

4.4 Aneuploidy and Implications on Human Health

Aneuploidy is a frequent cause of mental retardation, congenital malformations and pregnancy wastage in human beings. In addition, numerical chromosomal changes are also frequently observed in various stages of carcinogenesis in man. The survival of aneuploid germ cells leads to the formation of aneuploidy embryos. In humans, 30% of all conceptuses carry numerical chromosomal aberrations. Fortunately, most of these chromosomally abnormal embryos are lost, some before implantation (unrecognized abortuses), others after implantation (recognized and unrecognized abortuses), so that only 5% of the stillborn, and only 0.3% of the live born children are chromosomally abnormal (reviewed by Kirsch-Volders et al., 2002).

Aneuploidy in somatic cells is associated with the development of several cancers (Cavenee et al., 1991; ECETOC, 1997; Fearon and Vogelstein, 1990). Boveri, (1914) had suggested a relationship between numerical aberrations and cancer as early as 1914. The observation of extensive aneuploidy in cancer cells (Mertens et al., 1997) indicates that modifications of the fidelity of chromosome segregation leading to karyotype instability may be critical events in the aetiology of at least a proportion of human tumours. Chromosome instability at an early stage in cancer progression may act as a driving force leading to the alteration in the copy number of one or more genes controlling cellular growth, thus affecting the expression of oncogenes and/or tumour suppressor genes. There is evidence that aneuploidy

is also associated with later stages of the carcinogenesis process (e.g. chronic myeloid leukaemia, where the initiation event is a translocation producing the Ph1 chromosome and progression into the blastic phase appears to be correlated with the induction of specific hyperdiploidy changes) (ECETOC, 1997). An increased risk of tumour development has been observed with certain congenital aneuploidies: patients with Down's syndrome exert a greater risk for leukaemia (Porter and Paul, 1974), Klinefelter men show an increased incidence of breast tumour and gonadoblastoma (Simpson and Photopus, 1976), and Turner women often have tumours of neural crest origin (ECETOC, 1997).

5 Classical Methods for Aneuploidy Testing

Considering the deleterious consequences of aneuploidy in germ cells and somatic cells, it is important that validated assays for the detection of chemically induced aneuploidy *in vitro* and *in vivo* are available (reviewed by Dellarco et al., 1986; Parry and Natarajan, 1993). The most commonly used tests for chemically induced aneuploidy involve chromosome counting at metaphase, micronucleus induction or cell division aberrations.

5.1 Chromosome Counting

Aneuploidy induction can be measured by the identification and counting of whole chromosomes in second division cells following chemical treatment. Either the entire chromosome complement or specific chromosomes can be counted. Chromosome identification can be either visual or genetical. There are problems associated with all approaches, these issues were discussed by Parry et al. (1995). While chromosomes can sometimes be identified by their morphology or by banding methods, the cytogenetic identification of chromosomes has improved in recent years with the use of DNA hybridization probes. *In situ* hybridization frequently coupled with fluorescent labelling (FISH), combines the resolution of molecular biology techniques with conventional cytogenetics to allow the detection of nucleic acid sequences on chromosomes at either metaphase or interphase (Brandriff et al., 1991). The reaction involves a heteroduplex formation between a chemically modified, single strand DNA probe sequence and a denatured chromosome immobilised on a microscope slide.

The method of chromosome counting in MMII oocytes and spermatocytes has been used to determine the spontaneous (Mailhes and Yuan, 1987) and radiation (Tease and Fisher, 1996) or chemically (Miller and Adler, 1992) induced aneuploidy in mouse germ cells. In these studies,

the identification of individual chromosomes has been facilitated by c-banding which allows the differentiation of large acentric fragments and entire chromatids.

5.2 Micronucleus Test

The micronucleus (MN) assay is a method widely used to assess clastogenicity both *in vitro* and *in vivo* using interphase cells, having the advantage of allowing rapid screening of large numbers of cells (Heddle et al., 1991). The general principle is that MN are scored in cells that have divided once after chemical exposure. Since MN can result from chromosome breakage or lagging chromosomes, the detection of MN has the potential to be used as a screen for aneuploidy induction if methods are included to allow the identification of whole chromosomes inside of MN. Various techniques have been developed to distinguish MN induced by clastogens or by aneugens however, the most reliable method are the ones that mark centromeres (i.e. CREST-staining and FISH techniques).

When scoring MN in interphase cells in vitro as an indicator of induced aneuploidy, the quality and quantification of the assay is improved if those cells that have completed a cell division can be identified. This has been done by labelling cells with 5-bromodeoxyurinidine (BrdU) (Pincu et al., 1984) or [^{3}H]thymidine (Fenech and Morley, 1985), or treating cultured cells with cytochalasin B (CB), an inhibitor of cytokinesis (Fenech and Morley, 1985). CB is the most popular method, mainly because there are fewer doubts about its effect on aneuploidy induction or clastogenictiy. Cells that have divided in the presence of CB are binculeate and, therefore, easily recognisable. It is possible that some cell types are more resistant than others to this chemical. MN assays may be faster and more amenable to automation than chromosome counting. They enable detection of chromosome loss, which cannot always be assessed by chromosome counts, due to possible interference by technical artefacts. However, non-disjunction events are not detected by this technique.

5.3 Analysis of Cell-Division Aberrations

Mitotic alterations including C-mitosis, increases in mitotic metaphases, cell cycle delay, premature chromatid separation, chromosome lagging, multipolar spindles, and diplochromosomes have been used as indicators for the aneugenic potential of chemicals. Mitotic effects were first described by Levan, (1938) following treatment of *Allium cepa* root tips with COL (therefore termed C-mitotic effects) and various authors have suggested that

these effects are indicative of aneuploidy induction (Dulout and Natarajan, 1987; Önfelt, 1987; Parry et al., 1982).

Analysis of spindle morphology and chromosome alignment provides information on disturbances specific to a test compound. Most spindle-chromosome staining methods have been used in cultured somatic mammalian cells (Parry et al., 1982; Salassidis and Bauchinger, 1990; Salassidis et al., 1991) or on cultured mouse oocytes (Eichenlaub-Ritter et al., 1986). Gassner and Adler, (1995), adapted a differential and an immunofluorescent spindle staining method to male mouse germ cell preparations, where high numbers of meiotic cells are available for analysis. They found the differential staining with brilliant blue (spindle) and safranin O (chromosomes) was more effective to detect misplaced chromatin whereas the immunostaining with FITC-labelled anti-α-tubulin antibody was superior when scoring for alterations of the spindle structure.

6 Aneuploidy Assays in Somatic Cells

6.1 Erythrocyte Development (Erythropoieses)

The many types of blood cells all derive from a common pluripotent stem cell. In the adult individual, the stem cells are found mainly in bone marrow, where they normally divide infrequently to produce more stem cells (self-renewal) and various committed progenitor cells, which are irreversibly determined to produce only one or a few types of blood cells. The progenitor (unipotential) cells are stimulated to proliferate by specific growth factors but progressively lose their capacity for division and develop into terminally differentiated blood cells. In adult mammals, all of the cells shown develop mainly in the bone marrow except for T lymphocytes, which develop in the thymus, and macrophages and osteoclasts, which develop from blood monocytes. The pluripotent stem cells also give rise to various types of tissue cells, such as natural killer cells, mast cells, and a variety of classes of antigen-presenting cells (Zucker, 1980).

The erythrocyte is by far the most common cell type in the blood. When mature, it is packed full of hemoglobin and contains practically none of the usual cell organelles. In an erythrocyte of an adult mammal, even the nucleus, endoplasmic reticulum, mitochondria, and ribosomes are absent, having been extruded from the cell in the course of its development. The erythrocyte therefore cannot grow or divide; the only possible way of making more erythrocytes is by means of stem cells. Furthermore, erythrocytes have a limited life span -

about 120 days in humans or 55 days in mice. Worn-out erythrocytes are phagocytosed and digested by macrophages in the liver and spleen, which remove more than 10^{11} senescent erythrocytes in each of us each day.

In mouse bone marrow the maturing erythrocytes go through six or seven cell divisions with a cell-cycle length of about 10 h (Cole et al., 1979). About 10 h after the last mitotic division the expulsion of the main nucleus is completed and the resulting young erythrocyte still contains RNA, is basophilic and stains light blue or blue gray with Giemsa. This polychromatic erythrocyte (PCE) remains in the bone marrow for another 10 h (Fig. 5).

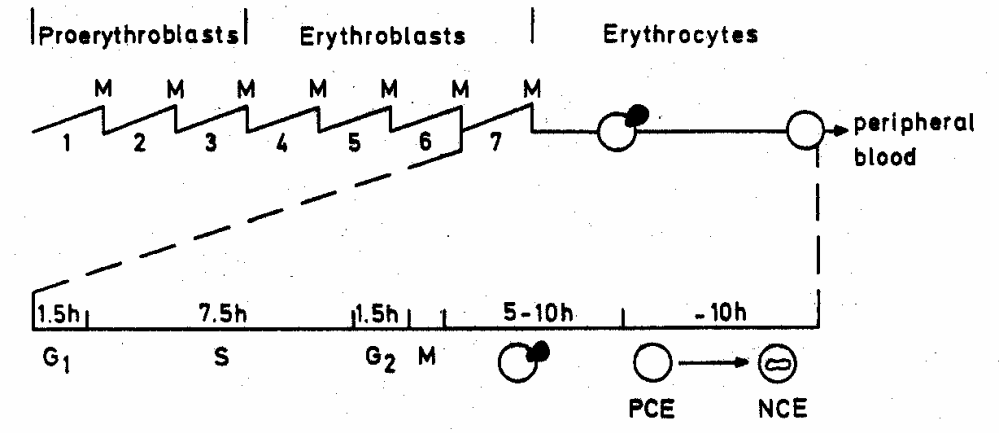


Fig. 5. Simplified schematic diagram of the timing of erythropoiesis in adult mouse bone marrow. The upper line shows the whole process, the lower line shows the last cell cycle and erythrocyte maturation. Adapted from Adler, 1984.

The PCEs, with time, lose ribosomes and contain primarily hemoglobin and become normochromatic erythrocytes (NCE), i.e. mature erythrocytes, which are somewhat smaller than PCE, are acidophilic and stain light orange or orange-pink with Giemsa. Within about 18-22 h after nuclear expulsion, the cells move into the peripheral blood compartment (Hayashi et al., 1984). Erythrocyte clones develop in the bone marrow on the surface of macrophages, which phagocytise and digest the nuclei discarded by the erythrocytes.

6.2 Bone-Marrow Micronucleus-Test

MN can be observed most reliably in cells that do not contain a nucleus, namely in erythrocytes (Schmid, 1973). During erythroblast proliferation, a given test agent administered to the test animal (mouse, rat, Chinese hamster) may cause chromosome damage, such as breaks and exchanges, and may also act on macromolecules related to the function of chromatid distribution, e.g., tubulin causing spindle dysfunction, depending on its mechanism

of action. These anomalies (an acentric fragment or an entire chromosome) may lag behind in the cell during division and may not become integrated into daughter nuclei, rather may eventually form MN, which can be seen in the cytoplasm. During maturation, when an erythroblast develops into a PCE and the main nucleus is expelled, micronuclei remain behind in the otherwise enucleated cytoplasm. Thus, visualization of MN is facilitated in these cells because they lack a main nucleus. An increase in the frequency of micronucleated PCE (MNPCE) in test agent-treated animals is an indication of induced chromosome damage.

In the MN assay, comparison of the PCE/NCE ratio in bone marrow of treated animals to vehicle-control animals provides an indication of cytotoxicity. When erythroblast proliferation is depressed, the ratio will decrease, when proliferation is increased to repopulate the PCE pool, the ratio is increased. Some cytotoxicity can provide a valuable indication of bone-marrow exposure by the test agent, but excessive toxicity is undesirable. The frequency of micronucleated PCE reflects the level of damage induced by agents that produce chromosome breakage (clastogens) and/or chromosome loss (aneugens). Many chemicals may have clastogenic as well as aneugenic potential. However, with the conventional MN test it is impossible to distinguish between these two events.

6.3 Centromere-Labelling Methods

MN containing entire chromosomes were first characterized by their large size (Yamamoto and Kikuchi, 1980), by c-banding (Verschaeve et al., 1988) or by measurement of DNA content (Vanderkerken et al., 1989). However, these methods were not very reliable. Recently, two molecular cytogenetic methods were developed to identify the presence of centromeres in MN and thereby differentiate between MN of clastogenic and aneugenic origin: (i) Immunofluorescent CREST-staining and (ii) FISH with pancentromeric DNA probes (Fig. 6). The CREST antibodies against centromeric proteins were found in sera of patients with the CREST syndrome, a form of progressive systemic sclerosis (Beck et al., 1963). The antibodies were characterized by Cox et al. (1983). They bind to three different centromeric proteins, designed CENP-A, CENP-B, CENP-C (Earnshaw et al., 1986). The CREST method applied to the bone marrow MN test is described in detail by Miller and Adler, (1990). Using CREST antibody along with propidium iodide, Krishna et al. (1992) described a method to differentiate immature erythrocyte, mature erythrocytes, and MN containing centromeres.

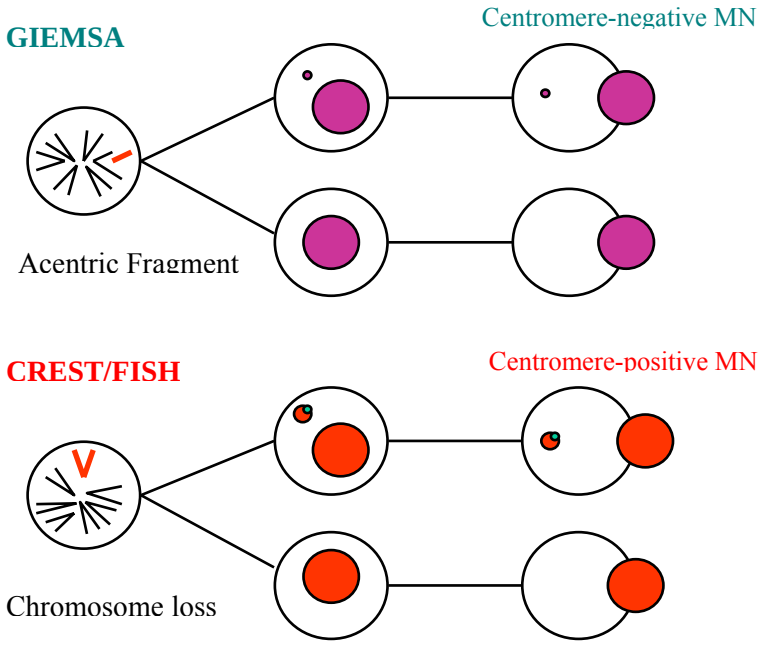


Fig. 6. Formation and classification of micronuclei (MN) in polychromatic erythrocytes: According to the presence or absence of a centromeric signal with CREST or FISH MN occurred by a clastogenic or aneugenic event.

With FISH, the first pancentromeric DNA-probe used for MN in mouse bone marrow erythrocytes was the major satellite DNA and the method was described by Miller et al. (1991). Pericentric heterochromatin of all chromosomes except the Y-chromosome is labelled with this major centromeric probe. Comparisons between the signal frequencies in mitomycin C (MMC)-induced MN showed that FISH with the major centromeric probe gave significantly more signals than CREST-staining. Because MMC is known to cause breaks within pericentric heterochromatin some of the FISH signals might have been produced by acentric fragments with pericentic heterochromatin causing a false positive response. In fact, by using the minor satellite DNA probe that hybridizes closer to centromere, Schriever-Schwemmer and Adler, (1994) could show that the frequencies of CREST- and minor-probe labelled MN induced by 1 mg/kg of MMC were identical. Therefore, the minor probe is preferable for FISH analysis of MN. The main deficiency of the FISH methods is that they do not differentiate between NCEs and PCEs. However, MN frequencies in NCE of the bone marrow are usually very small in control and treated animals. Thus, no discrimination between MN induced in PCE and NCE is required for the FISH analysis since the NCE only contributed minimally to the total number of

MN (Schriever-Schwemmer et al., 1997). The advantage of the in vivo MN test with centromer labelling is that clastogenic and aneugenic effects can be detected side by side.

7 Aneuploidy Assays in Germ Cells

7.1 Germ Cell Development

In all vertebrate embryos certain cells are singled out early in development as progenitors of the gametes. These primordial germ cells (PGCs) migrate to the developing gonads, called the genital ridges, which will form the ovaries in females and the testes in males. After a period of mitotic proliferation, these cells undergo meiosis and differentiate into mature gametes either sperm or oocytes. Later, the fusion of sperm and oocytes after mating initiates embryogenesis, with the subsequent production in the embryo of new PGCs, which begins the cycle again.

7.1.1 Spermatogenesis

Spermatogenesis is the production of sperm from the PGCs. Once the vertebrate PGCs arrive at the genital ridge of a male embryo, they become incorporated into the sex cords. They remain there until maturity, at which time the sex cords hollow out to form the seminiferous tubules, and the epithelium of the tubules differentiates into the Sertoli cells. The initiation of spermatogenesis during puberty is probably regulated by the synthesis of bone morphogenetic protein 8B (BMP8B; a member of the TGFbeta superfamily of growth factors), by the spermatogenic germ cells, the spermatogonia. When BMP8B reaches a critical concentration, the germ cells begin to differentiate. The differentiating cells produce high levels of BMP8B, which can then further stimulate their differentiation. Mice lacking BMP8B do not initiate spermatogenesis at puberty (Zhao et al., 1996). Each day, some 100 million sperm are made in each human testicle, and each ejaculation releases 200 million sperm. Unused sperm are either resorbed or passed out of the body in the urine. During his lifetime, a human male can produce 10^{12} to 10^{13} sperm (Reijo et al., 1995).

After reaching the gonad, the PGCs divide to form type A_1 spermatogonia. These cells are smaller than the PGCs and are characterized by an ovoid nucleus that contains chromatin associated with the nuclear membrane. The A_1 spermatogonia are found adjacent to the outer basement membrane of the sex cords. They are stem cells, and at maturity, they are thought to

divide to produce another type A_1 spermatogonium as well as a second, paler type of cell, the type A_2 spermatogonium. Thus, each type A_1 spermatogonium is a stem cell capable of regenerating itself as well as producing a new cell type. The A_2 spermatogonia divide to produce the A_3 spermatogonia, which then beget the type A_4 spermatogonia.

It is possible that each of the type A spermatogonia are stem cells, capable of self-renewal. The A_4 spermatogonium has three options: it can form another A_4 spermatogonium (self-renewal); it can undergo cell death (apoptosis); or it can differentiate into the first committed stem cell type, the intermediate spermatogonium. Intermediate spermatogonia are committed to becoming spermatozoa, and they divide mitotically once to form the type B spermatogonia. These cells are the precursors of the spermatocytes and are the last cells of the line that undergo mitosis. They divide once to generate the primary spermatocytes, the cells that enter meiosis. It is not known what causes the spermatogonia to take the path toward differentiation rather than self-renewal; nor is it known what stimulates the cells to enter meiotic rather than mitotic division (Dym, 1994).

Each primary spermatocyte undergoes the first meiotic division to yield a pair of secondary spermatocytes, each containing two sets of duplicated autosomal chromosomes and either a duplicated X or a duplicated Y chromosome. The two secondary spermatocytes proceed through meiotic division II to produce four spermatids, each with a haploid number of single chromosomes (Fig. 7). These haploid spermatids then undergo morphological differentiation into sperm, which are released into the lumen of the seminiferous tubule. The sperm subsequently pass into the epididymis, a coiled tube overlying the testis, where they are stored and undergo further maturation. The total duration of spermatogenesis in the tests is approximately 64 days in man, while in mouse and rat it takes about 35 and 50 days respectively (Adler, 1996) (Table 2).

Table 2. Comparison of the duration (days) of male germ cell development in mice, rats, and humans. Adapted from Adler, 1996.

Species	Differentiating spermatogonia (mitotic cells)	Spermatocytes (meiotic cells)	Spermatids (post-meiotic cells)	Testicular sperm	Total testicular spermatogenesis	Epididymal sperm
Mouse	6	14	9	6	35	4-6
Rat	10.5	19	12	8.5	50	7
Human	16	25	16	6.5	64	8-17

7.1.2 Oogenesis

Oogenesis is the formation, development and maturation of the female gametes, which begins in late embryonic and early foetal periods, and is not completed until after puberty. Females of the almost all mammals studied are born with their total pool of oocytes; during life no new oocytes are formed. In the developing embryo, the stem cells (oogonia) develop from PGCs that migrate into the developing gonad early in embryogenesis. After a number of mitotic divisions, oogonia begin meiotic division I, and are called primary oocytes. In mammals primary oocytes are formed very early (between 3 and 8 months of gestation in the human embryo) and remain arrested in prophase of meiotic division I until the female becomes sexually mature. The next phase of oocyte development is called oocyte maturation, and it usually does not occur before sexual maturity is stimulated by hormones.

Under these hormonal influences the cell resumes its progress through division I of meiosis. To end division I, the cytoplasm divides asymmetrically to produce two cells that differ greatly in size: one forms a small polar body, and the other is a large secondary oocyte, the precursor of the egg. At this stage each of the chromosomes is still composed of two sister chromatids. These chromatids do not separate until division II of meiosis, when they are partitioned into separate cells by a process that is identical to a normal mitosis. After this final chromosome separation at anaphase II, the cytoplasm of the large secondary oocyte again divides asymmetrically to produce the mature egg (or ovum) and a second small polar body, each with a haploid number of single chromosomes (Fig. 7). All of the polar bodies eventually degenerate. In most vertebrates oocyte maturation proceeds to metaphase of meiosis II and then arrests until fertilization. At ovulation, the arrested secondary oocyte is released from the ovary, and if fertilization occurs, the oocyte is stimulated to complete meiosis. The oocyte remains viable for about 24 hours and if not fertilized by then it degenerates.

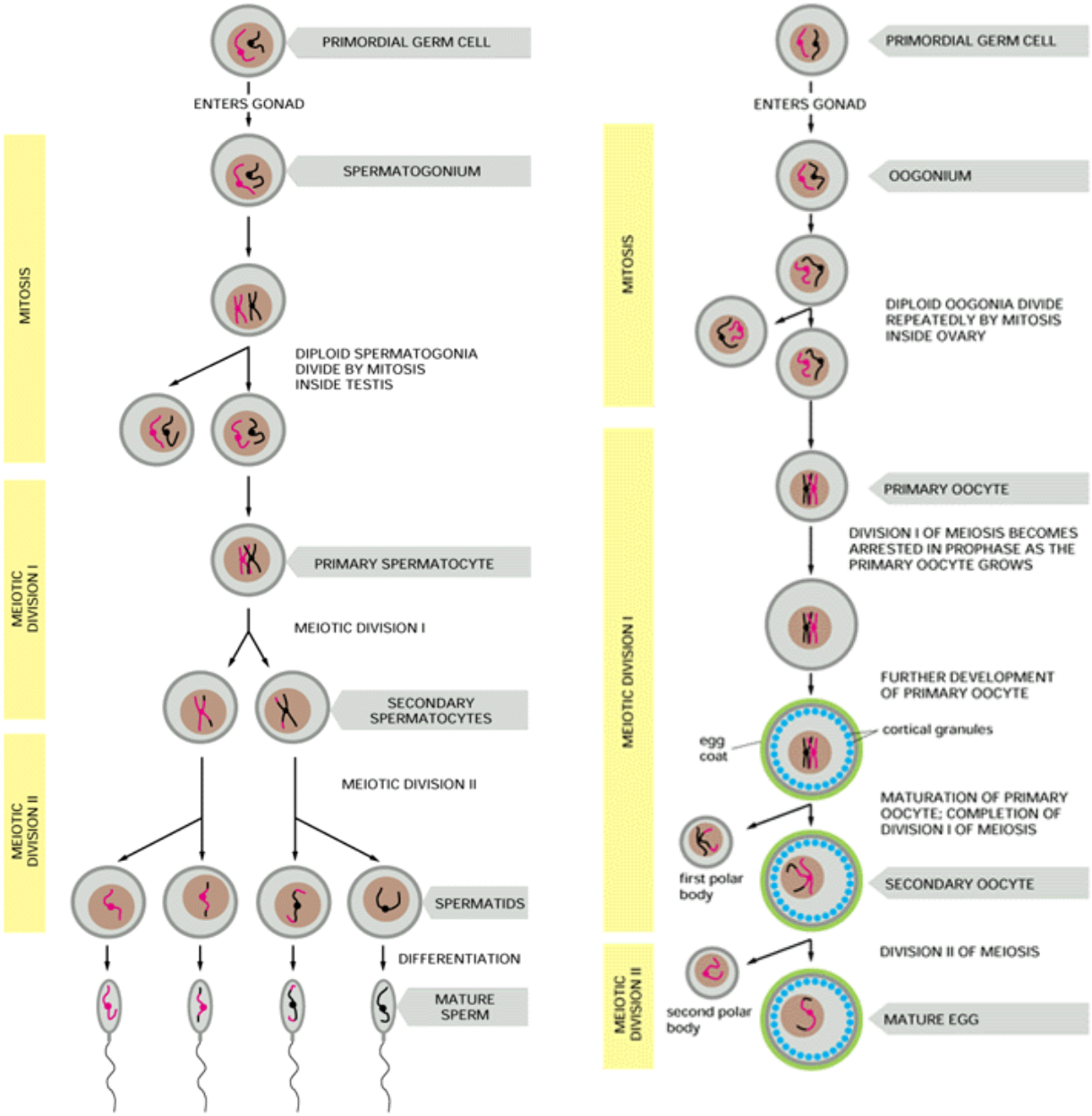


Fig. 7. The stages of spermatogenesis and oogenesis. Adapted from Alberts et al., 1994.

Ovulated oocytes represent only a small proportion of the potential eggs produced during oogenesis, the vast majority is eliminated from the ovaries by a process of degeneration which commences in the foetus and continues throughout reproductive life (Alberts et al., 1994).

7.2 Chromosome-Specific FISH in Sperm

An important advancement in sperm cytogenetics for detecting the consequences of chemically induced missegregation during meiosis was the adaptation of FISH with chromosome-specific DNA probes (Robbins et al., 1995; Wyrobek et al., 1990). Using FISH to score the numbers of signals in sperm, large numbers of sperm can be scored quickly. Furthermore, the use of multicolor FISH techniques made it possible to study several chromosomes simultaneously for hyperhaploidy (disomy) and to distinguish between diploid and disomic sperm (Robbins et al., 1997a; Spriggs et al., 1996). The method can be applied to sperm of experimental rodents and humans. Using the FISH aneuploidy assay, Robbins et al. (1997a) were able to demonstrate increased hyperhaploidy in the sperm of men undergoing cancer chemotherapy, while Robbins et al. (1997b) reported data, which indicate a significant association between aneuploidy induction and caffeine or alcohol consumption and suggestive evidence for a smoking effect. Given the difficulties in conducting sperm studies of exposed humans, Wyrobek et al. (1996) noted that complementary experimental approaches in animals are needed to identify aneugens, elucidate their modes of action and determine threshold concentrations.

Wyrobek et al. (1995) studied aneuploidy for one autosome and one sex chromosome in late testicular spermatids of mice. An improved method was applied to detect increased levels of aneuploidy in mice of advanced age by adding a DNA probe for the second sex chromosome (Lowe et al., 1995). However, to bridge the gap between experimental rodents and humans for comparative hazard evaluation, the FISH technology had to be adapted to rodent epididymal sperm, which involved the problem of localizing specific chromosomal subdomains within mature sperm. For this purpose, Lowe et al. (1996) developed a novel method to detect aneuploidy in epididymal sperm. Using this method with DNA probes specific for chromosomes X, Y and 8 and a three-color FISH technique, Adler et al. (1996) determined the spontaneous frequencies of aneuploid sperm of young adult mice and compared inter-laboratory variation for the method. The first set of data with possible aneugens tested in the sperm FISH assay was published by Schmid et al. (1999). The results of this study suggested that the sperm FISH methodology was a promising procedure for the detection of aneuploidy in mouse sperm.

The FISH with DNA probes specific for chromosomes X, Y and 8 applied to epididymal mouse sperm has offered the possibility to detect the consequences of chemically-induced missegregation during meiosis (reviewed by Adler et al., 2002). The assay had to be

refined and validated. In particular, the timing of sperm sampling needed verification. It was originally based upon the timing of spermatogenesis reported by Oakberg, (1956), where he determined that it takes about 22 days for the cells to develop from meiosis to epididydimal sperm. Therefore, an initial sampling time of 22 days after treatment was chosen (Schmid et al., 1999). Subsequently, an assay was developed to test if the chemical treatment altered the duration of the meiotic divisions (Schmid et al., 2001a). In this assay, primary spermatocytes were labelled with BrdU during the last S-phase before meiosis and the appearance of BrdU-containing sperm in the epididymis was then detected at various intervals after treatment with the test chemicals. Scoring of BrdU-labelled sperm was performed automatically using a laser scanning cytometer (LSC). It could be shown that the positive control compound COL prolonged the duration of the meiotic divisions by 1 or 2 days. This observation is important in order to select the appropriate sampling times for sperm from the epididymes in the sperm-FISH assay.

8 Topoisomerase II Inhibitors

8.1 Topoisomerases

The double-stranded nature of DNA poses important topological problems that arise during DNA metabolic processes such as replication, transcription and segregation of daughter molecules. An example is during the progress of the replication fork machinery along the DNA molecule, that inevitably results in the generation of positive supercoiling ahead of the fork, while the already replicated parental strands in its wake become negatively supercoiled.

DNA topoisomerases are conserved nuclear enzymes that catalyze a variety of interconversions that take place between topological isoforms of DNA through transient DNA cleavage, strand passing and religation (reviewed by Wang, 1996). The genomes of prokaryotes typically encode four topoisomerases. One of these enzymes, DNA gyrase, is the target of several antimicrobial agents, including the quinolone antibiotics (Froelich-Ammon and Osheroff, 1995). The genomes of higher eukaryotes typically encode four or more topoisomerases, with the human genome currently known to contain five family members: topo I and topo IIIα and IIIβ, which are type I, and topo IIα and IIβ, two isozymes belonging to the type II family (Nitiss, 1998; Wang, 1996) (Fig. 8). Type I enzymes, which do not require ATP, act by forming a transient single-strand break (ssb) through which the other

DNA strand passes to achieve relaxation; while type II, usually ATP-dependent, is able to do so with the two strands that make up duplex DNA, creating a DNA-linked protein gate through which another intact duplex passes (Giménez-Abián et al., 1995). Both type I and type II enzymes are proficient in relaxing supercoiled DNA, while only topo II can decatenate intertwined DNA molecules. Whereas the biological functions of topo III and IIβ are poorly understood, many investigations have dealt with the roles of both topo I and topo IIα.

DNA Topoisomerase	Type	Structure	M.W. (kD)	DNA cleavage	Gene localization (Human chromosome)	Function
I	IB	Monomer	100	ssb	20	Replication, transcription, recombination
IIIα	IA	2 isoforms (alternative splicing)	110	ssb	17	Recombination, rDNA metabolism
IIIβ	IA	3 isoforms (alternative splicing)	96	ssb	22	Recombination
IIα	II	Homodimer	170	dsb	17	Chromosome condensation and segregation. Replication
IIβ	II	Homodimer	180	dsb	3	Not well defined

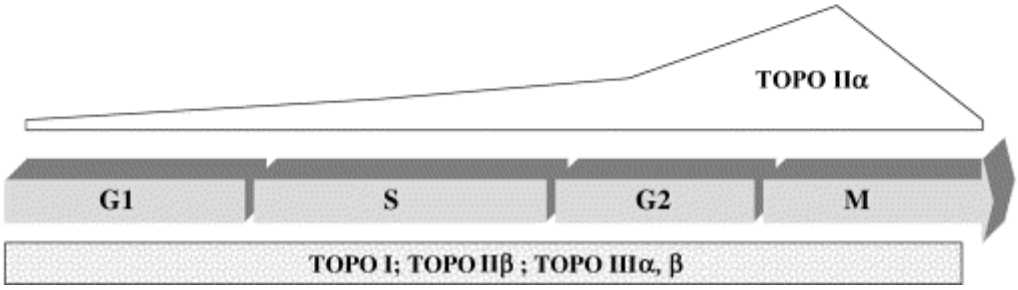


Fig. 8. Human DNA topoisomerases and their expression throughout the cell cycle. Adapted from Cortés et al., 2003.

Topoisomerases are required during DNA replication, transcription (mainly topo I) and homologous recombination (Giménez-Abián et al., 1995; Hatsuiker et al., 1998; Uemura et al., 1987) and a specific and unique role for topo II in segregation of daughter chromatids after DNA replication as well as in chromatin condensation and anaphase segregation during mitosis has been proposed (DiNardo et al., 1984; Sumner, 1995). In contrast with topo I and topo IIβ, for which the amount and stability show no significant fluctuations through the cell cycle, topo IIα protein levels vary naturally as a function of the proliferative stage (higher in cancer cells than in normal ones) and cell cycle position (Fig. 8).

Topo II acts by passing an intact segment of duplex DNA through a transient double-stranded break that it generates in a separate double helix (Fig. 9) (Fortune and Osheroff, 2000; Nitiss, 1998; Wang, 1996). The enzyme manufactures the double-stranded break by generating two staggered nicks (one on each strand) in the sugar-phosphate backbone (Sander and Hsieh, 1983). This scission reaction yields cleaved DNA molecules containing four-base single-stranded cohesive ends protruding on their 5'-termini (Sander and Hsieh, 1983). To maintain chromosomal integrity during the double-stranded DNA passage reaction, topo II forms covalent attachments with the newly created 5'-DNA termini via its two active site tyrosyl residues (one per each protomer subunit) (Zechiedrich et al., 1989). This covalent enzyme-cleaved DNA intermediate is known as the cleavage complex. Even though cells cannot survive without the double-stranded DNA passage activity of topo II, the formation of cleavage complexes is potentially toxic. When DNA-tracking enzymes such as polymerases or helicases attempt to traverse the topo II roadblock on the genetic material, transient cleavage complexes can be converted to permanent double-stranded DNA breaks (Fortune and Osheroff, 2000). Normally, cleavage complexes are present at very low levels and are tolerated by the cell. However, conditions that increase the cellular concentration of topo II-associated DNA breaks lead to DNA recombination and mutagenesis (Fortune and Osheroff, 2000; Li and Liu, 2001). When these breaks overwhelm the cell, they trigger programmed death pathways (Li and Liu, 2001).

This particular behaviour has made topo II the primary cellular target for a number of widely used anti-neoplastic drugs considerably more lethal to cells that contain high levels of topo II and which are undergoing high rates of DNA replication (Burden and Osheroff, 1998). To date, there are two general classes of topo II inhibitors that interfere with enzyme catalysis at distinct points of the enzyme reaction. DNA topo II inhibitors, such as VP-16, amsacrine, and genistein, stabilize cleaved DNA-topo II complexes. These drugs generate high levels of enzyme-mediated breaks, thus converting this essential enzyme into a potent cellular toxin. Hence, to distinguish their unique mechanism of action, they are referred to as topo II "poisons". Although topo II poisons encompass a number of structurally diverse classes, they all kill cells by elevating the level of enzyme-generated DNA breaks (Walker and Nitiss, 2002). They increase equilibrium levels of DNA cleavage complexes by two distinct, but not mutually exclusive mechanisms. Drugs such as VP-16 (and derivatives), amsacrine, and TAS-103 act primarily by inhibiting the ability of topo II to reseal (ligate) enzyme-linked DNA breaks. Conversely, drugs such as genistein, ellipticine and CP-115,953 (and other quinolones), have little effect on DNA ligation and appear to act primarily by stimulating the

forward rate of topo II-mediated DNA scission (Burden and Osheroff, 1998; Fortune and Osheroff, 2000). Furthermore, a variety of DNA lesions (including abasic sites and alkylated bases) also poison the enzyme without affecting rates of ligation (Sabourin and Osheroff, 2000).

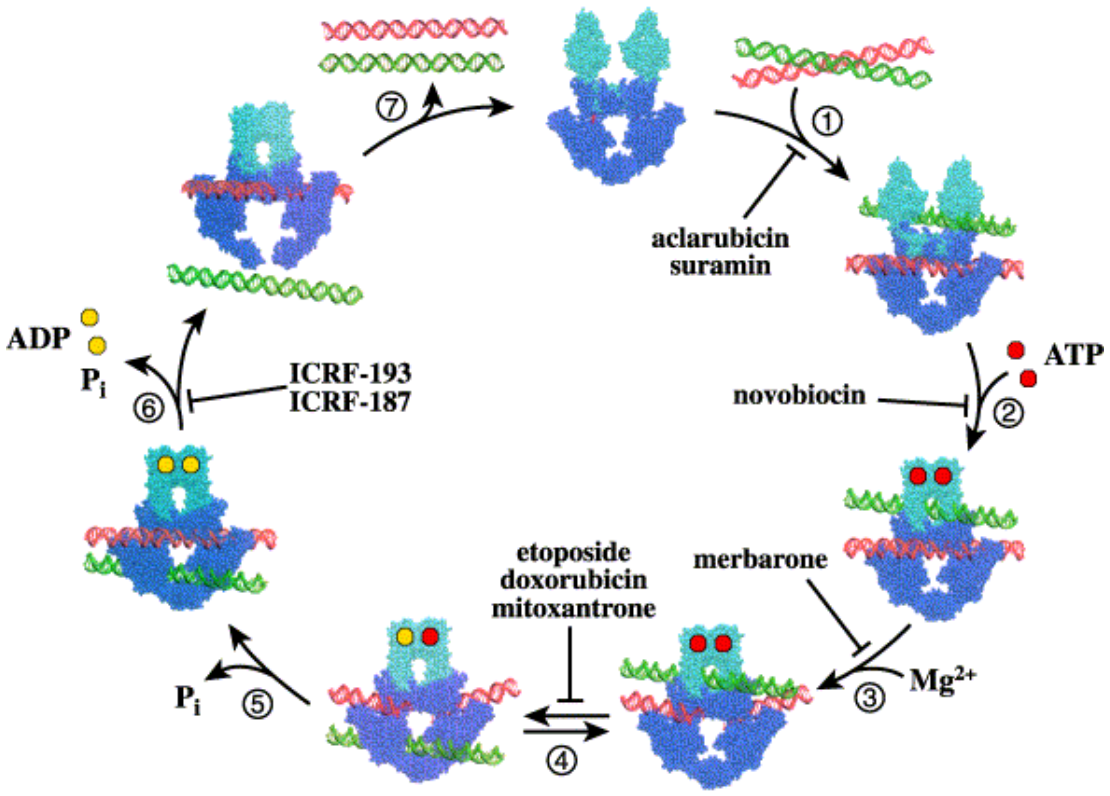


Fig. 9. The catalytic cycle of DNA topo II. The ATPase domains of topo II are shown in light blue, the core domain in dark blue, and the active site tyrosine residue in red. The C-terminal domain of the enzyme is not included in the diagram since its orientation, with respect to the rest of the molecule, is not known. The catalytic cycle is initiated by enzyme binding to two double-stranded DNA segments called the G segment (in red) and the T segment (in green) (Step 1). Next, two ATP molecules are bound, which is associated with dimerization of the ATPase domains (Step 2). The G segment is cleaved (Step 3) and the T segment is transported through the break in the G segment, which is accompanied by the hydrolysis of one ATP molecule (Step 4). The G segment is then religated and the remaining ATP molecule is hydrolyzed (Step 5). Upon dissociation of the two ADP molecules, the T segment is transported through the opening in the C-terminal part of the enzyme (Step 6) followed by closing of this gate. Finally, the N-terminal ATPase domains reopen, allowing the enzyme to dissociate from DNA (Step 7). The topo II poisons act by stabilising stage 4, where the DNA strand is cleaved. The catalytic inhibitors act either at stage 1 (aclarubicin and suramin) or at stage 2 (novobiocin) or at stage 3 (MER) or at stage 6 (bisdioxopiperazines). Adapted from Larsen et al., 2003.

In contrast to the complex-stabilizing topo II inhibitors, aclarubicin, the bisdioxopiperazines (e.g. ICRF-187 and -193) and MER block topo II at specific sites in its catalytic cycle prior to or subsequent to the formation or religation of DNA double-strand breaks (Andoh and Ishida, 1998). Aclarubicin, for example, blocks binding of the enzyme to its DNA substrate, the initial step of the topo II catalytic cycle (Sørensen et al., 1992). In contrast, ICRF-193 blocks the final step of the catalytic cycle, ATP hydrolysis (Andoh and Ishida, 1998). This action traps topo II on the DNA in its closed clamp form and prevents both enzyme release and regeneration. Finally, MER prevents cleavage of the gate-DNA strand acting at the same stage in the catalytic cycle as the topo II poisons (Fortune and Osheroff, 1998; Larsen et al., 2003) (Fig. 9). As of now, topo II poisons are, by far, the most frequently used class of topo II inhibitors in clinical use. Whereas topo II poisons are used for their antitumor activities, catalytic inhibitors are utilized for a variety of reasons, including their activity as antineoplastic agents (aclarubicin and MER), cardioprotectors (ICRF-187), or modulators in order to increase the efficacy of other agents (suramin and novobiocin) (reviewed by Larsen et al., 2003).

8.2 Etoposide (VP-16)

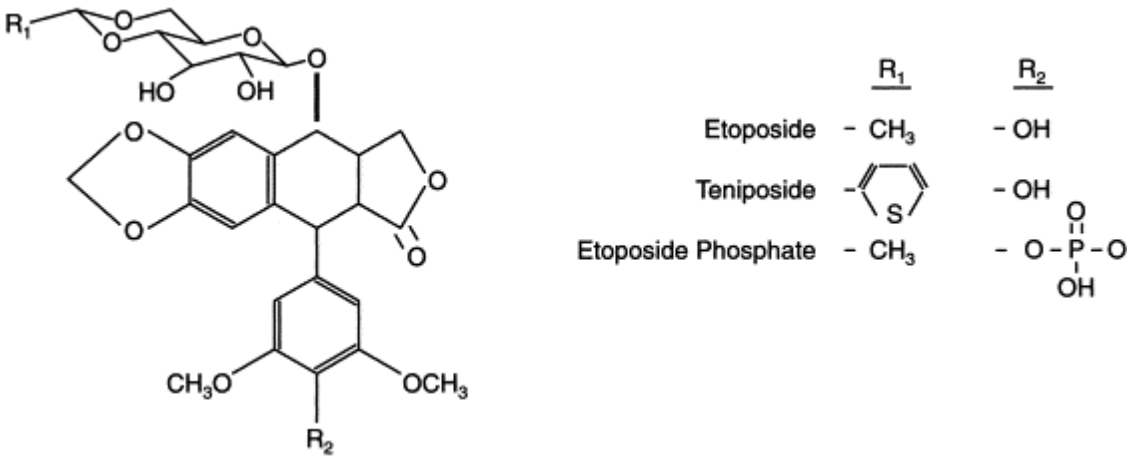


Fig. 10. Structural formula of epipodophyllotoxin and synthetic derivatives etoposide, teniposide and etoposide phosphate. Adapted from Hande, 1998.

History

The epipodophyllotoxins were synthesized in an effort to improve the activity of podophyllotoxin, an antimicrotubule agent that is present in mandrake plant extracts. A rather

subtle structural change (the removal of a methyl group from the pendant aromatic ring of podophyllotoxin) abrogates tubulin binding but confers the ability to poison topo II (Chen et al., 1984). The epipodophyllotoxins VP-16 and teniposide (VM-26) were in clinical use long before their mechanisms of action were understood. While early studies indicated that cells treated with these drugs exhibited DNA strand breaks (Wozniak and Ross, 1983), topo II was not identified as the target until the early 1980s (Long et al., 1985; Ross et al., 1984). VP-16 was the first anticancer drug to be demonstrated to work through inhibition of topo II.

Mechanism of Action

The primary cellular target for VP-16 is topo II (Heck et al., 1988; Liu, 1989). It kills cells by increasing the concentration of topo II-DNA cleavage complexes (Li and Liu, 2001; Walker and Nitiss, 2002). This action converts topo II to a potent cellular toxin that fragments the genome. Consequently, VP-16 is referred to as a topo II poison.

It has been known for more than a decade that VP-16 stabilizes topo II-associated double-stranded DNA breaks by inhibiting the ability of the enzyme to ligate cleaved nucleic acid molecules (Burden and Osheroff, 1998; Fortune and Osheroff, 2000). However, the mechanism by which it accomplishes this action is not well understood. Because there are two scissile bonds per enzyme-mediated double-stranded DNA break, it has been assumed that there are two sites for VP-16 in every cleavage complex. However, it is not known whether the action of VP-16 at only one scissile bond is sufficient to stabilize a double-stranded DNA break or whether both drug sites need to be occupied. A recent study that examined drug effects on human topo IIα indicated that VP-16 actions at both scissile bonds are required to stabilize double-stranded breaks in the genetic material (Bromberg et al., 2003). The presence of VP-16 at either scissile bond inhibits DNA ligation only on that strand, and hence stabilizes a single-stranded break in the double helix (Bromberg et al., 2003).

Clinical Pharmacology

VP-16 is approved in the United States for the treatment of testicular and small cell lung carcinomas. Etoposide phosphate is more water-soluble than VP-16 and is a prodrug that is rapidly converted to VP-16 in vivo (Schacter et al., 1994). Both intravenous (i.v.) and oral formulations of VP-16 and etoposide phosphate are available. Schedules using prolonged or frequent intermittent dosing appear to be more effective than single-dose regimens (Slevin et al., 1989). VP-16 is commonly administered as a one-hour i.v. infusion using multiple daily

doses of 100 mg/m^2. An elimination half-life of about 9 h is associated with this dose (Arbuck et al., 1986). However, daily doses up to 750 mg/m^2 also are used (Broun et al., 1997).

About 96% of VP-16 is bound to plasma proteins (Stewart et al., 1989), with a significant proportion of the drug excreted unchanged by the kidney (Sinkule, 1984). A 30% dose reduction is recommended in patients with impaired renal function (Joel et al., 1994). Although a small percentage of VP-16 and VP-16 metabolites are excreted in the bile, dose adjustment may not be required in patients with hepatic dysfunction (Hande et al., 1990). When administered intravenously, VP-16 should be infused slowly, since rapid infusion may result in hypotension. Etoposide phosphate can be administered more rapidly. When given orally, VP-16 is often administered daily for 14 to 21 days. Bioavailability of oral VP-16 is about 50%. Intestinal P-glycoprotein may efflux VP-16 and is thus important in the intestinal absorption of the drug (Leu and Huang, 1995).

Myelosuppression is the major toxicity associated with the administration of VP-16, either intravenously or orally. Infrequent toxicities include allergic or other infusional reactions manifested as fever, bronchospasm, and hypotension. Toxicities associated with oral VP-16 include nausea, vomiting, and mucositis (Hande, 1992). Most VP-16 side effects occur within 1-10 days of drug administration. However, the late (1-5 years) development of acute myeloid leukemia (t-AML) in 2-12% of patients treated with VP-16 has been reported, characterized by site-specific rearrangements in the mixed multiple leukemia (*MLL*) gene on chromosome 11q23 (Felix, 2001).

Activity

VP-16 is a highly successful anticancer drug that has been in clinical use for nearly 20 years. It is frequently used as a first-line drug for treating small cell lung cancer, germ cell tumours, lymphomas, and Kaposi's sarcoma associated with AIDS (Hande, 1998). It is also used to treat a variety of leukemias including acute lymphocytic leukemia. Until the emergence of the taxanes, VP-16 was the most widely prescribed anticancer drug in the world. It is used in combination with other antitumor drugs (Slevin, 1991), as well as irradiation (Evans et al., 1995).

8.3 Merbarone (MER)

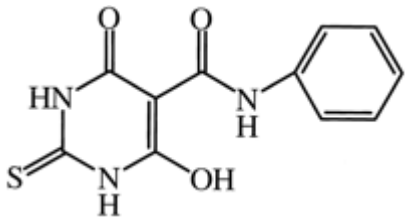


Fig. 11. Structural formula of merbarone. Adapted from Fortune and Osheroff, 1998.

History

MER 5 (N- phenyl carboxamide)-2-thiobarbituric acid is a nonsedating derivative of barbituric acid. With a molecular formula of $C_{11}H_9N_3O_3S$ and molecular weight 263.30. For preclinical and clinical use, it was formulated as the N-methyl-D-glucamine (meglumine) salt. MER was selected for clinical trials based upon its good antitumor activity that were observed in four intraperitoneally (i.p.) implanted murine tumor models, the P388 and L1210 leukemias, the B16 melanoma, and the M5076 sarcoma (Brewer et al., 1985). Although more than 700 barbiturates have been evaluated for anticancer activity by the National Cancer Institute (Bethesda, MD, USA) screening program (Driscoll et al., 1978), the high activity of MER was unique among barbiturate compounds. Retention of the antitumor efficacy of MER against the L1210 leukemia was observed when the tumour was implanted subcutaneously and MER was administered on a multiple i.p. or p.o. dose schedule, however, diminished efficacy was found upon i.v. injection of the drug (Brewer et al., 1985).

Mechanism of Action

The drug inhibits the catalytic activity of topo II with some selectivity toward the topo IIα isoform (Drake et al., 1989). In contrast, it has only weak activity toward topo I. MER showed no detectable interaction with nucleic acids and is not able to displace ethidium bromide from calf thymus DNA, as determined by fluorescence spectrometry (Cooney et al., 1985). A detailed study of the different steps in the catalytic cycle shows that MER has no effect on either DNA binding or ATP hydrolysis but is a potent inhibitor of enzyme-mediated DNA cleavage (Fortune and Osheroff, 1998). Furthermore, MER is able to compete with VP-16, suggesting that the two agents might be competing for similar binding sites on topo II. Exposure of living cells to MER leads to formation of DNA single-strand breaks, S phase

retardation, and G_2 arrest (Chen and Beck, 1993; 1995). Mitotic chromosomes are elongated and intertwined, consistent with inhibition of topo IIα (Chen and Beck, 1993; Kallio and Lahdetie, 1997). As expected, MER inhibits the induction of cleavable complexes by VP-16, amsacrine, and VM-26, but not by the topo I inhibitor camptothecin, in cellular models (Drake et al., 1989; Fortune and Osheroff, 1998). Recently, colony formation assays show that both MER and ICRF-187 are able to significantly potentiate the cytotoxicity of the alkylating agent melphalan (Hirota et al., 2002).

Clinical Pharmacology

Anti-tumour activity of MER has been described in several murine tumour cell lines including the L1210 leukemia, the M5076 sarcoma cell and the B16 melanoma cell lines (Brewer et al., 1985; Glover et al., 1987). Phase I studies have reported nephrotoxicity as the dose limiting toxicity of MER. Other mild and tolerated toxicities included hepatotoxicity, myelosuppression (Dimaggio et al., 1990) phlebitis (when the drug was administered through a peripheral vein), anorexia, nausea, and fatigue.
The antitumor activity of MER appears to be route and schedule dependent (Dimaggio et al., 1990). In the phase I trials where MER was administered on a 5-day continuous i.v. schedule, the maximum tolerated doses 1,000 mg/m^2 per day and 1,250 mg/m^2 per day were established (Kraut et al., 1988). MER is metabolised largely in the liver. In the schedule of 24 h continuous i.v. for 5 days, the two major metabolites are 4'-hydroxymerbarone and 2-oxo-desthiomerbaronethe (Supko and Malspeis, 1991).

Activity

Despite preclinical activity in predictive cell lines, phase II studies with MER did not demonstrate any appreciable activity in cervical, ovarian, pancreatic, and malignant brain tumours (Jones et al., 1993; Look et al., 1995; 1996; Malik et al., 1997).

9 Aim of the Experiments

Aneuploidy has a critical impact on human health. Chromosome studies of human gametes clearly demonstrated that most aneuploidies are attributable to meiotic segregation errors in germ cells (Kamiguchi et al., 1994). To decrease the risk of aneuploidy production, detection of aneugens and understanding of the causal mechanism of meiotic errors in gametes are essential. The experiments for this thesis were performed within the framework of the EU-Research Project "Protection of the European Population from Aneugenic Chemicals" (PEPFAC). In this project, five Universities and Research Groups collaborate to evaluate methods for the detection of aneugenic chemicals. The test organisms range from mammalian cells in culture (J.M. Parry, Univ. Swansea, UK, Coordinator), via somatic cells in vivo (mouse gut and bone marrow, M. Kirsch-Volders, Univ. Brussels) to mouse oocytes in vitro (U. Eichenlaub-Ritter, Univ. Bielefeld), mouse oocytes in vivo (F. Pacchierotti, ENEA, Cassacia, Rome) and male mouse germ cells in vivo (I.-D. Adler, GSF, Neuherberg). The chemicals to be studied in all test systems were selected by the Project Steering Committee on the basis of their molecular mechanisms of action. Topo II inhibitors were one group of chemicals of interest and of these, VP-16 and MER were chosen for the present experiments.

Since topo II is involved in chromosome condensation and chromosome segregation during mitosis and meiosis, the possibility exists that topo II interactive agents have potential clastogenic and aneugenic actions on mitotic as well as meiotic cells. In fact, VP-16 has been reported to cause both structural chromosome aberrations and aneuploidy in primary oocytes of the mouse (Mailhes et al., 1994; 1996) and the Chinese hamster (Tateno and Kamiguchi, 2001). Similar chromosomal effects of the inhibitor were found in mouse primary spermatocytes (Kallio and Lähdetie, 1996; Marchetti et al., 2001). Another topo II inhibitor MER, caused aneuploidy in mouse primary spermatocytes without formation of structural chromosome aberrations (Kallio and Lähdetie, 1997). The majority of the literature has described MER as having only modest or even no clastogenic activity using denaturing-type assays of DNA damage. In contrast, in a few studies measuring chromosomal alterations MER has been reported to produce significant genetic effects. Recently, the origin of VP-16 and MER-induced MN in TK6 cells was analysed by CREST-staining (Wang and Eastmond, 2002). The authors found that the majority of MN were of clastogenic origin. In bone marrow erythrocytes of mice treated with MER they found that only the frequencies of CREST-negative MN were increased dose dependently indicating only a clastogenic effect.

The objectives of this study were:

- Quantification of aneuploid sperm induced by two mechanistically different topo-II inhibitors, VP-16 and MER, during meiosis of male mice.
- Analysis of the induction of centromere-positive MN induced in mouse bone marrow erythroblasts by the two drugs.
- Interpretation of the dose-response curves.
- Comparison of the germinal and somatic effects of the two drugs.

The sperm-FISH assay and the bone marrow micronucleus test supplemented by FISH with the mouse minor satellite DNA probe were applied for these investigations.

B. MATERIALS AND METHODS

1 Animals

Experiments were performed with male (102/El x C3H/El)F1 mice aged 10-14 weeks and weighing 25-29 g. Animals bred in the mouse colony of the GSF-Research Centre were maintained on a 12 h light/dark cycle with mouse standard pellet food and water *ad libitum*. Each treatment group and vehicle control group consisted of 5 randomly assigned animals. The total number of animals in the current study was 120 treated mice and 75 vehicle control mice.

2 Test Chemicals

- Etoposide [VP-16, pure powder (Sigma, Steinheim, Germany)]:
  The drug solutions were formulated with DMSO in saline solution, mixed on a magnetic stirrer for at least 30 min prior to administration and administered by intraperitoneal (i.p.) injection within 1 h following preparation.

- Merbarone [MER; pure powder (Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD, USA)]:
  The drug solutions were formulated with DMSO in sterile water, mixed on a magnetic stirrer for at least 30 min prior to administration and administered by i.p. injection within 1 h following preparation.

- Colchicine [COL; pure powder (Sigma, Deisenhofen, Germany)]:
  The drug solution was prepared by dissolving the pure powder in sterile water and used at a concentration of 3.0 mg/kg as positive control aneugen.

- Mitomycin C [MMC; pure powder (Sigma, Steinheim, Germany)]:
  The drug solution was prepared by dissolving the pure powder in sterile water and used at a concentration of 1.0 mg/kg as positive control clastogen.

The choice of dosing for the topo II inhibitors tested the present experiments was based on human therapeutic dose levels for VP-16 and toxicology data from the literature for

MER. In human chemotherapy, VP-16 is administered typically at doses of 50-150 mg/m^2 per day for 3 to 5 days (Einhorn, 1997). The mouse has a body weight/surface area ratio of ≈3 kg/m^2. Thus, the highest dose of 50 mg/kg used in our study corresponds to ≈150 mg/m^2 and is within the dose range used for human chemotherapy. In a pre-clinical toxicity trial with MER, Glover et al. (1987), reported excitement, ataxia, decreased activity, laboured breathing, hyperextension of limbs, muscle tremors and lying motionless for mice exposed to single i.v. doses of 47-64 mg/kg or 5 daily i.v. doses of 41-57 mg/ kg MER. These toxicity symptoms were either followed by death or completely reversed. In our BrdU-incorporation experiment, five of 21 mice (23.8%) that were i.p. treated with 80 mg/kg of MER died within 2 h. Therefore, we performed the sperm FISH studies with 60 mg/kg MER as the highest dose.

3 BrdU-incorporation Assay for Meiotic Delay

3.1 Principle

It has been often discussed that cell cycle delay and aneuploidy induction are closely if not causally related (Chen et al., 1981; Miller and Adler, 1992). Therefore, Schmid et al. (2001a) developed the BrdU-assay to determine the time course for the appearance of BrdU-labelled sperm. In this assay, primary spermatocytes were labelled with 5-bromo-2`-deoxyuridine (BrdU) and the appearance of BrdU-containing sperm in the epididymis was then detected at various intervals after treatment with the test chemicals. BrdU is an analogue of thymidine (derivative of uridine) and can be incorporated specifically into DNA in place of thymidine. Cells can be pulse-labelled with BrdU because those cells that are synthesizing DNA (in S-phase during preleptotene of meiosis) will incorporate BrdU into the DNA. Anti-BrdU antibody is used to identify cells that underwent DNA synthesis during exposure to BrdU. The proportion of cells in S-phase of the cell cycle can be determined either manually by fluorescence microscopy or automatically by Laser Scanning Cytometry (LSC).

3.2 Treatment of Animals

The mice were i.p. injected with 100 mg/kg of BrdU at S-phase during preleptotene of meiosis. During meiosis I and II, 13 days later, the animals were treated with 50 mg/kg VP-16 in 6% DMSO or 80 mg/kg MER in 20% DMSO. Control mice received similar amounts of

DMSO in 0.9% NaCl, and the injected volume was 0.01 ml per 1 g body weight. The chosen doses represent the maximum tolerated doses for our hybrid mouse stock. In our experiments, five of the 21 mice (23.8%) that were i.p. treated with 80 mg/kg MER died within two hours. The cause of death was not ascertained. Five treated and 5 solvent control mice were sacrificed per day between 20-24 days after treatment and sperm were collected from the *Caudae epididymes* for the quantification of BrdU-labelled sperm by meiotic delay assay.

3.3 Sampling of Epididymal Sperm

The preparation technique for epididymal sperm was based on the procedure by Schmid et al. (1999). Briefly, both *caudae epididymes* of each animal were dissected and incisions were made. Then they were placed individually into Eppendorf cups filled with 300 µl of fetal calf serum (FCS). The cups were placed on an Eppendorf incubator at 32°C for 30 min to allow the sperm to actively leave the epididymes. The tissue residuals were removed from the cups and the sperm suspensions were stored at -20°C. Fresh or thawed sperm suspensions (7 µl) were pipetted onto microscope slide that have been cleaned with detergent and thoroughly rinsed in distilled water and 70% ethanol. Unfixed cells were spread across the slide and the slides were dried by first placing them on an Eppendorf incubator at 70°C for 5 min and then drying at room temperature overnight. The slides were coded and stored at -20°C for later use. The slide processing can be performed also directly after drying. The remaining sperm suspensions were stored at -80°C.

3.4 Sperm-Slide Processing

After heating the slides for 5 min at 70°C on a hot plate, the sperm nuclei were decondensed by placing the slides in a Coplin jar containing 10 mM DTT in 0.1 M Tris-HCl buffer at pH 8.0 for 30 min on ice. Thereafter, the slides were transferred to a Coplin jar containing 4 mM LIS in 0.1 M Tris-HCl buffer at pH 8.0 for 30 min on ice or at room temperature. The slides were dried by heating for 5 min at 70°C on a hot plate before being stored at -20°C. The immunofluorescence staining can be performed also directly after decondensation.

3.5 Immunofluorescence Staining

Immunofluorescence staining was performed according to a modified technique of Zink et al. (1998). The slides were denatured in 2N HCl at room temperature for 60 min and washed two times for 10 min in a Coplin jar with PBS at room temperature to neutralize the acid. Indirect immunodetection was carried out in two steps in a humidified chamber: slides were incubated with 40 µl per slide of a rat anti-BrdU antibody for 30 min at room temperature. After washing for 10 min with PN solution at room temperature, the slides were incubated with 40 µl per slide of a fluorescein-isothiocyanate (FITC)-conjugated anti-rat IgG antibody for 30 min at room temperature and washed again for 10 min in PN solution at room temperature. The nuclei were counterstained with 40 µl per slide of propidium iodide (PI; 2.0 µg/ml) for 10 min at room temperature. Then the slides were submerged shortly in PN solution and cover-slipped in Vectashield mounting medium. Slides were stored at -20°C in the dark before scoring.

3.6 Analysis by Laser Scanning Cytometry (LSC)

The microscope-based LSC manufactured since mid-1990 in the United States by CompuCyte Corp. (Cambridge, MA) and in Japan by Olympus Optical Co. (Tokyo), offers many advantages of flow cytometry (FC) combined with fluorescence microscopy. The analytical capabilities of LSC, therefore, complement those of FC, and extend the use of cytometry in many applications for specimen on microscopic slides. The instrument itself, its analytical capabilities, and similarities and differences between LSC versus FC are explained elsewhere (Grabarek and Darzynkiewicz, 2002; Tárnok and Gerstner, 2002). It will only be described briefly in this section. The instrument consists of a laser unit connected to a fluorescence microscope with two charge-coupling device (CCD) cameras (black-white and colour) to record scatter and fluorescence signals on a pixel-by-pixel basis.

The microscope (Olympus DX50) is the key part of the instrument, and it provides essential structural and optical components (Fig 12). The specimen on the stage of the microscope is illuminated by laser beams that rapidly scan the slide. The beams from two lasers (argon ion and helium-neon) spatially merged by dichroic mirrors are directed onto the computer-controlled scanning mirror producing a saw tooth motion at a nominal rate of 370 Hz, creating a line scan at the microscope slide.

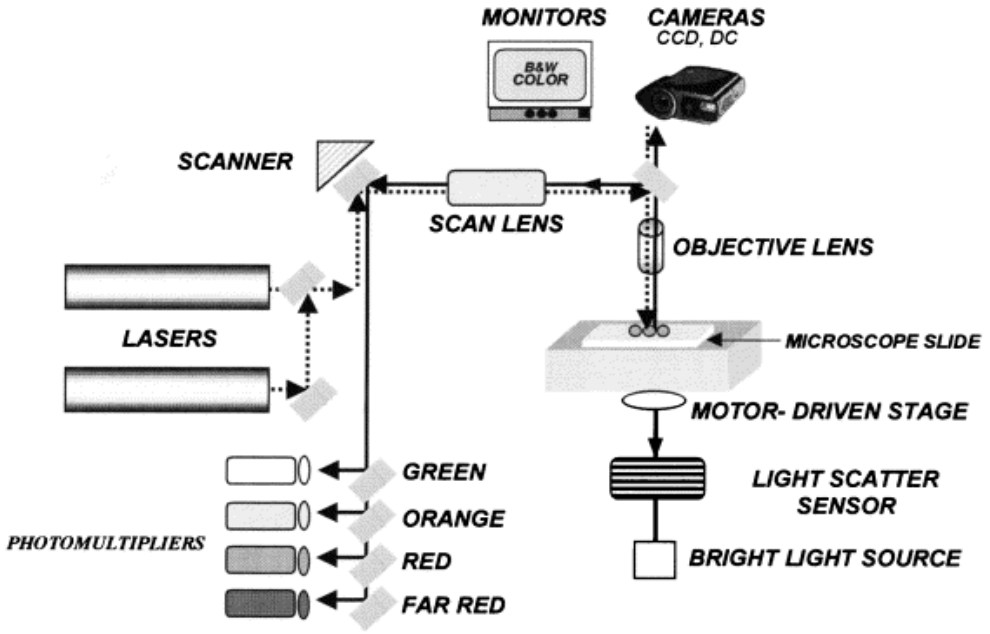


Fig. 12. Scheme representing major components of the laser scanning cytometer (LSC). See text for explanation. Adapted from Grabarek and Darzynkiewicz, 2002.

After passing through a scan lens, the beam enters the epi-illumination port of the microscope and is imaged by the objective lens onto the slide. The produced beam spot size varies depending on the lens magnification, from 2.5 (at 40x) to 685 μm (at 10x). The slide, with its *xy* position monitored by sensors, is placed on the computer-controlled motorized microscope stage, which moves at 0.5 μm steps per each laser scan, perpendicularly to the scan. Laser light scattered by the cells is imaged by the condenser lens and its intensity recorded by sensors. The specimen-emitted fluorescence is collected by the objective lens and part of it is directed to a CCD camera for imaging. Another part is directed to the scanning mirror. Upon reflection, it passes through a series of dichroic mirrors and optical emission filters to reach one of the four photomultipliers. Each photomultipler records fluorescence at a specific wavelength range, defined by the combination of filters and dichroic mirrors. In the standard instrument, the following bandpass filters are used: 530/DF30 (green), 580/DF30 (orange), 625/DF28 (red); a 650EFLP longpass filter (far red) is also used. A light source, additional to the lasers, provides transmitted illumination to visualize the objects through an eyepiece or the CCD camera. The light signals are converted to electric signals, which are then digitized. After every scanning step, a digital image is created for each PMT (i.e. colour) on a pixel-to-pixel basis. These images are analyzed by software (WinCyte) according to the operators' settings as schematically shown in Figure 13.

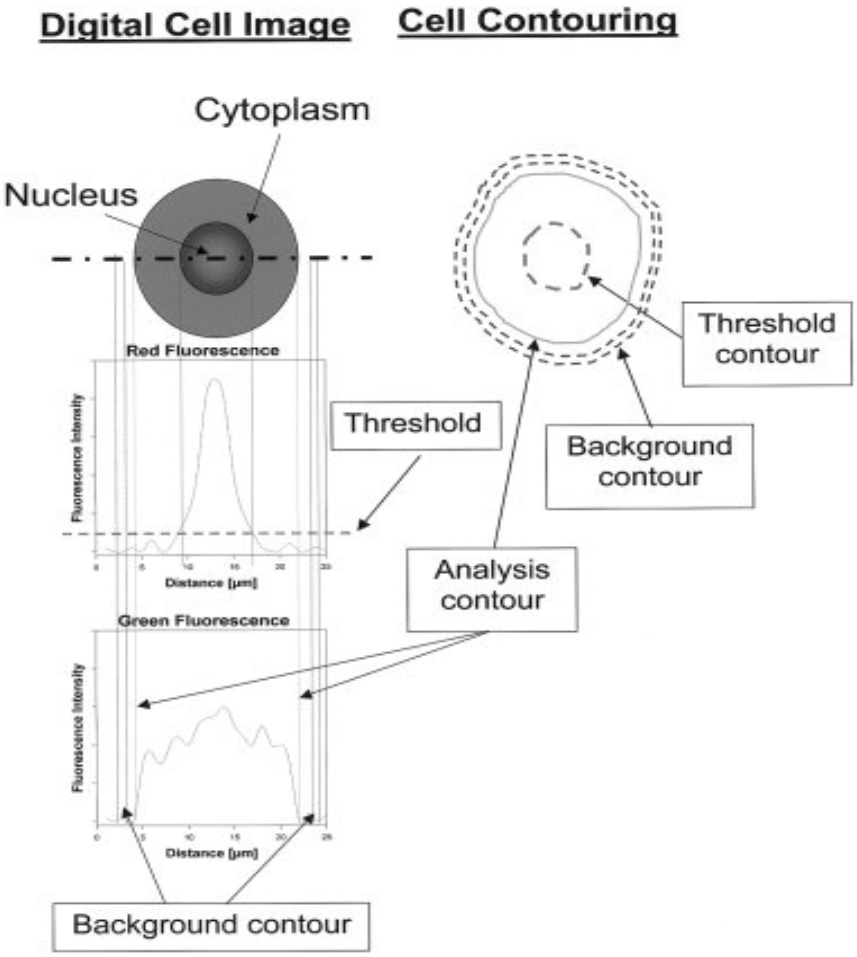


Fig. 13. Schematic outline of cell analysis by laser scanning cytometry (LSC). In this example, the cell nucleus is stained with propidium iodide (PI, red) and the cytoplasm is stained with fluorescein (FITC, green). The fluorescence image is acquired by LSC (top left). The fluorescence intensity distribution along the hatched line is exemplified in the two histograms below for the red and the green fluorescence, respectively. In this example, the red signal was used for triggering; the threshold contour (top left) is drawn around the pixels that exceed the operator-set threshold value. Only cells with areas inside the threshold contour above an operator-set minimal area are further analyzed. The analysis contour is then calculated at an operator-set pixel distance from the threshold contour. Background contours are generated automatically at operator-set pixel distances. Adapted from Tárnok and Gerstner, 2002.

An average of 10,000 BrdU-positive (green fluorescence) and BrdU-negative (red fluorescence) cells per animal and a total of 555,877 sperm were scored automatically with the LSC. The parameters “red integral” and “green integral” as well as “green maximum pixel” were registered for each sperm, and the data were displayed as a dot plot or histogram (Fig. 14).

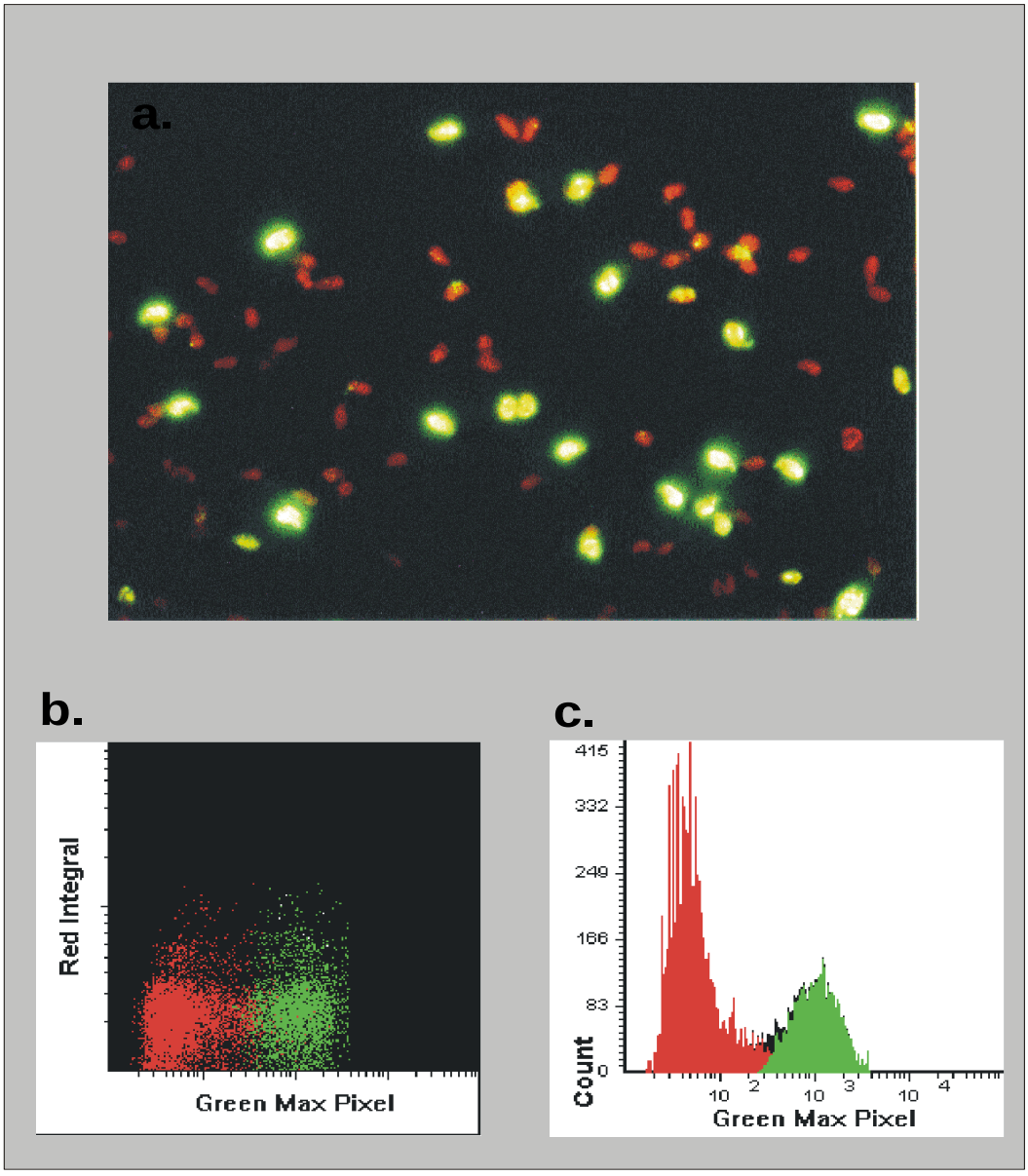


Fig. 14. (a) Microscopic photograph of the BrdU-positive and negative sperm. The sperm chromatin was labelled with a fluorescein anti-BrdU antibody (FITC, green sperm) and counterstained with propidium iodide (PI, red sperm). (b) Dot plot (red integral against green integral). (c) Histogram (green maximum pixel) of green (FITC) fluorescent sperm scored with the LSC.

3.7 Statistical Analysis

The differences between the frequencies of BrdU-positive sperm in treated animals and the concurrent solvent controls on each day were tested for significance using Student's *t*-test (Ott, 1988).

4 Fluorescence *in situ* Hybridization (FISH)

4.1 Principle

FISH is used for many purposes, including analysis of chromosomal damage, gene and cosmid mapping and ordering, clinical diagnostics, molecular toxicology and cross-species chromosome homology. FISH allows to identify the presence and location of a region of cellular DNA or RNA within morphologically preserved chromosome preparations, fixed cells or tissue sections. Several reviews have already described the history of FISH and its potential and range of applications (for example Levsky and Singer, 2003; Moter and Göbel, 2000; Swiger and Tucker, 1996). Recently, the range of FISH applications was complemented by a diversity of probes, new fluorochromes and detection systems. As a result, an increasing number of different DNA probes can be hybridized and analyzed simultaneously. This led to a multitude of approaches, strategies and technologies to address different diagnostic and biological problems. FISH detects nucleic acid sequences by a fluorescently labelled probe that hybridizes specifically to its complementary target sequence within the intact metaphase and interphase cell (Fig. 15).

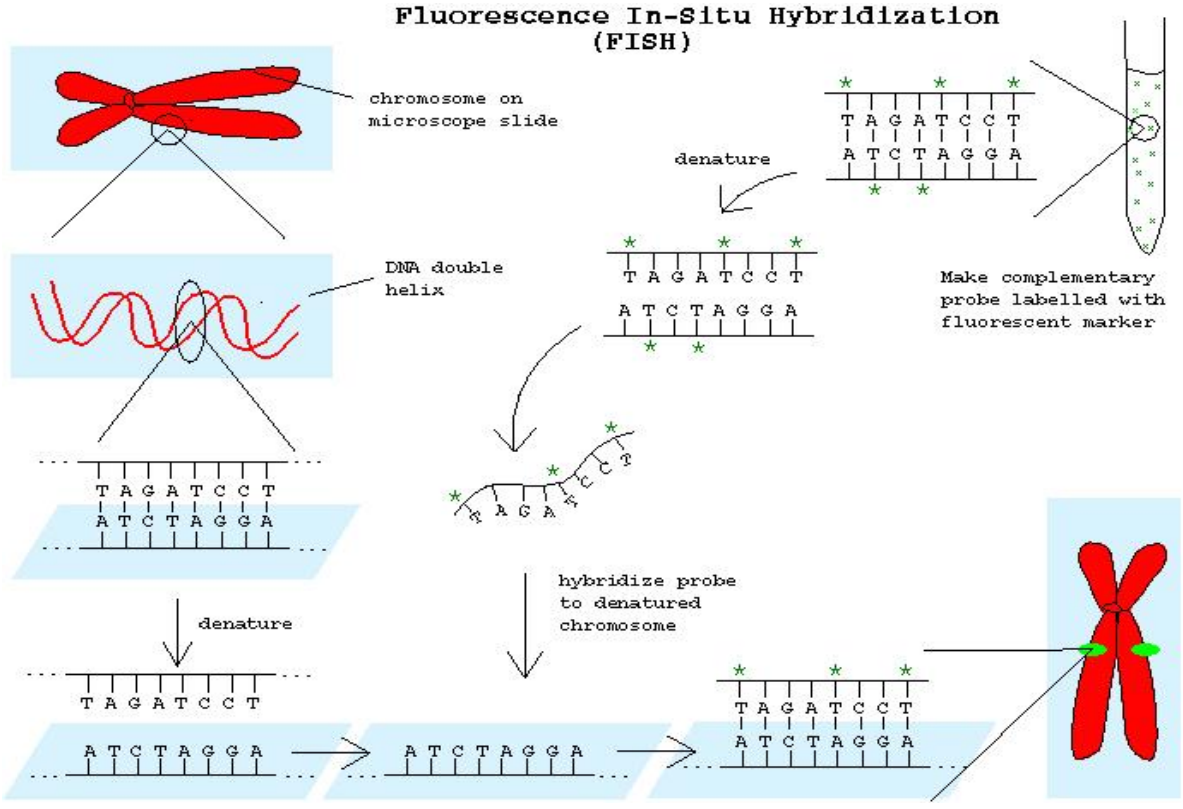


Fig. 15. Flow chart of major steps of the FISH assay.

A typical FISH protocol includes the following steps: fixation of the specimen; permeabilization of the sample; hybridization with the respective probes for detecting the respective target sequences; washing steps to remove unbound probes; mounting, visualization and documentation of labelled cells by microscopy or flow cytometry. In the following section we concentrate on the most commonly used techniques only.

Hybridization Reaction: Kinetics and Components

Hybridization is a dynamic reaction where denaturated target sequences and complementary single stranded DNA or RNA probes anneal forming stable double stranded hybrid molecules. During hybridization, stable and fleeting interactions between target and probe occur. Four types of duplexes are formed including target: target, probe: probe, stable (specific) hybrids, and unstable (non-specific) hybrids. The first three types are more stable and their duplexes can withstand more stringent conditions during hybridization and subsequent washes due to a high degree of complementary base pairing. Probe: probe or target: target duplexes are not detectable or desirable because the complex is either not bound to the slide and are removed during the washing steps, or are not labelled and can not be seen. Non-specific interactions are usually eliminated during stringent washing steps.

The kinetic molecular theory is useful for predicting the parameters of the hybridization process. The concentration of probe and target, temperature and salt content, as well as pH, significantly determine the rate of annealing. Most DNA samples exist at ground state conformation in continuous double helices with minor looping, breaks and imperfections (non-complementary base pairing). Duplex DNA is asymmetrical in sequence, excluding repetitive elements, and remains at ground state in cellular conditions. However, under alkaline (pH >10) or low salt concentrations (0.01 M NaCl) in the presence of heat and/or formamide (FA), DNA may be denatured (Darnell et al., 1990). Upon returning to cellular conditions, the denatured DNA will form bonds between complementary base pairs, returning to its native conformation.

Hybridization reactions are usually performed in a dark humid chamber at 37°C for 12-72 h in the presence of 2x SSC, 50% FA, blocking, carrier and probe DNA, and intermolecular crowders (e.g. dextran sulfate), at pH = 7.0. FA is used during hybridization because it destabilizes hydrogen bonding across base pairs and lowers the melting temperature (T_m) of target and probe. Within the range of experimental conditions, each percent FA present lowers the T_m by about 0.7°C (Polak and McGee, 1991). FA destabilizes

AT-rich sequences disproportionately more than GC-rich hybrids. DNA from distantly related species (salmon or herring sperm) is often used as blocking and carrier DNA during hybridization in order to reduce the non-specific binding of a hybridization probe to nonbiological sites in the absence of target (Sambrook et al., 1989). It is sized to approximately 400 pb and usually 70 to 170 ng/ml (up to 30-fold the concentration of probe) is used during hybridization. Crowders such as dextran sulfate facilitate the hybridization process by decreasing the free space in the hybridization reaction. Dextran sulfate is commonly used because it is more hydrophilic than DNA or RNA, and is strongly hydrated in aqueous solutions. Thus, the nucleic acids have less access to water and hybridize at a faster rate.

Probes, Labelling and Detection

In molecular cytogenetics, three different types of probes are generally used: probes specific for repeated sequences (centromeric or telomeric probes), whole chromosomes (chromosome panting probes), or single copy sequences (used for identifying structural chromosome changes). The optimal probe length and concentration should be determined experimentally. Typical probe length is between 250 bp and 1 kb, depending on the application. Probes exceeding 1 kb usually result in increased non-specific background, thus decreasing the signal to noise ratio. Probes less than 200 pb may have insufficient hydrogen bonding to the target and require lower stringency during hybridization and washes for adequate signal detection. Probe concentration often affects the signal to noise ratio. If the probe concentration is too high, non-specific background hybridization is increased and decreased signal to noise is observed. Alternatively, if the probe concentration is too low, hybridization is insufficient for detection.

Much of the FISH analysis used today uses cloned DNA. Cosmids, plasmids, YACs and BACs may be used to produce suitable probes. Often, the insert together with a vector may be labelled using nick translation, PCR or random-primer extension. Alternatively, the cloned insert may be removed with restriction enzymes prior to labelling. There are different ways of labelling. The direct labelling method is most commonly used and is also the fastest, cheapest and easiest one because it does not require any further detection by antibodies or (strept)avidin after hybridization. Common fluorochromes include fluorescein, rhodamine, Texas red, AMCA and, increasingly, carbocyanine dyes like Cy3 or Cy5. One or more fluorescent dye molecules are directly bound to the nucleotide either chemically during

synthesis through an aminolinker at the 5'-end of the probe (Fig. 16a), or enzymatically using terminal transferase to attach fluorescently labelled nucleotides at the 3'-end (Fig. 16b) (Moter et al., 1998). Coupling of FITC to the nucleotide via an 18-carbon spacer may increase signal intensity as compared to a directly conjugated probe (Deere et al., 1998). An increase in fluorescence signal has also been reported by labelling probes at both ends, one fluorescent molecule at the 3'-end and four molecules at the 5'-end using appropriate spacers to prevent quenching of fluorescence (Spear et al., 1999). Directly labelled nucleotides are now commercially available from a variety of sources and come in many colours. They can be stored at -20°C for several months.

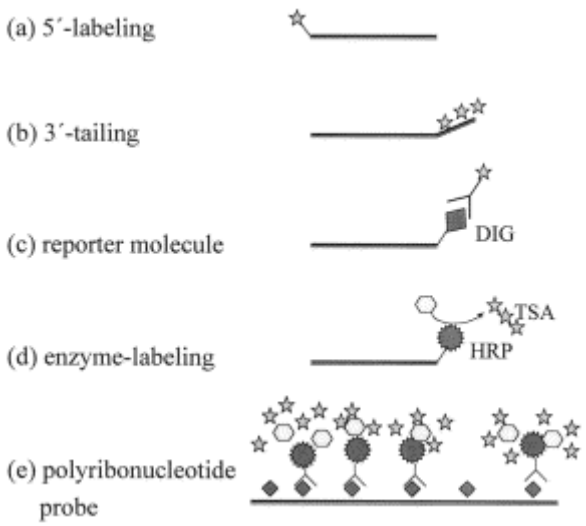


Fig. 16. Direct, (a) and (b), and indirect, (c)-(e), labelling of probes using digoxygenin (DIG), horseradish peroxidase (HRP) or Tyramide Signal Amplification (TSA). Adapted from Moter and Göbel, 2000.

In some cases, indirect detection is helpful; it has been shown that the sensitivity of the FISH assays can be increased by linking the probe to a reporter molecule like digoxygenin (DIG) that is then detected by a fluorescent antibody (Fig. 16c) (Zarda et al., 1991). Sensitivity of FISH can be further increased using enzymatic signal amplification. This method was developed by Kagiyama et al. (1993), for cytogenetic investigations and later adapted for the detection of bacteria by Yamaguchi et al. (1996). A DIG-labelled nucleotide is detected by an Anti-DIG antibody that is coupled to alkaline phosphatase. This enzyme converts 2-hydroxy-3-naphtoic acid-2'-phenylanilide phosphate to its dephosphorylated form that generates bright-red fluorescence in combination with Fast Red TR. Fluorescence was reported to be eight times more intensive than that of nucleotides carrying a single label.

This approach of enzymatic signal amplification was further improved by a technique called `Tyramide Signal Amplification (TSA)' system. Nucleotides were labelled with

horseradish peroxidase (HRP) that used fluorescein–tyramide as substrate (Fig. 16d) (Schönhuber et al., 1997). The sensitivity of FISH in natural samples was significantly increased using polyribonucleotide probes labelled with several fluorochrome molecules (DeLong et al., 1999). Probably the most sensitive approach is the combined use of polyribonucleotide probes, internally labelled with DIG, with the tyramide signal amplification system. This technique was successfully used for the detection and visualization of a virulence factor in *Listeria* bacterial cells (Fig. 16e) (Wagner et al., 1998). A standard indirect labelling (nick translation) is used in our laboratory to label 1 µg of DNA and to cut it to an average fragment length of 273-9,000 bp, which is optimal to obtain a uniform and intense hybridization. Fragment lengths of genomic probes are estimated by electrophoresis in 1% agarose gels and compared with a DNA molecular weight marker.

DIG and biotin molecules are covalently bound to the C-5 position of dUTP that can be incorporated by polymerases into nucleic acid probes. To detect bound probe, macromolecular reporter molecules [e.g. antibodies, (strept)avidin] that are conjugated with a fluorochrome tag (e.g. FITC, Cy3) are then applied to the microscope slide. Biotinylated probes are detected by (strept)avidin conjugated Cy3, and can be amplified with successive layers of biotinylated anti-(strept)avidin antibody. DIG is detected with Anti-DIG antibodies coupled with fluorochrome and the hybridization signal can be enhanced by adding labelled secondary antibodies such as fluorescein-anti sheep IgG (RAS). Blue fluorescent counterstaining can be performed with aromatic diamidines like DAPI that non-intercalatively binds to DNA with great affinity (Zimmer and Wähnert, 1986). PI, which is similar in structure to DAPI, is a non-specific duplex DNA and RNA binding dye. PI is also commonly used as a counterstain for fluorescein because both molecules are excited at the same wavelength (494 nm). However, they emit at different wavelengths, with PI and fluorescein appearing red and yellow, respectively. Dyes and fluorochromes with differing excitation and emission maxima may be observed simultaneously by using multi-bandpass filters, which are being used with increasing frequency. Most fluorochromes will fade rapidly upon ultraviolet excitation. To alleviate this problem, chemicals that prolong the intensity of the photon emissions have been routinely used for many years and are now commercially available (Vectashield, Vector Labs).

Detection of flurochrome in direct or indirect labelling systems is accomplished by passing a beam of light from a suitable light source (e.g. a mercury vapour lamp) through an appropriate colour filter (excitation filter) designed to transmit light at the desired excitation wavelength. In the fluorescence microscopy (Fig. 17) this light is reflected onto the

fluorescently labelled sample on a microscope slide using a dichroic mirror. The light then excites the fluorophore to fluoresce and as it doses so, it emits light at a slightly longer wavelength, the emission wavelength. The light emitted by the fluorophore passes back up and straight through the dichroic mirror, through an appropriate barrier filter and is then transmitted into the eyepiece of the microscope, and can also be captured using a suitable CCD camera.

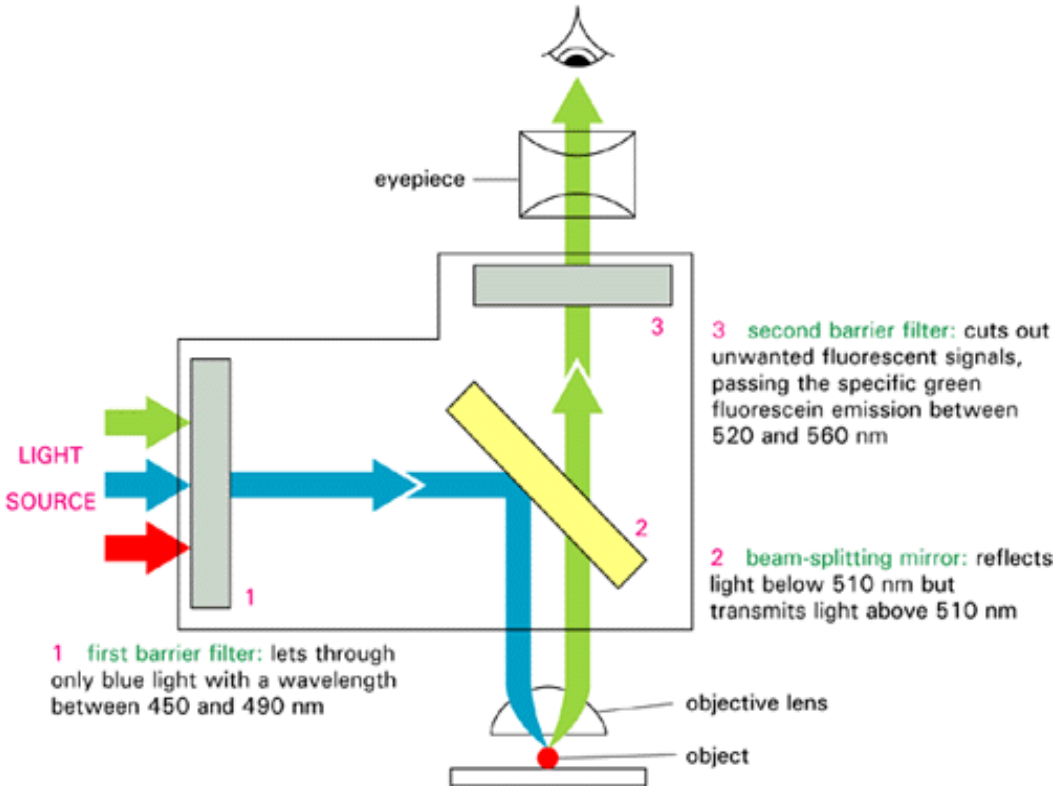


Fig. 17. The optical system of a fluorescence microscope. A filter set consists of two barrier filters (1 and 3) and a dichroic mirror (2). In this example, the filter set for detection of the fluorescent molecule FITC (green) is shown. Adapted from Alberts et al., 1994.

Mouse DNA-probes specific for chromosomes X, Y and 8 as well as mouse minor satellite DNA probe were used in our experiments. An overview of the labelling and maximum emission and excitation wavelengths for fluorochromes used for detection of the probes are shown in Table 3.

Table 3. Probes and fluorochromes.

Probes for	Reporter Molecule	Fluorochrome	Wavelength maxima *		Colour
			Excitation	Emission	
Chromosome 8	Biotin-16-dUTP	Cy3	552 nm	565 nm	Red
Chromosome X	Biotin-16-dUTP and Digoxigenin-11-dUTP	Cy3 FITC	552 nm 494 nm	565 nm 523 nm	Yellow
Chromosome Y	Digoxigenin-11-dUTP	FITC	494 nm	523 nm	Green
Centromere	Biotin-16-dUTP	Cy3	552 nm	565 nm	Red
Counterstaining	DAPI	-	359 nm	441 nm	Blue

*Wavelength may vary slightly depending on the respective manufacturer. Emission spectra might change in different solvents or sample conditions (Cullander, 1999).

4.2 Sperm-FISH Assay for Aneuploidy Detection

The sperm-FISH assay is a multistep procedure involving slide processing, denaturation of target DNA, hybridization of labelled probe, washing and probe detection. The technique for sperm FISH assay was based on the procedure described by Schmid et al. (1999).

4.2.1 Treatment of Animals

Four experiments were performed with the sperm-FISH assay. In the first experiment, random groups of five mice each were treated by i.p. injection of 25 or 50 mg/kg VP-16 in 6% DMSO or 3 mg/kg COL as positive control aneugen.

Since it was observed that a high percentage of animals died after a dose of 80 mg/kg of MER in the BrdU-incorpration assay, we performed the second experiment with 60 mg/kg MER in 15% DMSO to assess its effects on aneuploidy induction. To determine a dose-dependent effect of VP-16 or MER on aneuploidy induction random groups of 5 mice were treated with 12.5 mg/kg VP-16 and 15 and 30 mg/kg MER in the third experiment.

To determine if subacute treatment would have an effect because prophase stages would be included in VP-16 treatment, doses of 1, 2 and 4 mg/kg in 6% DMSO were injected on 13 consecutive days in the fourth experiment and sperm were sampled 23 days after the last treatment.

Concurrent solvent controls received equal amounts of DMSO in 0.9% NaCl were included in every experiment in order to code the slides and avoid scoring biases. The injected volume was 0.01 ml per 1 g body weight. After drug administration, the animals were maintained with food and water *ad libitum* until being sacrificed.

Males were sacrificed 24 days after administration of VP-16 or COL and 22 days after administration of MER and sperm were collected from the *Caudae epididymes*. The times of sampling were chosen on the basis of previous BrdU-studies for VP-16. Since MER did not cause meiotic delay, the sampling of epididymal sperm were designed according to the timing of spermatogenesis (Oakberg, 1956). A prolongation of the duration of the meiotic divisions was observed in mice treated with 3 mg/kg COL in earlier meiotic delay studies by Schmid et al. (2001a). Therefore, the optimum time for sperm sampling after COL-treatment was concluded to be 24 with COL.

4.2.2 Sperm Sampling and Slide Processing

The Preparation of sperm and slide processing were the same as in the BrdU-incorpration assay. The slides were coded by an investigator who was not involved in the scoring.

4.2.3 DNA-Probes for Chromosomes 8, X and Y

Mouse DNA-probes for chromosomes 8 (Boyle and Ward, 1992) and X (Disteche et al., 1985) were kindly supplied by A. Wyrobek (LLNL, California) and the Y-probe (Bishop and Hatat, 1987) was supplied by U.-H. Weier (LBNL, California). Plasmid DNA for chromosome X [clone DXWas70 (9 kb)], Y [clone pY353/B (1.5 kb)], and 8 [clones 8-4a (4.6 kb) and 8-5e (5.3 kb)] were transformed in *Escherichia coli* (E. coli) XL1-blue and extracted using the Qiagen Plasmid Maxi Kits according to the manufacturer's instructions. An indirect labelling of the probes with the fluorescent dyes FITC and Cy3 was performed by nick translation using the biotin or DIG systems.

Cloning, Isolation and Determination of DNA-Probes

The transformed cells were always stored at -80°C in 87% Glycerol. The cells were plated out by spreading on an agar surface containing 50 μg/ml ampicillin. The agar plates were incubated at 37°C for 24-48 h in order to ensure the growth of well-separated cell colonies.

Subsequently, individual colonies was picked from a plate and inoculated in 100 ml [for high-copy plasmids (8 and Y)] or 500 ml [for low-copy plasmids (X)] LB medium containing 50 µg/ml ampicillin. The bacterial cells were grown at 37°C for 12-24 h with vigorous shaking ≈300 rpm and harvested by centrifugation at 6,000 x g for 15 min at 4°C. The pellet was resuspended by vortexing in 10 ml of resuspention buffer containing 100 µg/ml RNase A solution, then 10 ml of lysis buffer were added and mixed gently by inverting 4-6 times before incubation at room temperature for 5 min. Ten ml of chilled neutralization buffer were added and mixed gently by inverting 4-6 times then incubated on ice for 20 min. The samples were centrifuged at ≥13,000 x g for 30 min at 4°C and the supernatant containing plasmid DNA was removed promptly and re-centrifuged again for 10 min at 4°C.

The supernatant was applied to equilibrated Qiagen-tip 500 and allowed it to enter the resin by gravity flow, then washed two times by washing buffer to remove all contaminants. The plasmid DNA was eluted with 15 ml elution buffer then precipitated by 10.5 ml isopropanol and centrifuged immediately at ≥13,000 x g for 30 min at 4°C. The glassy DNA pellets were washed with 70% ethanol and centrifuged at 13,000 x g for 10 min at 4°C, then the supernatant was carefully decanted. The pellets were dried in speed vac and resuspended in a suitable volume of TE-buffer (pH 8.0) and then stored at -20°C until ready to use. For the Qiagen-tip 500, the expected yields are 300-500 µg for high-copy plasmids and 100-500 µg for low-copy plasmids. The concentration of plasmid DNA in solution was determined by both UV spectrophotometry and quantitative analysis on an agarose gel. Some aliquots were removed and saved during the purification procedure (120 µl from the cleared lysate supernatant, 120 µl from the flow-through, 240 µl from the combined wash fractions and 60 µl of the eluate) in order to monitor the procedure on an analytical gel when plasmid DNA is of low yield or quality.

UV Spectrophotometry

To each microcentrifuge tube containing 99 µl dH_2O, 1 µl of plasmid DNA was added. The tubes were mixed gently and the absorbance was measured at 260 nm against a blank using Eppendorf BioPhotometer AG 22331. The concentration of plasmid DNA was expressed as µg/ml according to the following equation:

A x OD x F = µg/ml, where by A is the absorbance, OD is the optical density (1 OD = 50 µg DNA), and F is the dilution factor (100).

Agarose Gel Analysis

Hundred ng of DNA probe, 2 µl of *Eco* RI buffer and 0.5 µl of restriction endonuclease *Eco* RI were added to a 1.5 ml microcentrifuge tube placed in ice. The volume was completed to 20 µl with dH_2O, mixed gently but thoroughly by centrifugation for 5 seconds and incubated at 37°C for at lest 2 h. 0.5 gm agarose was melted in 50 ml 1 x TAE buffer using a microwave. A suitable volume of melted agarose was poured onto the gel platform after cooling to 55°C in a water bath. After the gel has hardened, the tape and the gel comb were removed. A sufficient volume of 1 x TAE buffer were added until the tops of the sample wells were submerged. Two µl of 10 x loading buffer were added to each digested DNA probe, mixed gently and 10 µl of DNA probe or 1 kb DNA ladder were loaded into the sample wells with a micropipet. Electrophoresis was performed at 80 volts.

During the electrophoresis, DNA fragments, which are negatively charged because of the phosphate groups, are repelled from the negative electrode towards the positive electrode, and sieved through the porous gel. Smaller DNA fragments move faster. The power supply was turned off when the bromophenol blue from the loading buffer had migrated a distance judged sufficient for separation of the DNA fragments (1-2 h). Gel containing DNA probes was then stained for 20 min by covering in dH_2O containing 0.5 µg/ml ethidium bromide. DNA fragments were photographed using a Polaroid camera while the DNA was visualized using a 366 nm UV light under a transillumination. The DNA ladder yields the following 14 discrete fragments (in bp): 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250 (the bright band at 3000 serves as a reference).

4.2.4 Labelling of DNA-Probes

Principle

The nick translation method is based on the ability of DNase 1 to introduce randomly distributed nicks into DNA at low enzyme concentrations in the presence of Mg^{2+}. *E. coli* DNA polymerase 1 synthesizes DNA complementary to the intact strand in a 5′→3′direction using the 3′-OH termini of the nick as a primer. The 5′→3′ exonucleolytic activity of DNA polymerase 1 simultaneously removes nucleotides in the direction of synthesis (Rigby et al., 1977). The polymerase activity sequentially replaces the removed nucleotides with hapten-labelled deoxyribonucleoside triphosphates (e.g. DIG-11-dUTP or Biotin-16-dUTP). At low temperature, newly synthesized labelled DNA thus replaces the unlabelled DNA in the reaction.

Procedure

The probes were labelled with DIG-11-dUTP or Biotin-16-dUTP by using a nick translation kit (GIBCO Invitrogen, USA) according to the manufacturer's protocol with slight alterations. Ten µl of dNTP-Mix (minus dTTP), 0.8 µl DIG-11-dUTP or Biotin-16-dUTP and 1.2 µl of 1mM dTTP were added to a 1.5 ml microcentrifuge tube placed in ice. A suitable volume of DNA probe containing 2 µg DNA was added and the volume was completed to 90 µl with dH_2O. 10 µl Polymerase I/DNase I Mix was added to each tube, mixed gently but thoroughly by centrifugation for 5 seconds and incubated at 15°C overnight. The reaction was stopped by addition of 10 µl stop buffer, the DNA plasmid was precipitated by 275 µl (2.5 volume) cooled absolute ethanol, 2 µl glycogen and incubation at -20°C for 1 h. The labelled DNA plasmid was centrifuged at 15,000 x g for 20 min at 4°C, the pellets were dried in speed vac and resuspended in 40 µl of TE-buffer pH 8.

4.2.5 Preparation of Hybridization Mix

The probe mix, containing two parts of salmon sperm DNA, twenty parts of the biotinylated 8-probe, fifteen parts of biotinylated X-probe, twenty fife parts of DIG labelled X-probe and thirty parts of DIG labelled Y-probe, was placed into a microcentrifuge tube cooled on ice. The probes were precipitated with 230 µl (2.5 volume) ice-cooled absolute ethanol and 2 µl glycogen at -20°C for 30 min and centrifuged at 15,000 x g for 30 min at 4°C. The pellets were dried in speed vac and resuspended in 9 µl dH_2O and 21 µl master mix 2.1. After denaturation at 78°C for 8 min, the probes were incubated at 37°C before application to denatured sperm slides.

4.2.6 Hybridization Techniques

The hybridizations were performed according to a modified technique of Pinkel et al. (1986). To denature the sperm nucleus DNA, the slides were placed in 70% FA at 78°C for 5 min, dehydrated in an alcohol series (70, 90 and 100%, 2 min each at 4°C) and dried on a slide warmer at 37°C for about 3 min. Aliquots of 30 µl of the denatured hybridization mix were added to each slide and the hybridization areas were covered with cover slips (24 x 50 mm) and sealed with rubber cement. Then, the slides were incubated at 37°C for 24-48 h in a moist chamber. Post-hybridization washing consisted of two steps: 30 min in 50% FA at 45°C and 30 min in pre-warmed PN-buffer at 37°C. The purpose of these steps is to slightly

denature DNA, which allow the loosely bound probe that does not match very well the target sequence to be carried away and to reduce the signal background.

4.2.7 Detection of Fluorescence Signals

The hybridized probes were detected by 40 µl each per slide of streptavidin-Cy3 for chromosome 8 and X and Anti-DIG-FITC for chromosome Y and X. The slides were covered with parafilm and incubated at 37°C for 30 min in a humidified chamber. The slides were then rinsed in PN buffer for 20 min. The hybridization signals were amplified by two rounds of incubation with biotinylated anti-streptavidin followed by streptavidin-Cy3 and FITC-anti-sheep made in rabbit. Between every amplification step the slides were washed in PN buffer for 20 min. The nuclei were counterstained with DAPI (0.05-0.1 µg/ml in PBS) for 10 min at room temperature and cover-slipped in Vectashield mounting medium. Slides were stored at 4°C in the dark before scoring.

4.2.8 Scoring Criteria

For this study, at least 10,000 sperm per animal and a total of 501,029 sperm were manually examined for aneuploidy on a Zeiss Axioplan Fluoescence Photo Microscop equipped with HBO 100 W/2 mercury lamp (Osram). The following filters were used: triple filter (Chroma, USA) for simultaneous visualization of green (FITC), yellow (FITC + Cy3), red (Cy3) fluorescence hybridization domains and the blue (DAPI) fluorescence of the sperm nuclei, and individual filters for each of the fluorochromes [FITC, HQ 480/40 and HQ 535/50; Cy3, HQ 535/50 and HQ 610/75 (Chroma, USA); DAPI, BP 365 and LP 397 (Zeiss, Deutschland)] to digitise the image. Records of every sperm with an abnormal number of hybridization domains were taken by digitizing the microscopic image with a CCD camera and the computer program ISIS3 (MetaSystems, Deutschland). The three applied DNA probes are identified by a yellowish white domain for the X chromosome (labelled with Cy3 + FITC), a large green domain for the Y chromosome (labelled with FITC) and a red domain for chromosome 8 (labelled with Cy3) (Fig. 18).

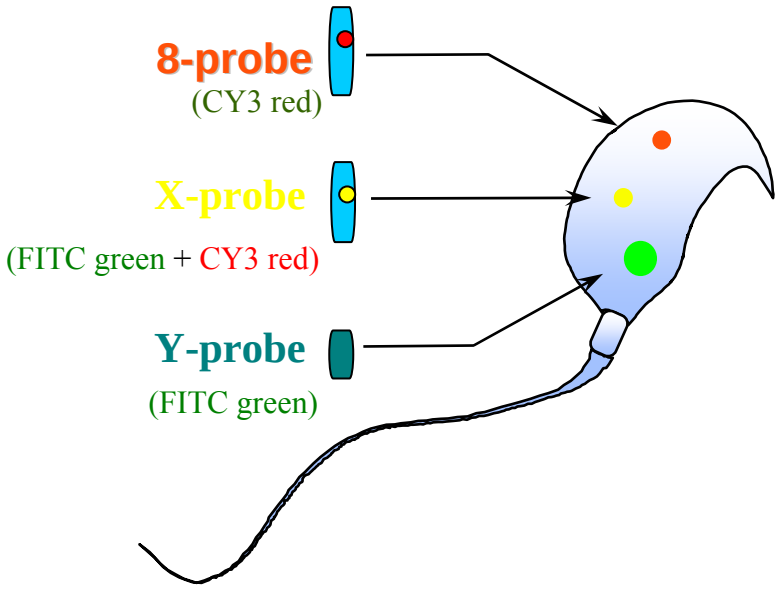


Fig. 18. Schematic representation of the three-colour FISH on sperm of mice.

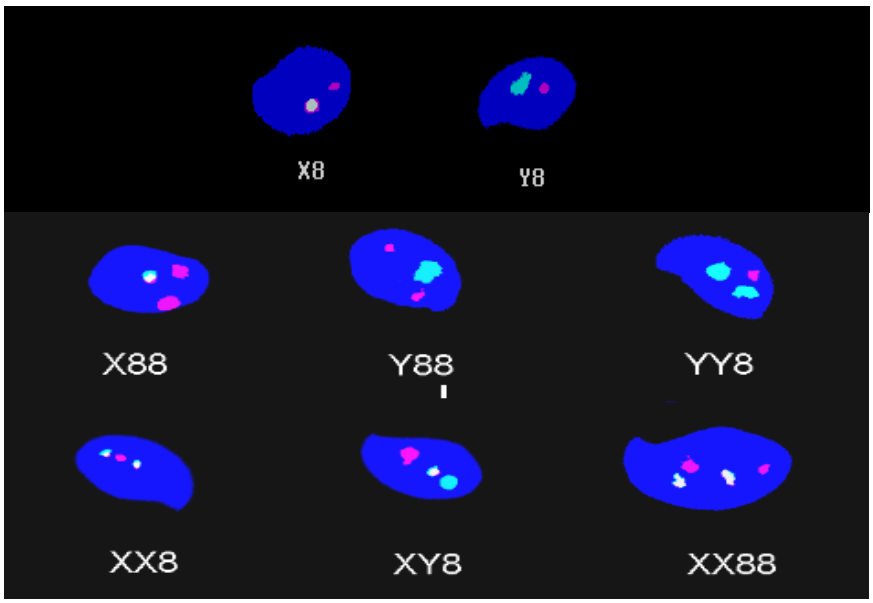


Fig. 19. Illustration of the various signal combinations in epididymal sperm of mice. X8 and Y8 are normal haploid sperm, X88 to XY8 are hyperhaploid (disomic) sperm and XX88 is a diploid sperm. The cells were digitized using the computer program ISIS3 (MetaSystems, Deutschland).

The sperm were assigned to the specific fluorochrome phenotypes as determined by the combinations of fluorescence signals in each nucleus: X8, Y8 as normal sperm, XX8, YY8, XY8, X88, or Y88 as disomic sperm, XY88 as autodiploid sperm resulting from first meiotic division suppression, XX88 or YY88 as diploid sperm resulting from second meiotic

division suppression. The patterns of fluorescent signals in epididymal mouse sperm visualized by multicolour FISH and examples of normal, five classes of disomic sperm and one class of diploid sperm are shown in Figure 19.

Strict scoring criteria were applied to avoid misinterpretation of signal numbers. Cells were scored as having two domains of the same colour if the two signals were (a) similar in size and intensity, (b) separated by at least the distance of half the diameter of the domain, and (c) regular in appearance, not diffuse and clearly positioned within the sperm head. We scored for disomies and diploidies but not nullisomies, since failure to detect a signal could be due to technical difficulties as well as to non-disjunction and, therefore, cells with the hypohaploidy phenotypes were not considered to be a reliable measure of aneuploidy. The terms diploidy and autodiploid were used rather than the term double disomy, because these cells appear larger in size than normal cells, which is suggestive that they contain a duplicated set of all autosomes.

4.2.9 Statistical analysis

For a statistical overall comparison of the frequencies of disomic or diploid sperm the χ^2 test with Yate's correction was used and comparisons on an individual animal basis were carried out with the Mann-Whitney *U*-test (Sachs, 1984).

4.3 Bone Marrow Micronucleus Test Combined with FISH-assay

4.3.1 Treatment of Animals

Two experiments were performed with the mouse bone-marrow micronucleus test. In the first experiment, animals were treated with 1 mg/kg of VP-16, 3 mg/kg of COL as positive control aneugen or 1% DMSO as solvent control I.

In the second experiment, mice were treated with 60, 30, 15 or 7.5 mg/kg of MER, 1 mg/kg of mitomycin C (MMC) as positive control clastogen or 15% DMSO as solvent control II. The doses for the positive controls were chosen by reference to earlier studies in our laboratory (COL: Schmid et al., 1999; MMC: Kliesch et al., 1981). The dose of 1 mg/kg of VP-16 was selected based on the data by Ashby et al. (1994). The highest dose of MER was selected based on our previous studies in mouse sperm FISH assay. The injected volumes were 0.1 ml per 10 g body weight.

4.3.2 Sampling and Preparation of Bone Marrow Cells

Erythrocytes

In the micronucleus test, polychromatic and normochromatic erythrocytes were scored. The animals were sacrificed 24 h after treatment with the test chemicals or solvent and both femurs were removed. The bone marrow cells were collected in FCS, centrifuged at 1,100 rpm for 10 min, and the pellet was carefully resuspended in as little supernatant as possible before slide preparation according to the standard technique (Adler, 1984). At least four slides were made for each animal and allowed to dry overnight. The slides were coded by an investigator who was not involved in the scoring. Two slides per animal were stained with May-Gruenwald/Giemsa solutions for conventional assessment of the MN frequencies. The remaining unstained slides from the animals treated with VP-16, COL, 60 mg/kg of MER, and MMC as well as from the two vehicle-controls were stored at -20ºC for 1 day to several weeks before the FISH analysis with the mouse minor satellite DNA probe to discriminate MN of clastogenic and aneugenic origin.

Mitotic Cells

Metaphase chromosomes were used to evaluate the specificity of the minor probe and the hybridization procedure. The preparation technique for metaphases cells was based on the procedure by Adler, (1984). Briefly, 24 h before the preparation, the mice were s.c. injected with a yeast suspension in the neck squirt, in order to release an immune responses of the marrow cells which leads to high mitotic activity. The animals were sacrificed by CO_2 inhalation and both femurs were removed. The bone marrow cells were collected in HBSS, centrifuged at 1,000 rpm for 5 min, and the pellet was treated with hypotonic solution (0.56% KCl) to swell the cells. After 15 min incubation at room temperature cells were spun down again and the pellet was fixed with freshly prepared cold Carnoy's fixative (methanol/glacial acetic acid, 3 + 1). The fixative was changed after 10 min and the third fixation step lasted for at lest 1 h incubation in refrigerator. A final cell suspension dropped onto ethanol-clean dry glass slides in such a way that chromosomes from mitotic cells are spread well. Good results were obtained by dropping two separate drops onto a grease-free slide, adding two drops of fixative and blowing gently across the slide until the drops have dried. The slides were stored at -20°C until use.

4.3.3 May-Gruenwald-Giemsa Staining

For May-Gruenwald-Giemsa staining according to the standard technique (Adler, 1984) the slides were incubated for 3 min in undiluted May-Gruenwald solution, washed with water diluted May-Gruenwald solution (1:1) for 1 min, stained for 20 min in 5% Giemsa solution, rinsed with water and allowed to dry for at least 1 hour in the dark. The slides were immersed twice in xylene for 5 min each, cover-slipped in Eukitt mounting medium and stored at room temperature in the dark until scoring.

4.3.4 Centromeric DNA-Probe

The mouse minor satellite DNA probe p^{MKB6} (Wong and Rattner, 1988), a 273 bp fragment that represents approximately two tandem repeats, in plasmid p^{TZ19U}, was a gift from H. Zitzelsberger (GSF-Institute of Radiation Biology).

Cloning, Isolation, Labelling of Probe and Preparation of Hybridization mix

It was propagated in *E. coli* JM101, cloned, extracted using the Qiagen Plasmid Maxi Kits and labelled with biotin-16-dUTP as in sperm-FISH assay. The hybridization mix contained twenty parts of master mix 1.0, three parts of the biotinylated DNA probe (0.15 µg per slide), three parts of salmon sperm DNA (30 µg per slide) and four parts of distilled water. It was denatured at 78°C for 8 min and chilled on ice.

3.4.5 Hybridization Technique

The hybridization was performed as described by Schriever-Schwemmer and Adler, (1994) with slight modifications. For interphase nuclei, the frozen slides were brought to room temperature and rehydrated for 10 min in 2 x SSC in a Coplin jar at room temperature prior to treatment with 0.1% Triton X-100 in 2 x SSC for 3 min in a moist chamber. After washing the slides in 2 x SSC, the cells were fixed in 4% paraformaldehyde (PA) for 10 min at room temperature and washed again in 2 x SSC for 3 min. Then, the slides were denaturated for 10 min in 70% FA at 78°C and dehydrated in an ice-cold ethanol series of 70%, 90% and 100% for 2 min each. After air-drying, the slides were brought to 37°C for less than 3 min on a slide warming plate prior to mixing with the denaturated hybridization mix. For metaphase chromosomes, the frozen slides were brought to room temperature and rehydrated for 10 min in 2 x SSC in a Coplin jar at room temperature. The cells were fixed in

1% PA for 10 min at room temperature, then the slides were incubated for 10 min in a pre-warmed pepsin solution at 37°C. The cells were dehydrated in an ice-cold ethanol series of 70%, 90% and 100% for 2 min each and denaturated for 10 min in 70% FA at 78°C, then dehydrated again in an ice-cold ethanol series of 70%, 90% and 100% for 2 min each. The slides were brought to 37°C on a slide warming plate prior to mixing with the denaturated hybridization mix.

The next steps were identical for both cells (interphase and metaphase slide cells). Aliquots of 30 µl of denaturated hybridization mix were added to each slide, the hybridization areas were covered with cover-slips (24 x 50 mm) and sealed with rubber cement. Hybridization was carried out overnight in a moist chamber at 37°C. Post-hybridization washing consisted of two steps: 40 min in 50% FA at 45°C and two washes for 15 min in PN buffer at 37°C.

3.4.6 Detection of Fluorescent Signals

After incubation with 40 µl PNBR buffer for 10 min, the biotin-labelled probe was detected with 40 µl per slide of streptavidin-Cy3 in PNBR buffer for 30 min at room temperature in a moist chamber. Thereafter, the slides were washed twice in PN buffer for 10 min each. When necessary, the hybridization signals were amplified with biotin-conjugated anti-streptavidin followed by streptavidin-Cy3 in PNBR buffer. Between every amplification step, the slides were washed twice in PN buffer for 10 min each. The cells were counterstained with 40 µl of DAPI (for interphase cells; 0.1-0.5 µg/ml PBS) or PI (for metaphase chromosomes; 2.0 µg/ml dH_2O) per slide for 10 min at room temperature and cover-slipped in Vectashield mounting medium. The slides were scored immediately or following storage for several days at 4°C in the dark.

4.3.7 Scoring Criteria

Conventional MN test

First, the slides were screened for regions of suitable technical quality, where the cells were well spread and perfectly stained. The stained slides were analysed by light microscopy at 1250x magnification for the presence of MN inside of polychromatic young erythrocytes (PCE). Mature normochromatic erythrocytes (NCE) and PCE were distinguished by a strong bluish tint in the young, immature PCE. Debris and possible nuclear fragments of ruptured apoptotic nucleated cells were clearly distinguished from true MN by shape and colour of PCE as well as MN (Fig. 20). For each of five animals per dose group and concurrent solvent controls, 1,000 PCE were scored per slide (2,000 per animal, 10,000 PCE per group). Numbers of PCE were counted in microscopic fields, which contained 2,000 NCE and expressed as PCE/ (PCE+NCE) in percent to determine a reduction of erythroblast proliferation.

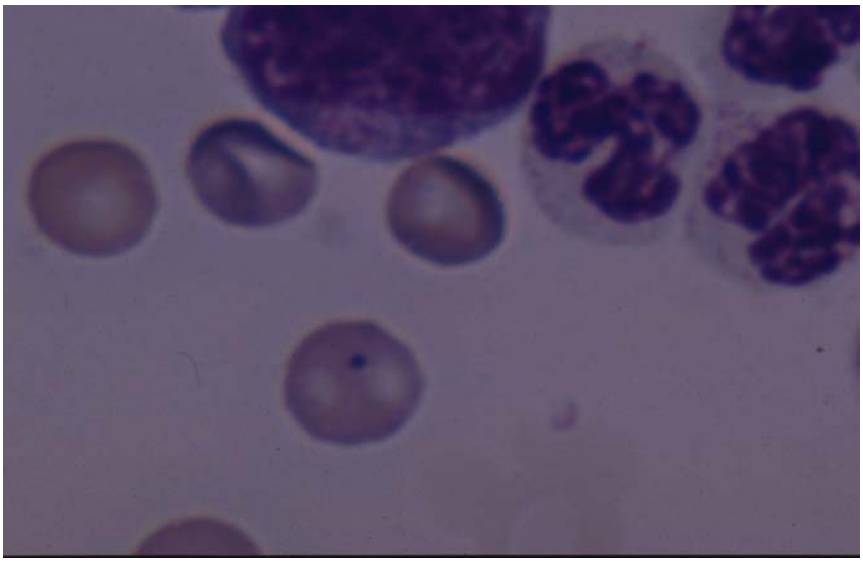


Fig. 20. Microscopic image of a bone marrow preparation after May-Gruenwald-Giemsa staining showing nucleated as well as enucleated cells. One polychromatic erythrocyte (PCE) contains a micronucleus (MN).

FISH analysis of MN

Before hybridization of MN, the specificity of the minor satellite DNA probe and the hybridization procedure were evaluated using mouse bone marrow cells at metaphase. It was confirmed that the minor satellite DNA probe hybridizes to the centromere region of all

mouse chromosomes except the Y chromosome (Fig. 21). In Figure 22 examples of signal positive (red) and signal negative MN after FISH with pancentromeric DNA probe are given.

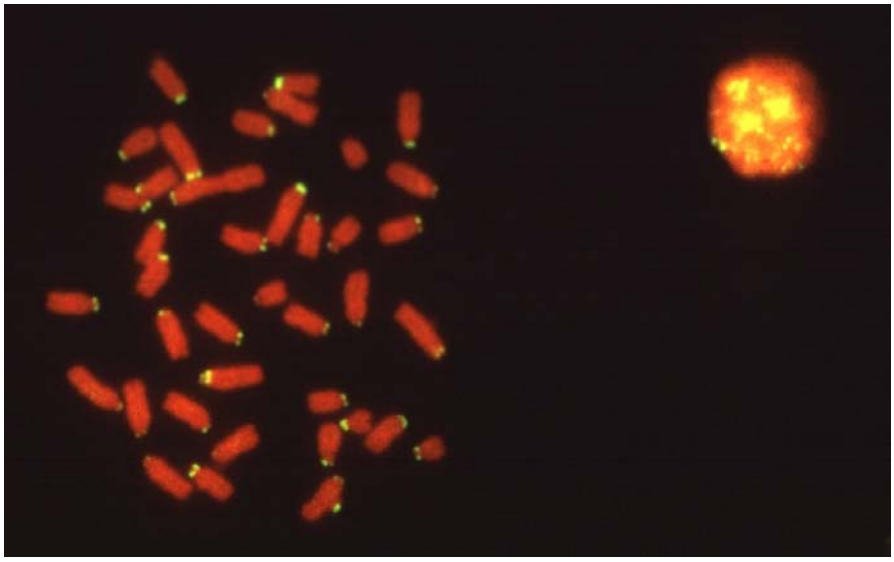


Fig. 21. Mouse bone marrow cell at metaphase after *in situ* hybridization with the mouse minor satellite DNA probe. The slide was counterstained with PI for visualization of the 40 chromosomes. Hybridization signals are seen on 39 of the 40 mouse chromosomes; the Y chromosome is not labelled. The FITC signals appear as double spots, corresponding hybridizaed centromeric domains on both sister chromatids.

First, the slides were screened for regions of suitable technical quality, where the cells were well spread and showed a sufficient centromere labelling in the nucleated cells. The slides were microscopically examined at 1250x magnification using a Zeiss Axioplan Fluorescence Photo Microscope (Zeiss, Deutschland). A dual band pass filter was used for analysis of fluorescent signals in MN; one filter had a wavelength of 365-450 nm for observation of DAPI fluorescence and a second had a wavelength of 546-590 nm for observation of Cy3-streptavidin fluorescence. Following the observation with a blue filter of an erythrocyte containing a MN, a red filter for the red Cy3-signal was used to detect the fluorescent signals of the minor satellite DNA probe and to count the number of signals. Records of all signal-containing erythrocytes (Fig. 22) were taken by digitizing the microscopic image with a CCD camera and the computer program ISIS3 (MetaSystems, Deutschland).

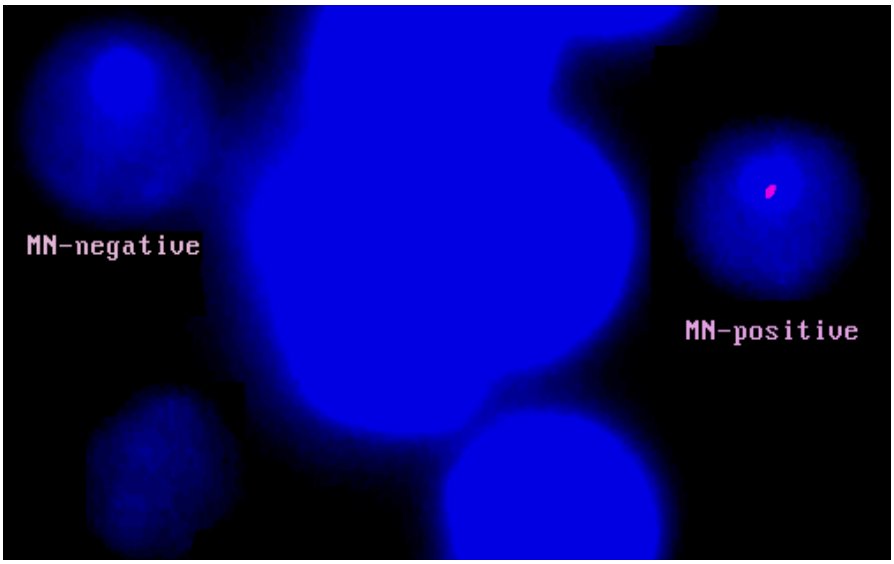


Fig. 22. Examples of mouse erythrocytes with signal positive (Cy3-red) and negative MN after *in situ* hybridization with the mouse minor satellite DNA probe. The cells were counterstained with DAPI (blue), and digitized using the computer program ISIS3 (MetaSystems, Deutschland).

4.3.8 Statistical analysis

Significant differences of mean frequencies of MNPCE between individual treatment groups and corresponding solvent controls or between the two solvent control groups were calculated on an animal to animal basis by the non-parametric Mann-Whitney *U*-test (Sachs, 1984). To compare the frequencies of signal-positive MN between the two treatment groups (VP-16 and MER), the χ^2 test was used (Sachs, 1984). Results were considered significantly different if the p value was ≤ 0.05.

C. RESULTS

A series of three related but separated studies were conducted to assess the aneugenic effects induced in mouse sperm and bone marrow following VP-16 and MER administrations. First, the possibility of a cell cycle delay caused by the test chemicals was assessed using the meiotic delay assay in order to optimise the test protocol and to avoid missing an effect by inappropriate timing of the sperm samples. Second, the sperm-FISH assay with DNA-probes specific for mouse chromosomes X, Y and 8 was used to determine the frequency of hyperhaploidy and diploidy in mouse sperm induced during meiotic divisions of spermatocytes. Lastly, the conventional bone marrow MN assay was applied and the origin of the MN was determined with FISH using the minor satellite DNA probe.

1 Studies on the Duration of Meiosis

1.1 Meiotic Delay with VP-16

The data for VP-16 are shown in Table 4 and Figure 23a. After the treatment with 50 mg/kg VP-16, the peak of BrdU-labelled epididymal sperm occurred significantly later than in the control group. On day 22, the frequencies of BrdU-labelled sperm in the control group was 41.1%, the corresponding frequencies of the VP-16 group was 14.2% ($p< 0.001$). On days 23 and 24, there were no significant difference between the frequencies of BrdU-labelled sperm from VP-16-treated and control animals. Therefore, the optimum time for sperm sampling in a sperm-FISH assay was concluded to be 23 or 24 days after VP-16 treatment.

Table 4. Results of the BrdU-incorporation assay with 50 mg/kg of VP-16 and 80 mg/kg of MER.

	BrdU-positive epididymal sperm (%) (mean ± SD)				
	Day 20[a]	Day 21	Day 22	Day 23	Day 24
Solvent Control	16.9 ± 6.8	30.3 ± 8.2	41.1 ± 7.7	31.3 ± 2.5	31.2 ± 5.6
VP-16	11.7 ± 3.4	17.4 ± 9.2	14.2 ± 5.6*	25.8 ± 13.1	23.7 ± 10.7
Solvent Control	-	27.7 ± 1.1	37.2 ± 9.6	29.8 ± 9.9	31.0 ± 4.9
MER	-	25.2 ± 2.2	32.1 ± 6.7	29.7 ± 10.8	27.9 ± 2.7

[a] Days of sperm sampling after treatment.
VP-16 = etoposide, MER = merbarone.
*$P< 0.01$, compared with the concurrent control (*t*-test).

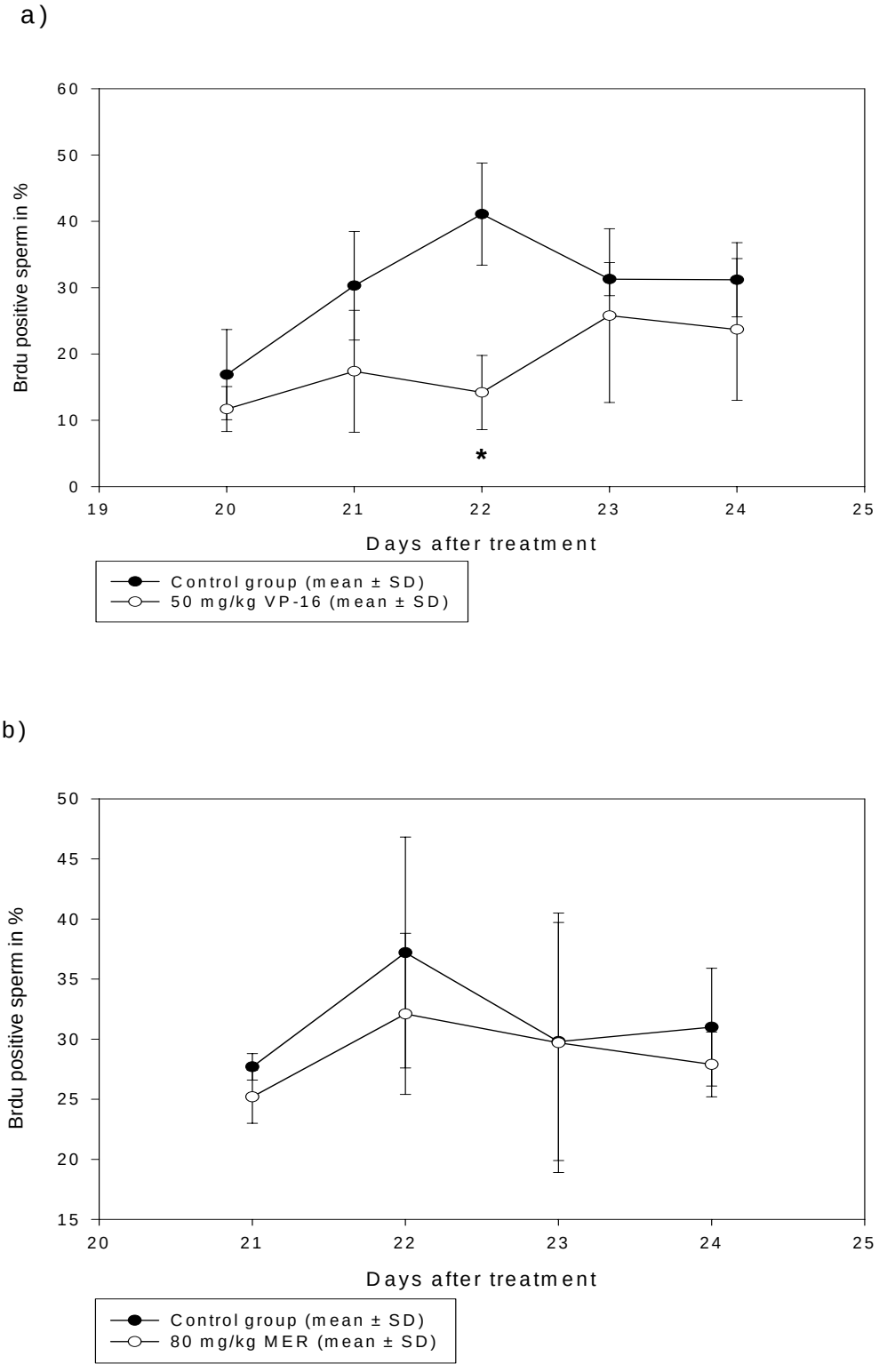


Fig. 23. Time course of appearance of BrdU-labelled sperm in the epididymes.
(a) Control versus 50 mg/kg VP-16. (b) Control versus 80 mg/kg MER.
VP-16 = etoposide, MER = merbarone,
*$P < 0.01$ compared with the concurrent solvent control (*t*-test).

1.2 No Meiotic Delay with MER

Treatment of male mice with 80 mg/kg of MER did not cause a meiotic delay (Table 4 and Fig. 23b). The frequencies of BrdU-labelled sperm in the control and MER groups were similar on all days. Thus, the 22 days sampling was chosen for the sperm FISH assay according to the original protocol.

2 Sperm-FISH Studies

The results of the FISH analysis of disomic and diploid sperm in mice after exposure to COL, VP-16 and MER are shown in Tables 5-8 together with the concurrent control data. A total of ≈500,000 epididymal sperm were evaluated from 50 animals. The ratio of X and Y carrying sperm (X8 versus Y8) was 1:1 for all mice, as expected. Furthermore, there were no significant differences in the frequencies of diploid (0.0, 0.002 and 0.004%) or disomic sperm (0.052, 0.044 and 0.040%) among the three control groups.

2.1 Experiment 1 (COL 3mg/kg, VP-16 25 and 50 mg/kg)

The results of the first experiment are shown in Table 5. Treatment of male mice with 25 and 50 mg/kg of VP-16 induced hyperhaploid (disomic) sperm. A significant increase in the frequency of disomic sperm by a factor of 2.3 was caused by treatment with 50 mg/kg VP-16, i.e. 0.122% compared with the corresponding control value of 0.052% [$p<0.01$ (χ^2 test and Mann-Whitny *U*-test)]. Treatment with 25 mg/kg VP-16 increased the disomy frequency slightly but the result was significantly different to the corresponding control using the animal-to-animal comparison with the Mann-Whitny *U*-test, i.e. 0.074% compared with the corresponding control value of 0.052% ($p<0.05$).

The frequency of disomic sperm induced by 3 mg/kg COL was significantly increased by a factor of 1.7, i.e. 0.088% compared with the corresponding control values of 0.052% ($p<0.05$). However, in contrast to the topo II inhibitors, COL did not significantly increase the frequency of diploid sperm (Table 5).

It is noteworthy that XX8 and YY8 bearing hyperhaploid sperm were significantly more frequent than XY8 bearing sperm in the controls and all treatment groups, i.e. control: 11 versus 2 ($p<0.01$), 3 mg/kg of Col: 16 versus 3 ($p<0.01$), 25 mg/kg of VP-16: 20 versus 3 ($p<0.01$), 50 mg/kg of VP-16: 27 versus 7 ($p<0.01$). Furthermore, treatment with 25 and 50

mg/kg VP-16 induced diploid sperm, i.e. 0.032% and 0.056%, respectively, compared with the corresponding control value of 0.004 and 0%, respectively ($p<0.01$). Among the diplod sperm, the chromosomal constitutions XX or YY were significantly more frequent than XY, i.e. 25 mg /kg of VP-16: 15 versus 1 ($p<0.01$), 50 mg/kg of VP-16: 24 versus 4 ($p<0.01$).

Table 5. Result of the three-colour FISH with epididymal sperm of mice treated with COL and VP-16 (Experiment 1).

	Control (6% DMSO)	COL (3 mg/kg)	VP-16 (25 mg/kg)	VP-16 (50 mg/kg)
No. of animals	5	5	5	5
Sperm scored	50087	50058	50123	50170
X/Y bearing sperm	1.0307	0.9944	1.0023	0.9745
Disomic sperm				
X-X-8	9	8	15	17
Y-Y-8	2	8	5	10
X-Y-8	2	3	3	7
X-8-8	9	10	3	14
Y-8-8	4	15	11	13
Total	26	44	37	61
% Disomies ± SD	0.052 ± 0.013	0.088* ± 0.018	0.074$^{\#}$ ± 0.013	0.122** ± 0.011
Diploid sperm				
X-Y-8-8	0	0	1	4
X-X-8-8	0	1	11	19
Y-Y-8-8	0	1	4	5
Total	0	2	16	28
% Diploidies ± SD	0.000 ± 0.000	0.004 ± 0.005	0.032** ± 0.005	0.056** ± 0.005

COL = colchicine; VP-16 = etoposide.
*$P< 0.05$, **$P< 0.01$, compared with the concurrent solvent control (χ^2 test),
$^{\#}P<0.05$, compared with the concurrent solvent control Mann-Whitney *U*-test.

2.2 Experiment 2 (MER 60 mg/kg)

The results of the second experiment are shown in Table 6. Treatment with 60 mg/kg MER significantly increased the frequency of disomic sperm by a factor of 2.2, i.e. 0.098% compared with the corresponding control values of 0.044% ($p<0.01$). Again the hyperhaploid sperm with two X or two Y chromosomes were significantly more frequent than with X and Y in the solvent control group and the MER-treated group, i.e. control: 12 versus 2 ($p<0.01$), 60 mg/kg of MER: 16 versus 2 ($p<0.01$). The frequency of diploid sperm was also significantly

increased with 60 mg/kg MER, i.e. 0.05% in the treated and 0.002% in the control group (p<0.01) and XX plus YY bearing diploid sperm were significantly more frequent than XY bearing sperm, i.e. 25 versus 1 (p<0.01).

Table 6. Result of the three-colour FISH with epididymal sperm of mice treated with 60 mg/kg of MER (Experiment 2).

	Control (15% DMSO)	MER (60 mg/kg)
No. of animals	5	5
Sperm scored	50113	50078
X/Y bearing sperm	0.9839	0.9871
Disomic sperm		
X-X-8	6	10
Y-Y-8	6	6
X-Y-8	2	2
X-8-8	2	13
Y-8-8	6	18
Total	22	49
% Disomies ± SD	0.044 ± 0.009	0.098** ± 0.013
Diploid sperm		
X-Y-8-8	0	1
X-X-8-8	1	18
Y-Y-8-8	0	7
Total	1	26
% Diploidies ± SD	0.002 ± 0.004	0.05** ± 0.008

MER = merbarone.
**P< 0.01, compared with the concurrent solvent control (χ^2 test),

2.3 Experiment 3 (VP-16 12.5 mg/kg, MER 15 and 30 mg/kg)

The results of the third experiment are shown in Table 7. Treatment of male mice with 12.5 mg/kg of VP-16 as well as with 15 and 30 mg/kg MER did not induce significant increases in disomic sperm, i.e. 0.064, 0.046 and 0.058% compared with the corresponding control values of 0.044%, respectively. However, treatment with 12.5 mg/kg of VP-16 and 30 mg/kg MER induced diploid sperm, i.e. and 0.024% and 0.022%, respectively, compared with the corresponding control value of 0.004% (p<0.01 and p<0.05, respectively.

Table 7. Result of the three-colour FISH with epididymal sperm of mice treated with lower doses of VP-16 and of MER (Experiment 3).

	Control (15% DMSO)	VP-16 (12.5 mg/kg)	MER (15 mg/kg)	MER (30 mg/kg)
No. of animals	5	5	5	5
Sperm scored	50078	50107	50066	50149
X/Y bearing sperm	0.9808	0.9876	0.9953	1.0008
Disomic sperm				
X-X-8	7	11	11	9
Y-Y-8	8	9	7	6
X-Y-8	1	1	2	5
X-8-8	3	5	1	6
Y-8-8	1	6	2	3
Total	20	32	23	29
% Disomies ± SD	0.04 ± 0.007	0.064 ± 0.011	0.046 ± 0.009	0.058 ± 0.0045
Diploid sperm				
X-Y-8-8	2	2	1	2
X-X-8-8	0	7	1	5
Y-Y-8-8	0	3	1	4
Total	2	12	3	11
% Diploidies ± SD	0.004 ± 0.005	0.024** ± 0.005	0.006 ± 0.009	0.022* ± 0.0084

VP-16 = etoposide; MER = merbarone.
*P< 0.05, **P< 0.01, compared with the concurrent control (χ^2 test).

2.4 Dose-Response Curves for VP-16 and MER

The data from the three experiments are combined in the dose-response curves for VP-16 (Fig. 24a) and for MER (Fig. 24b). In these figures, the control values were averaged from the respective experiments because they were not significantly different. The dose response curves for VP-16-induced disomic and diploid sperm can be described by the linear equations $y = 0.044+0.0015D$ ($r = 0.99$) and $y = 0.006+0.001D$ ($r = 0.98$), respectively. The dose response curves for MER-induced disomic and diploid sperm can be described by the linear equations $y = 0.037+0.00094D$ ($r = 0.96$) and $y = 0.002+0.008D$, respectively.

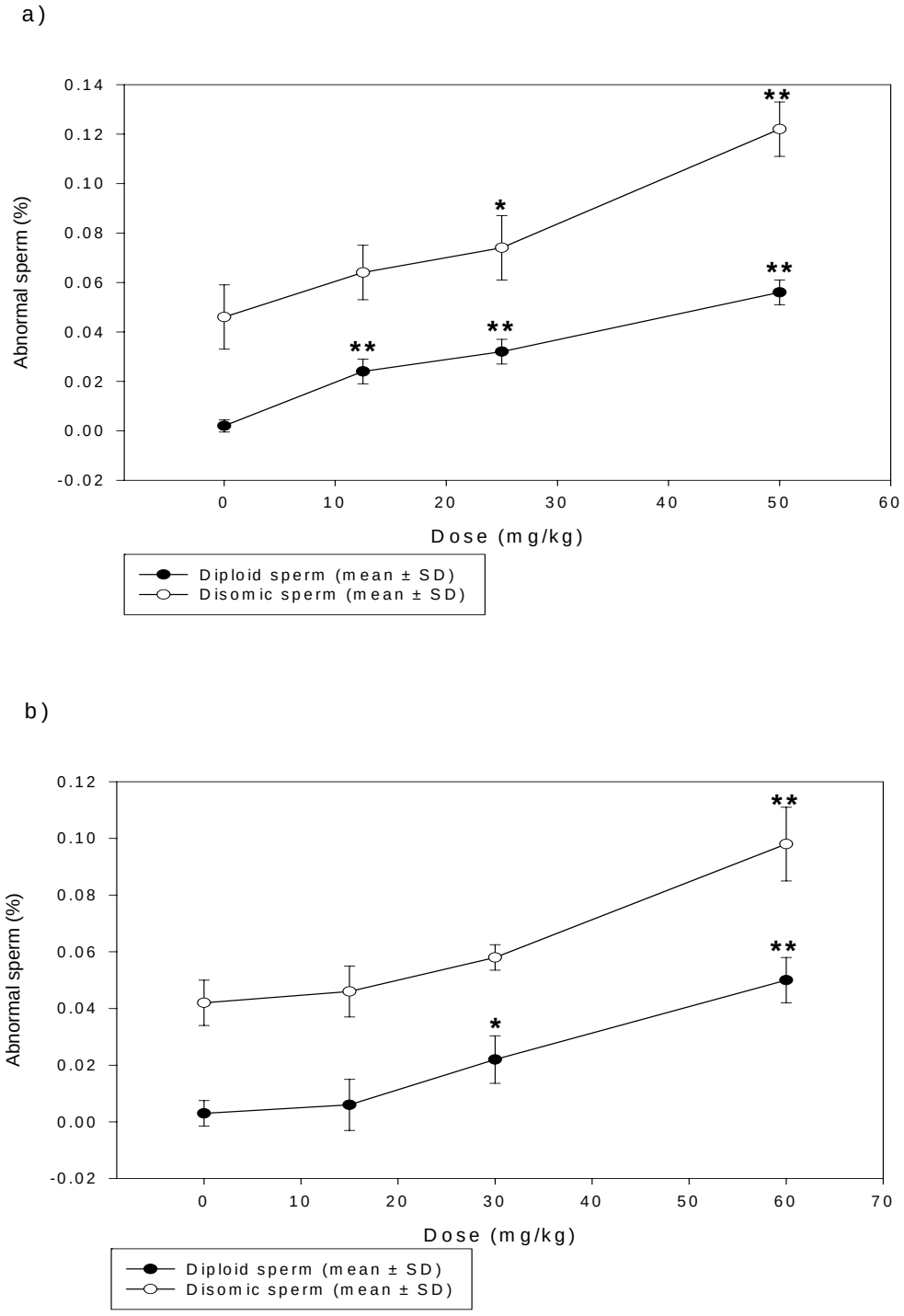


Fig. 24. Dose-response curves of the frequencies of abnormal sperm from mice after treatment with etoposide (VP-16, a) and merbarone (MER, b). The sampling time was 24 days after treatment with VP-16 and 22 days after treatment with MER.
$^{**}P < 0.01$ compared with the concurrent solvent control (χ^2-test), $^{*}P < 0.05$ compared with the concurrent solvent control (Mann-Whitney *U*-test). Control values were averaged from experiments 1 and 3 (a) or 2 and 3 (b).

2.5 Experiment 4 (13 x VP-16 1, 2, and 4 mg/kg)

The results of the fourth experiment are shown in Table 8 and Figure 25. Treatment of male mice with 1 and 2 mg/kg of VP-16 on 13 consecutive days did not induce significant increases of disomic (0.058% compared with the corresponding control values of 0.04%) or diploid sperm (0.008% compared with the corresponding control values of 0.004%). However, the highest dose group (4 mg/kg/day), which received a total of 52 mg/kg, induced significant increases in disomic (0.088% compared with the corresponding control value of 0.04%) and diploid sperm (0.03% compared with the corresponding control value of 0.004%, (p<0.05 and p<0.01, respectively).

Table 8. Result of the three-colour FISH with epididymal sperm of mice treated with VP-16 (Daily exposure for 13 consecutive days) (Experiment 4).

	Control (6% DMSO)	VP-16 (1 mg/kg/day)	VP-16 (2 mg/kg/day)	VP-16 (4 mg/kg/day)
No. of animals	5	5	5	5
Sperm scored	50099	50201	50171	50185
X/Y bearing sperm	1.0077	0.9962	0.9758	0.9794
Disomic sperm				
X-X-8	5	6	10	13
Y-Y-8	5	5	5	9
X-Y-8	2	2	2	3
X-8-8	3	6	5	13
Y-8-8	5	10	7	6
Total	20	29	29	44
% Disomies ± SD	0.04 ± 0.0122	0.058 ± 0.0083	0.058 ± 0.013	0.088* ± 0.0083
Diploid sperm				
X-Y-8-8	0	0	1	3
X-X-8-8	1	1	2	8
Y-Y-8-8	1	3	1	4
Total	2	4	4	15
% Diploidies ± SD	0.004 ± 0.005	0.008 ± 0.0044	0.008 ± 0.0083	0.03** ± 0.0122

VP-16 = etoposide.
*P< 0.05, **P< 0.01, compared with the concurrent control (χ^2 test).

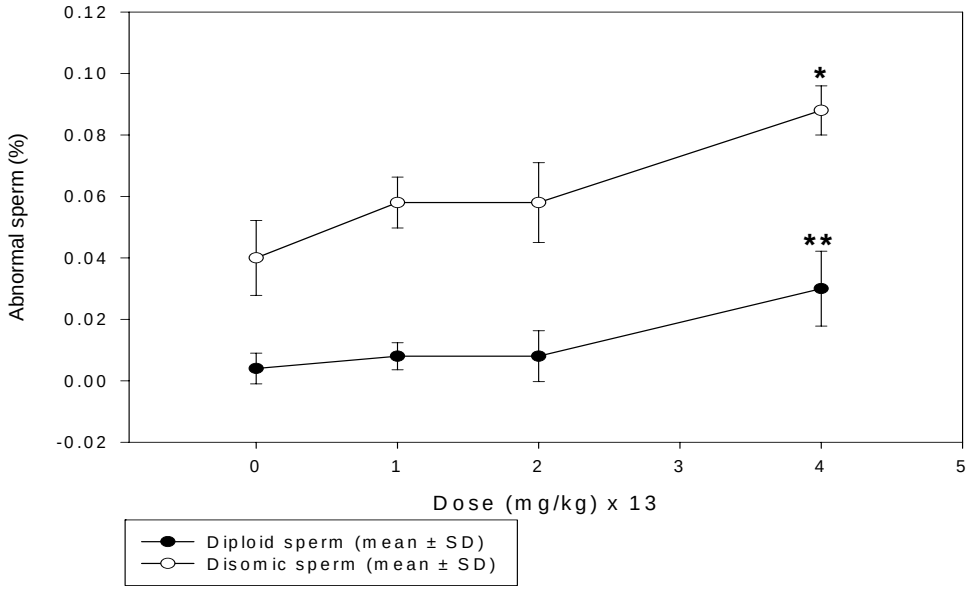


Fig. 25. Dose response curve of the frequencies of abnormal sperm from mice 24 days after the last treatment with VP-16 (etoposide) (daily exposure for 13 consecutive days).
*P< 0.05 and **P< 0.01 compared with the concurrent solvent control (χ^2-test).

3 Bone marrow Micronucleus Studies

3.1 Conventional Micronucleus Test

The results of the conventional MN test are presented in Table 9. The frequencies of micronucleated polychromatic erythrocytes (MNPCE) in control I of 0.37% and in control II of 0.31% were not significantly different. In the first experiment, the positive control aneugen COL showed the expected significant increase in MNPCE. At a dose of 3 mg/kg, the frequency of MNPCE was 1.14% compared to 0.37% in the corresponding solvent control (p<0.01). Similarly, VP-16 at a dose of 1 mg/kg significantly increased the frequency of MNPCE from 0.37% in the control to 3.44% (p<0.01). VP-16 also significantly decreased the percent PCE from 48.12% in the control to 45.10% (p<0.01) indicating a reduction in erythroblast proliferation most likely by mitotic arrest.

In the second experiment, the positive control clastogen MMC showed the expected significant increase in the frequency of MNPCE (Table 9). At a dose of 1mg/kg of MMC the frequency of MNPCE was 2.02% compared to 0.31% in the corresponding negative control group (p<0.01). As seen in Table 9 and Figure 26, MER (7.5-60 mg/kg) increased the

MNPCE frequencies in a dose-dependent manner. The dose response can be best described by the linear-quadratic equation $Y = 0.33-0.015D+1.35x10^{-3}D^2$. The lowest positive dose was 15 mg/kg and significantly increased the frequency of MNPCE from 0.31 to 0.50% (p<0.05, Mann-Whithey *U*-test). Furthermore, MER caused a significant dose-dependent decrease of the PCE/NCE+PCE ratios which can be described best by the linear equation of Y = 48.37-0.057D. This indicates a suppression of bone marrow proliferation at all doses tested.

Table 9. Frequencies of MNPCE and PCE in bone marrow of mice 24 h after treatment with the indicated doses of VP-16, MER, COL and MMC.

Chemical	Dose (mg/kg)	Individual animal data (MNPCE/ 2000 PCE)	% MNPCE (mean ± SD)	% PCE[a] (± SD)
First experiment				
Control I	0	9, 8, 7, 8, 5	0.37 ± 0.10	48.12 ± 1.18
COL	3	18, 23, 25, 30, 18	1.14 ± 0.20 **	47.06 ± 1.48
VP-16	1	75, 62, 62, 66, 79	3.44 ± 0.42 **	45.10 ± 1.03 **
Second experiment				
Control II	0	5, 6, 5, 9, 6	0.31 ± 0.09	49.27 ± 0.79
MMC	1	43, 33, 44, 42, 40	2.02 ± 0.27 **	47.24 ± 1.03 *
MER	7.5	6, 7, 4, 5, 5	0.27 ± 0.06	47.29 ± 0.90 *
MER	15	6, 16, 8, 8, 12	0.50 ± 0.20 *	47.13 ± 0.86 *
MER	30	21, 17, 26, 24, 13	1.01 ± 0.28 **	46.60 ± 0.90*
MER	60	97, 86, 87, 71	4.26 ± 0.72 **	45.19 ± 0.94 **

PCE = polychromatic erythrocytes, MNPCE = micronucleated PCE (conventional staining), VP-16 = etoposide, MER = merbarone, COL = colchicine, MMC = mitomycin C, Control I (1% DMSO in dH_2O), Control II (15% DMSO in dH_2O).

[a]Numbers of PCE were counted in microscopic fields which contained 2000 normochromatic erythrocytes (NCE), percent PCE were calculated as % PCE = [PCE/(PCE+NCE)] x 100.

*P< 0.05 and **P< 0.01, compared with the concurrent solvent control (Mann-Whitney *U*-test).

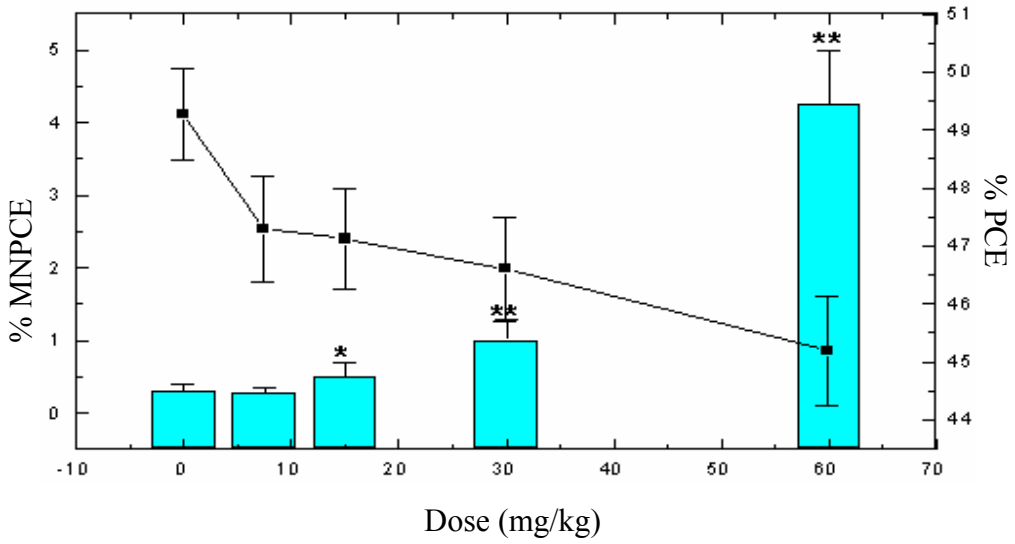


Fig. 26. Illustration of the dose-response obtained in the conventional micronucleus test with MER (Table 9, second experiment).
MER = merbarone, PCE = polychromatic erythrocytes, MNPCE = micronucleated PCE, shown as blue bras (mean percent ± SD). Reduction of %PCE [(PCE/NCE+PCE) x 100] is shown as solid line (mean percent ± SD).
**P*< 0.05; ***P*< 0.01, compared with the solvent control (Mann-Whitney *U*-test),

3.2 FISH Analysis of Induced Micronuclei (MN)

The results of the FISH analysis of MN are shown in Table 10 and Figure 27. In the first solvent-control group, a total of 52 MN were analysed and 23 (44.3%) of them were signal-positive. One signal per MN was observed in 34.8%, two signals were seen in 43.5% and ≥3 signals were seen in 21.7% of the 23 MN. In the second solvent-control group, 44 MN (43.1%) out of 102 MN scored were signal-positive. Of these, 24 MN (54.6%) had one signal, 17 MN (38.6%) contained two signals, and 3 MN (6.8%) had ≥3 signals. Thus, the two solvent controls were very similar despite the different DMSO concentrations used.
After treatment of male mice with the positive control aneugen COL, 69 (74.1%) of the 93 MN scored were signal-positive as expected, and the distribution of signals per MN was similar to control I.

After treatment with the positive control clastogen MMC, only 21 MN (23.8%) of 88 MN analysed were signal positive confirming the predominantly clastogenic effects of MMC. Of the signal-positive MN, 12 MN (57.1%) had one signal, 6 MN (28.6%) contained two signals, and 3 MN (14.3%) had ≥3 signals. In the VP-16 group, a total of 208 MN were

analysed by FISH and 83 MN (39.9%) were signal-positive. Out of these 83 MN, 49 (59.0%) had one signal, 26 (31.3%) contained two signals, and 8 (9.6%) had ≥3 signals. A total of 199 MN from the MER group were analysed by FISH and 92 MN (46.2%) were signal-positive. Of these signal-positive MN, 41 MN (44.6%) had one signal, 41 MN (44.6%) contained two signals, while 10 MN (10.8%) had ≥3 signals (Table 10).

Table 10. Results of FISH analysis with the mouse minor satellite DNA probe in MN induced with COL, VP-16, MER, and MMC.

Chemical	Dose (mg/kg)	No. of MN scored	No. of signal-positive MNPCE		Distribution of FISH-signals per MN		
			Total	%	1 signal	2 signals	≥ 3 signals
Control I	0	52	23	44.3	8 (34.8%)	10 (43.5%)	5 (21.7%)
COL	3	93	69	74.2	24 (34.8%)	33 (47.8%)	12 (17.4%)
VP-16	1	208	83	39.9	49 (59.0%)	26 (31.3%)	8 (9.60%)
Control II	0	102	44	43.1	24 (54.6%)	17 (38.6%)	3 (6.80%)
MER	60	199	92	46.2	41 (44.6%)	41 (44.6%)	10 (10.8%)
MMC	1	88	21	23.8	12 (57.1%)	6 (28.6%)	3 (14.3%)

VP-16 = etoposide, MER = merbarone , COL = colchicine, MMC = mitomycin C, MNPCE = micronucleated polychromatic erythrocytes, Control I (1% DMSO in dH_2O) and control II (15% DMSO in dH_2O).

3.3 Combination of Conventional and FISH Data

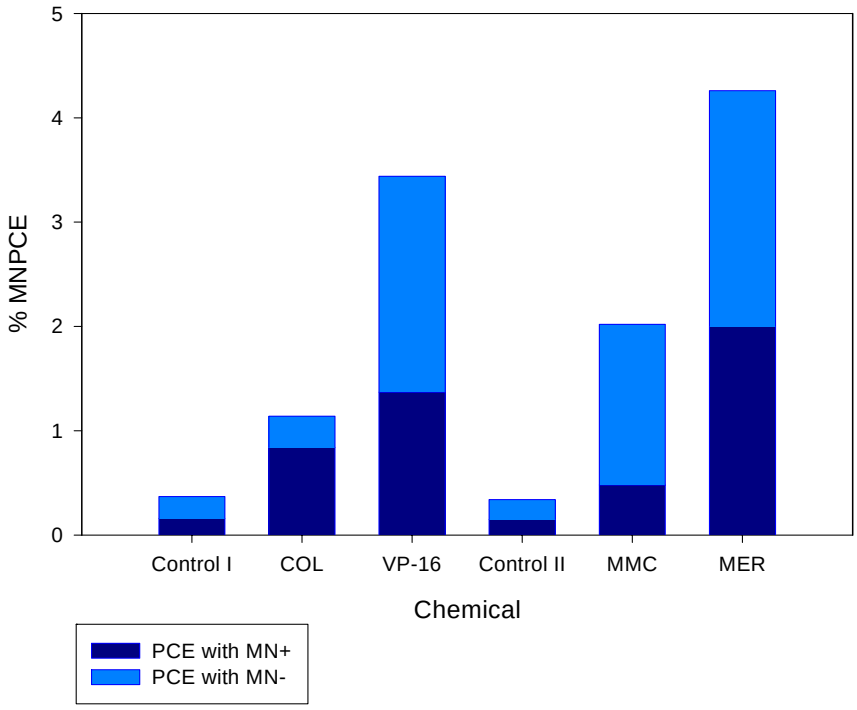
To correlate the FISH data with the conventional MN data, the expected percent of PCE with minor-positive and -negative MN were calculated (Table 11). For example, after treatment with COL, 1.14% MNPCE were found in the conventional MN test and 74.1% MN were signal-positive in the FISH analysis, thus 0.84 % of the 1.14% MNPCE were calculated to be signal-positive and, correspondingly, 0.3% MN were calculated to be signal-negative. The data for the model aneugen showed that the majority of MN were due to chromosome loss. In the same way, the expected frequencies of signal-positive and -negative MN were calculated for MMC to be 0.48% and 1.54%, respectively, which indicates that the MN were predominantly due to acentric fragments, as expected. For VP-16, the calculated frequencies of signal-positive and -negative MN were 1.37% and 2.07%, respectively. For MER, the valus

Table 11. Summery of the micronucleus test with centromere labelling by FISH with the mouse minor satellite DNA probe.

Chemical	Dose (mg/kg)	Total MNPCE (%)	PCE with MN^+ (%)	PCE with MN^- (%)
Control I	0	0.37	0.16	0.21
COL	3	1.14 ***	0.84 ***	0.3
VP16	1	3.44 ***	1.37 ***	2.07 ***
Control II	0	0.34	0.15	0.19
MMC	1	2.02 ***	0.48 **	1.54 ***
MER	60	4.26 ***	2.00 ***	2.26 ***

Control I (1% DMSO in dH_2O) and control II (15% DMSO in dH_2O).
MNPCE = micronucleated PCE (conventional staining), MN^+ = FISH-signal-positive MN, MN^- = FISH-signal negative MN.
$^{**}P< 0.01$, $^{***}P< 0.001$, compared with the solvent control (χ^2 test).

Fig. 27. Illustration of the contribution of clastogenicity and aneugenicity to the induced MN frequencies (Table 11).



VP-16 = etoposide, MER = merbarone , COL = colchicine, MMC = mitomycin C, Control I (1% DMSO in dH_2O) and control II (15% DMSO in dH_2O).
PCE = polychromatic erythrocyte, MNPCE = micronucleated PCE (conventional staining), MN^+ and MN^- = signal-positive and -negative MNPCE, respectively (FISH).

were 2.0% and 2.26%, respectively. Thus, the topo II inhibitor-induced MN were formed from acentric fragments and lagging chromosomes by similar proportions.

The data of Table 11 are illustrated in Figure 27. It demonstrates that COL induced predominantly signal-positive MN and MMC induced predominantly signal-negative MN, as expected. On the other hand, both topo II inhibitors significantly increased the frequencies of signal-positive as well as signal-negative MN. Thus, despite their different mode of action, both topo II inhibitors displayed clastogenic and aneugenic potential.

D. DISCUSSION

Most of the antitumour agents currently used in the treatment of human malignancies are targeted at topo II. The function of this enzyme is that of relaxing a supercoiled DNA by allowing double-stranded DNA chains to pass one through another following a cleavage generating blunted DNA ends. Notwithstanding DNA breaks, genome integrity is maintained because topo II attaches to the newly generated DNA termini forming transient enzyme-DNA complexes (Fortune and Osheroff, 2000). The topo II inhibitors referred to as "poisons" (e.g. VP-16, genistein and amsacrine) convert this crucial enzyme into a potent cellular toxin that eventually drives cells to death (Wang et al., 1997). In fact, they do not inhibit the topo II cleavage activity, rather they increase the physiologic concentration and/or lifetime of the cleavage complexes, thus converting transient DNA breaks into permanent fractures (Anderson and Berger, 1994). A consequence of this peculiar mode of action is that the higher the enzyme levels, the more lethal are the effects of topo II poisons (Madden and Champoux, 1992). This explains why topo II poisons behaviour particularly efficacious against aggressive cancers, which possess high enzyme levels, whereas they are ineffective against, slow growing cancers, which possess low topo II levels (Wang et al., 1997).

Compounds interfering with the catalytic activity of the enzyme (MER and bisdioxopiperazines) are expected to be active against both fast and slow growing cancers (Fattman et al., 1996). Among them, MER is particularly interesting for the following reasons: (i) it acts primarily by blocking topo II-mediated DNA cleavage without intercalating into DNA or binding to the minor groove (Fortune and Osheroff, 1998); (ii) it inhibits in vitro topo II decatenation and relaxation activities (Drake et al., 1989) and causes G_2/M blockade (Chen and Beck, 1995); (iii) it induces apoptosis (Khelifa and Beck, 1999) by a mechanism distinct from that reported for the topo II poisons VP-16 and doxorubicin.

Due to their importance, evidence is accumulating that topo II inhibitors are genotoxic (Boos and Stopper, 2000), and that they can induce secondary tumours (Felix, 2001) and impair fertility (Anderson and Berger, 1994). In the current study, we used a mouse model to asses the ability of the two mechanistically different topo-II inhibitors, VP-16 and MER to induce aneuploidy in mouse sperm and bone marrow cells. Three types of experimental studies were applied: the BrdU-incorporation assay to test if the chemical treatment altered the duration of the meiotic divisions, the sperm-FISH assay for aneuploidy induction during male meiosis and the bone marrow MN test complemented by FISH with a pancentromeric DNA probe to determine the clastogenic or aneugenic origin of MN.

1 Effects of Topo II Inhibitors on Cell Cycle Progression

It has been often discussed that chemicals with aneugenic properties can alter the progression of cell division in both mitotic and meiotic cells (Adler, 1993). Such agents can induce cell-cycle delay in somatic cells (Chen et al., 1981). Miller and Adler, (1992) discussed a possible correlation between induction of aneuploidy and meiotic delay in mouse spermatocytes. The relationship between cytotoxicity of topo II inhibitors and their effects on cell cycle has been described. If the drug-induced DNA-topo II-complexes interfere with the functions of topo II during DNA synthesis, a G_2 arrest might be caused that allows damaged DNA to be repaired before cells move to the next cell cycle stage (Murray, 1992; Stewart et al., 1995).

1.1 The Topo II Poison Etoposide (VP-16)

VP-16 and VM-26 are known to prevent cells from entering mitosis and to inhibit cdc2 kinase activity in cultured mammalian cells (Lock, 1992; Roberge et al., 1990). These observations are in line with earlier reports on synchronised cell populations that VP-16 and VM-26 block cells in the late S or G_2 phases of the cell cycle and prolong the cell cycle time (Erba et al., 1992; Misra and Roberts, 1975). Treatment of HL-60 cells with VP-16 in subcytotoxic concentrations resulted in a massive accumulation of the cells in the G_2/M phase of the cell cycle (Humeniuk et al., 2003). In vivo, VP-16 was shown to significantly prolong the cell cycle time in Swiss albino mouse bone-marrow cells (Agarwal et al., 1994). In mouse spermatocytes, VP-16 caused arrest of cells during the first meiotic division evidenced by anaphase/telophase stages in tubular sections where normally only post-meiotic spermatids are found (Kallio and Lähdetie, 1996). Exposure of living cells to MER leads to formation of DNA single-strand breaks, S phase retardation and G_2 arrest (Chen and Beck, 1995). Exposure of sensitive CEM human leukemia cells to ICRF-187 is associated with G_2/M arrest. In contrast, ICRF-187-resistant CEM cells exhibit either transient or complete lack of the catenation checkpoint. This is not due to a general lack of the G_2 checkpoint, since both parental and ICRF-187-resistant cells are arrested in G_2/M by VM-26 and MER (Morgan et al., 2000). Furthermore, it has been reported that cell cycle arrest in G_2/M for ICRF-154-treated cells was followed by cell death (Kizaki and Onishi, 1997).

Inhibition of topo II function at S phase by topo II inhibitors, such as amsacrine (Haldane et al., 1993), VP-16 (Smith et al., 1994), VM-26 and MER (Chen and Beck, 1995)

slows down cell cycle progression through the S phase and causes cells to arrest at the G_2 phase, which delays entry into mitosis. This is probably a consequence of activation of a G_2 phase-checkpoint machinery that halts the cell cycle and allows damaged DNA to be repaired before cells move to M stage (Murray, 1992; Stewart et al., 1995). In addition, various topo II inhibitors have been reported to produce severe defects in chromosome condensation when introduced before entry into the M phase (Gorbsky, 1994; Roberge al., 1990). When administered at M phase, topo II-directed drugs prolong the metaphase, interfere with chromosome segregation in PtK2 and HeLa cells (Downes et al., 1991) and induce a metaphase block and polyploidy in Chinese hamster ovary cells (Sumner, 1995).

Cell cycle arrest in G_2 can be due to different mechanisms, including DNA damage (e.g., following irradiation or exposure to VP-16), incomplete DNA replication (exposure to hydroxyurea), or incomplete chromatid decatenation (exposure to MER, aclarubicin, and bisdioxopiperazines). The catenation checkpoint was first described for ICRF-193 (Downes et al., 1994) and, subsequently, was shown to be induced also by other catalytic topo II inhibitors, including MER and aclarubicin (Anderson and Roberge, 1996). The catenation checkpoint differs from the DNA damage checkpoint, as it is independent of ATM and does not involve phosphorylation of hChk1 or hChk2 kinases. Furthermore, the G_2 arrest seems to be due to nuclear export of the cdc2/cyclin B complex, whereas the DNA damage checkpoint principally is mediated by preventing the cdc25C phosphatase to activate of cdc2/cyclin B. However, both functional ATR (ATM and rad3-related) and BRCA1 (a tumour suppressor gene mutated in 50% of familial breast and ovarian cancers) are needed to regulate the catenation checkpoint as they are for the DNA damage and the replication checkpoints (Deming et al., 2001).

A number of different compounds are able to overcome the catenation checkpoint, including caffeine, fostriecin, and okadaic acid (Anderson and Roberge, 1996; Downes et al., 1994). In addition, the catenation checkpoint can be overcome by induction of the RCC1 (regulator of chromosome condensation) gene product, which functions as a guanine nucleotide exchange factor for the nuclear G-protein Ran, which regulates transport of proteins across the nuclear envelope (Anderson and Roberge, 1996). Clifford and colleagues investigated the role of p53 in the G_2 arrest that occurs in response to the topo II inhibitors VP-16 and MER. In HT1080 cells expressing a dominant-negative form of p53, treatment with VP-16 still caused G_2 arrest, but the arrest could be overcome by additional treatment with caffeine, which inhibits the damage-responsive kinases ATM and ATR. However, caffeine could not overcome VP-16-induced G_2 arrest in HT1080 cells with functional p53

(Clifford et al., 2003). These authors concluded that VP-16 activates two pathways, one of which depends on p53 and the other of which is sensitive to caffeine, and that either pathway is sufficient to activate G_2 arrest. Inhibition of topo II with MER, which does not stabilize a cleavage complex, causes G_2 arrest by a checkpoint that monitors the decatenation of chromatin. Clifford and colleagues found that caffeine can abrogate MER-induced G_2 arrest even in cells with functional p53, indicating that p53 does not contribute to the decatenation-sensitive response. Thus, p53 has a differential role in effecting G_2 arrest in response to topo II inhibitors, depending upon the mechanisms of action of the inhibitors tested.

VP-16 stabilizes the cleavage complex of topo II and DNA, presumably creating more DNA damage. Consistent with this comparison, Clifford et al. (2003), observed ≈2-fold higher levels of histone H2AX phosphorylation in cells treated with VP-16 than with MER. However, VP-16 was ≈50-fold more toxic than MER. One possible explanation for the much larger difference in toxicity than phosphorylation of H2AX is that the damage caused by MER is easier to repair than that caused by VP-16. Both types of damage may induce the phosphorylation of histone H2AX, but only VP-16-induced damage might be highly toxic. Thus, different foci that are positive for p139H2AX may differ drastically in their ability to be repaired and their ultimate contribution to cell death. Previous work with catalytic inhibitors of topo II have suggested that because these agents do not directly create DNA damage, some other signal such as catenated chromatin, must be responsible for G_2 arrest. In these studies, MER was not used, however, results of Clifford and colleagues suggest that MER can also trigger the decatenation checkpoint because it inhibits topo II without directly causing DNA damage, and it causes G_2 arrest, which can be abrogated with caffeine. These studies with MER also suggest that p53 does not contribute to the decatenation checkpoint under the conditions studied.

In the sperm-FISH assay, a crucial prerequisite for the timing of sperm sampling is a precise knowledge of both the duration of the meiotic stages and the possible temporal changes of meiotic divisions induced by aneugens. Schmid et al. (2001a), concluded that the effect of a test chemical on meiotic division progression should be determined before designing the sampling time of sperm in aneuploidy studies with the sperm-FISH assay in order to optimize the test protocol and to avoid missing an effect by inappropriate timing of the sperm samples. We performed the BrdU-incorporation assay to determine the time course for the appearance of BrdU-labelled sperm. In cytogenetics, manual scoring for cell analyses is mainly utilized, which tends to be both time-consuming and labour intensive. A scorer has to evaluate a minimum of 10,000 sperm per animal manually under a fluorescence

microscope, using strict scoring criteria. Depending on the skill and experience of the scorer, it will take 30-60 min to evaluate 1,000 sperm. Therefore, automation of the detection of fluorescence signals in large numbers of cells would be a desirable improvement. The LSC analysis applied by Schmid et al. (2001a) has many advantages over manual scoring, including high-speed analysis and improved throughput. As this is usually coupled with the analysis of larger cell numbers LSC analysis has the potential for improved sensitivity and consistency.

The present results show that the treatment with topo II poison VP-16 (50 mg/kg) prolonged the duration of the meiotic divisions in mouse spermatocytes for at least 24 hours (Table 4 and Fig. 23a). These observations therefore confirm previous results that inhibition of topo II function during different phases of the cell cycle by topo II inhibitors slows down cell cycle progression and cause cell to arrest at the G_2 phase. We also performed the BrdU-incorporation assay with topo II poisons amsacrine and genistein in our laboratory. Genistein gave negative results at the dose used while amsacrine delayed the appearance of BrdU-labelled sperm by 24 h (unpublished results). Probably, the differences in the p21(WAF1/Cip1)-expression induction between VP-16, and genistein, as discussed by Ding et al. (2003), were reflected in their different effects on cell-cycle progression.

Ding et al. (2003) showed that topo II inhibitors, genistein and VP-16, induce p21(WAF1/Cip1) expression mainly in a p53-dependent manner in the human lung cancer cell line A549. However, although p53 accumulated, p21(WAF1/Cip1) expression did not depend on the level of Ser15 phosphorylation of p53. Caffeine, an ATM and ATR inhibitor, abrogated genistein-induced p21(WAF1/Cip1) and largely blocked VP-16-induced p21(WAF1/Cip1) expression. Wortmannin, an ATM- and DNA-dependent protein kinase inhibitor, partially inhibited p21(WAF1/Cip1) expression induced by genistein and VP-16, whereas the cell cycle abrogator UCN-01, a Chk1 inhibitor, partially blocked VP-16, but not genistein-induced p21(WAF1/Cip1) expression. These data suggest that both genistein and VP-16 induce p21(WAF1/Cip1) expression in a p53-dependent manner.

Genistein appears to stimulate p21(WAF1/Cip1) expression through p53 via ATM, whereas VP-16 may activate both ATM and ATR pathways. These results suggest that different mechanisms participate in genistein and VP-16 induced p21(WAF1/Cip1) expression. On the other hand, Balabhadrapathruni et al. (2000) found that the cell cycle progression of human breast cancer cells is arrested at G_2/M by genistein. These authors suggested that the cell cycle arrest by genistein is possibly due to perturbation of the synthesis or degradation of cyclin B1 protein and $p34^{cdc2}$ kinase, that promote microtubule spindle

formation. A microtubule inhibitor, colcemid, induces lower amounts of cyclin B1 protein and G_2 arrest (Sherwood and Schimke, 1995). Thus, genistein may indirectly interfere with mitotic spindle formation, and lead to incorrect segregation of chromosomes. The basis of the discrepancy is not clear but may be the result of differences in testing systems, treatment conditions, or technique sensitivity.

1.2 The Catalytic Topo II Inhibitor Merbarone (MER)

Information on the effects of MER on cell cycle progression is limited, but certain other inhibitors of topo II, which like MER, do not induce DNA strand breaks, have been shown to prevent anaphase segregation of chromosomes (Gorbsky, 1994), and induce the formation of tetraploid nuclei (Ishida et al., 1991). Chen and Beck, (1995), have found that MER like VM-26 can inhibit chromosome separation and cell division without preventing cells from exiting mitosis. VP-16 but not MER greatly reduce the rate at which the chromosomes pass from metaphase through anaphase to the subsequent interphase, with a much greater proportion of cells remaining in metaphase than in the control CHO cell cultures (Sumner, 1995). The author suggested that this failure might well be due to an inability of the MER to penetrate the cells adequately. It is, however, worth noting that MER inhibits topo II in a different way than VP-16. MER inhibits topo II without binding to DNA, inducing strand breaks, or stabilizing the cleavable complex (Drake et al., 1989; Fortune and Osheroff, 1998). Moreover, the different role of p53 in effecting G_2 arrest in response to MER (Clifford et al., 2003), and these differences may pertain to the effect on cell cycle progression.

In the spermatid MN studies of Kallio and Lähdetie, (1997) with MER, significant increases of arrested metaphase and anaphase cells were observed. However, in the present BrdU-experiments, MER 80 mg/kg did not cause meiotic delay. After MER-treatment, the frequencies of BrdU-labelled sperm in the control and MER groups were similar on all days (Table 4 and Fig. 23b). Thus, the differences in the mechanisms of action between VP-16, amsacrine, MER and genistein as discussed here and in the Introduction were reflected in their different effects on cell cycle progression in spermatocytes of mice, i.e. only VP-16 and amsacrine caused a meiotic delay. A possible explanation for the differences in the cell cycle effects is that the DNA damage caused by MER and genistein is easier to repair than that caused by VP-16 and amsacrine.

2 Germ-Cell Aneuploidy

Studies in humans have shown that certain chemotherapy regimens increase the frequencies of aneuploid sperm (Martin et al., 1999), suggesting that such patients may be at higher risk for abnormal reproductive outcomes. Therefore, it is of general concern to prevent increases in aneuploidy due to exposure of humans with agents that may cause alterations in the fidelity of chromosome segregation. Levels of topo IIα peak in late S and G_2/M several-fold (normally over 10 times) over the amount detected in G_1 cells (Fig. 8). These high levels are consistent with the idea that topo IIα is required mainly during the final stages of DNA replication to facilitate chromosome untangling, condensation and mitotic segregation (reviewed by Cortés et al., 2003). A variety of drugs that interfere with topo II have been reported to induce polyploidy to different degrees (Kallio and Lähdetie, 1997; Sumner, 1995), providing indirect evidence that the enzyme is required for separation of sister chromatids.

We applied the multicolour sperm-FISH analysis in the mouse model to detect the frequencies of hyperhaploidy for chromosomes 8, X, and Y, and diploidy. The frequencies of disomic and diploid sperm in the three vehicle control groups compare well with the published data by Schmid et al. (1999). The known spindle poison COL, which inhibits polymerisation of tubulins (Liang and Brinkley, 1985) was used in the present study as the positive control aneugen with a 24-day sampling interval. Whereas the induced frequencies of disomic sperm were significantly higher after COL-treatment than the corresponding control, there was no similar tendency for the induction of diploid sperm indicating that no complete meiotic arrest occurred. These results are in accord with previous data published by Schmid et al. (1999).

2.1 The Topo II Poison Etoposide (VP-16)

In the present experiments, significant increases above the concurrent controls in the frequencies of hyperhaploid sperm were found after treatment with 25 and 50 mg/kg of VP-16. The induction of disomies showed a linear dose-response between 0 and 50 mg/kg of VP-16. Furthermore, VP-16 caused a dose-dependent significant increase in the frequencies of diploid sperm and the increase was statistically significant at all three doses tested (Fig. 24a).

Previous studies reported that VP-16 might prevent the segregation of the homologous chromosomes because topo II is required for the resolution of recombined chromosomes and the proper segregation of sister chromatids (Rose et al., 1990). VP-16 treatment of germ cells of male mice induced fragmentation of centromeric DNA in chromosomes of first meiotic divisions as well as MN with and without centromeric (CREST) signals (Kallio and Lähdetie, 1996). Lahtedie et al. (1994), found that the clastogenic effect of VP-16 in mice was greatest during diplotene-diakinesis of primary spermatocytes, reduced during late pachytene and low during preleptotene stages; a very different pattern from that seen with DNA alkylating chemicals. These authors suggested that VP-16 caused a failure of resolution of recombined chromosome arms, probably associated with cell cycle arrest and triggering of the apoptotic pathway. Furthermore, VP-16 reduced meiotic recombination in male mice (Russell et al., 2000).

VP-16 was different from model aneugens such as COL and vinblastine, for which male germ cells seem to be less sensitive than female germ cells (Mailhes, 1995). Marchetti et al. (2001) reported that the frequency of hyperhaploidy found in MMII spermatocytes was similar to that found in MMII oocytes with VP-16 treatment (Mailhes, et al., 1994).

Marchetti et al. (2001), analyzed first and second meiotic metaphases (MMI and MMII) as well as first cleavage divisions at several intervals after treatment of male mice with 80 mg/kg of VP-16 using FISH with painting probes for three different autosomes plus X and Y. They confirmed the observations by Kallio and Lähdetie that VP-16 treatment of meiotic prophase stages caused structural chromosome aberrations and showed that these could be transmitted to the zygotes. Marchetti et al. (2001) also stated that VP-16-treatment of pachytene to diakinesis caused the highest frequencies of hyperhaploid MMII cells ever found for an aneugen in male germ cells. However, only one third of the corresponding first cleavage zygotes displayed hyperploidies. This indicates that aneuploid sperm do not survive the second meiotic division or are at a disadvantage during fertilization.

Counting of MMII chromosomes is limited to the detection of chromosome malsegregations that occurs during the first meiotic division. The sperm-FISH assay is capable of detecting non-disjunction during both meiotic divisions of male meiosis. By taking into account the distribution of sex chromosomes among the hyperhaplod sperm, errors in the first and second meiotic division can be distinguished from each other. Sperm with two different sex chromosomes (XY) originate in first meiotic metaphase (MMI) whereas sperm with two identical sex chromosomes (XX or YY) represent errors during MMII. In our experiments, MMII was consistently more sensitive than MMI. In comparison to the data of Marchetti et al. (2001), our observations support the conclusion that hyperhaploid sperm produced during MMI have a lower chance to survive MMII, i.e. meiotic selection exists against aneuploidy spermatocytes. The reduced aneuploidy rate in first cleavage zygotes reported by Marchetti et al. (2001) suggests that, additionally, aneuploid sperm may be at a disadvantage to participate in fertilization.

Since our data and those of Kallio and Lähdetie, (1996) could demonstrate that VP-16 prolonged the meiotic cell cycle, it seems likely that the effects seen in the first cleavage divisions by Marchetti et al. (2001) in the 24.5-day mating group may have actually been induced at a later stage, i.e. during meiotic diakinesis, metaphase I or II instead of pachytene as the authors suggested. Therefore, we planned sperm-FISH study to determine if subacute treatment with low doses would have an effect because earlier prophase stages would be included in VP-16 treatment. Individual doses of 1, 2 and 4 mg/kg were injected on 13 consecutive days and sperm were sampled 23 days after the last treatment. We found that a total of 52 mg/kg applied to the entire prophase of meiosis significantly increased disomic and diploid sperm frequencies, while a total dose of 26 mg/kg was negative (Fig. 25). In contrast, a single acute dose of 25 mg/kg applied to spermatocytes during MMI/MMII gave a positive result (Fig. 24a). These data suggest that earlier prophase stages contribute relatively less to VP-16-induced aneuploidy in male germ cells. This conclusion is distictly in contrast to the report by Marchetti et al. (2001) where the aneuploidy was highest in first cleavage zygotes derived from sperm supposedly treated during preleptotene.

2.2 The Catalytic Topo II Inhibitor Merbarone (MER)

MER induced increased frequencies of hyperhaploid and diploid sperm in a dose-dependent manner (Fig. 24b). The positive response of the sperm-FISH assay to MER was in accord with the results of Kallio and Lähdetie, (1997). The authors found that treatment of mice with 80 mg/kg MER 18 or 48 hours before analysis (pro-metaphase I and diplotene-diakinesis) induced diploid and hypohaploid secondary spermatocytes but no hyperhaploid secondary spermatocytes. Treatment with 40 or 80 mg/kg MER during these stages also significantly increased the micronucleus frequencies in early spermatids. About 80% of these MN were CREST-positive indicating the presence of misplaced chromosomes. In the same slides, many arrested metaphases showed one or more chromosomes at the spindle poles and many anaphases showed lagging chromosomes at the spindle equator.

The magnitude of effect seen with MER at a comparable dose range by Kallio and Lähdetie, (1997) and in the present experiments was very similar. However, the two studies determined aneuploidy induction by different modes of action. In the present study, the sperm-FISH assay evaluated non-disjunction while the spermatid MN test estimated loss of chromosomes. Therefore, it has to be expected that the total effect of MER on aneuploidy induction in male germ cells is the sum of non-disjunction and chromosome loss.

2.3 Comparison with other Topo II Inhibitors

Inactivation of topo II by amsacrine induced aneuploidy during meiosis in spermatocytes of male mice that results in disomic sperm and caused meiotic arrest that results in diploid sperm (Attia, unpublished data). This in vivo observation is in line with earlier report on the GM10115A cell line (a Chinese hamster line containing a single human chromosome 4) that amsacrine led to polyploid cells, usually containing an uneven number of chromosome 4 (Ferguson et al., 1996b). Among other classes of topo II inhibitors, genistein has been studied in our laboratory by the sperm FISH assay to analyze their effect on chromosome segregation during meiosis of male mice. Genistein did not induce significant increases in disomic or diploid sperm at the doses of 250 and 500 mg/kg (Ruthsatz, unpublished data). On the other hand, genistein generated positive results in in vitro MN test, comet assay and the tk-mutation assay in L5178Y mouse lymphoma cells (Boos and Stopper, 2000). In contrast to VP-16 and amsacrine the mechanism of enhancing DNA cleavage is mediated by increasing the rate of cleavage as discussed in the Introduction chapter. There are

also reports of the induction of MN, DNA strand breaks and a weak mutagenicity in V79 cells (Kulling and Metzler, 1997), and the induction of chromosomal structural aberrations in human lymphocytes (Kulling et al., 1999), numerical and structural chromosome aberrations in Syrian hamster embryo cell line (Tsutsui et al., 2003) by genistein. The required concentration for induction of genetic toxicity were about 1000 x higher than those needed with VP-16 and amsacrine. Such high concentrations might not be reached in target tissues in vivo. This idea is supported by the fact, that genistein is a natural constituent of soy food, which is not considered detrimental, but is even presumed to be chemopreventive (Stoll, 1997).

2.4 Sensitivity Comparison of MMI and MMII

The sperm-FISH assay for aneuploidy or diploidy is capable of detecting effects induced during both meiotic divisions and to compare the sensitivity of both meiotic divisions. In the sperm FISH assay, we found that VP-16 caused significant increases above the concurrent controls of autodiploid sperm (XX88 and YY88) at all three doses tested. MER induced this effect only at the two higher doses. These autodiploid sperm were caused by complete arrest of the second meiotic division. After treatment with VP-16 and MER, autodiploid sperm resulting from arrest of MMII were significantly more frequent than diploid sperm resulting from arrest during MMI (XY88; 85 in 300,693 versus 11 in 300,693, $p<0.01$). Thus, the second meiotic division was more sensitive to VP-16 and MER treatment than the first meiotic division. A similar observation was made for other aneugens in the sperm-FISH assay, i.e. diazepam (Schmid et al., 1999) and griseofulvin (Shi et al., 1999).

The conclusion that second meiotic divisions were more sensitive than first meiotic divisions is supported by the observed frequencies of sex chromosome hyperploidies. Sperm with signals of XX8 or YY8 (67/150,400 in the VP-16 and 49/150,293 in the MER experiment) were more frequent than sperm with signals of XY8 (11/150,400 in the VP-16 and 9/150,293 in the MER experiment) for both chemicals. A similar observation was made for the other aneugens positive in the sperm-FISH assay (Table 12). This general higher sensitivity of MMII spermatocytes to induction of aneuploidy and diploidy can be explained in two ways. Firstly, it is a real effect and indicates meiotic selection or, secondly, it is a result of the timing of sperm sampling, i.e. inherent in the present test protocol, because the first wave of sperm in the epididymis is generally sampled. If samplings were obtained at later time intervals (25-27d after treatment), the contribution of cells exposed to the aneugen

during MMI could be greater. However, MMI and MMII occur within less than 24 h so that the latter explanation is rather unlikely.

An additional, general observation shall be discussed critically. Disomic sperm bearing two X chromosomes were more frequently scored than disomic sperm with two Y chromosomes. This may be due to a certain bias inherent in the strict scoring criteria since the Y probe is a large painting probe and sometimes two Y signals may be dismissed because they are too close in proximity. To avoid such problem, it would be desirable to use a centromeric Y probe, however, it may be difficult to obtain due to the heterochromatic nature of the entire Y chromosome.

2.5 Sperm-FISH Database

We have adapted and validated the sperm-FISH assay in our laboratory to detect the induction of numerical chromosome changes in male mammalian germ cells by chemical mutagens. The database now contains results for 16 known or suspected aneugens (Table 12). Four of these chemicals gave negative results: acrylamide, omeprazol, taxol, and genistein. Four chemicals induced only disomic sperm: COL, trichlorfon, nocodazol and albendazole. Another two chemicals induced only diploid sperm, which indicates that they suppressed the first or second meiotic division completely. These compounds were carbendazim and thiabendazole. Disomies and diploidies were induced by diazepam, griseofulvin, VP-16, MER and amsacrine. The other positive control substance, vinblastine, gave a similar response as COL in that the disomic sperm were significantly more frequent than in the controls at the two higher doses tested. Some of the vinblastine slides from the 1.0 mg/kg group were rescored at another laboratory (LLNL, USA) whereby the positive result by 1.0 mg/kg was not confirmed (Schmid et al., 2001b).

The database will be expanded by another two chemicals, which are presently being tested (Bisphenol A and Sodium orthovanadate). However, it seems timely to suggest that this reasonably simple assay could be of general use to determine the aneugenic potential of chemicals in male germ cells of mammals. It is mandatory for the reliability of the assay, that strict scoring criteria are observed and that concurrent control values stay within the range of our historical controls [0.049 ± 0.016 (mean value for disomic sperm ± SD); Adler et al., 2001]. We also advise that before starting a sperm-FISH assay, to perform a BrdU-incorporation assay to determine a possible delay in meiotic cell division induced by a given test chemical in order to chose an adequate sampling time for epididymal sperm.

Table 12. Summary of Results with the Sperm-FISH Assay

Chemical	Doses	Aneuploidy	Diploidy	Reference
Acrylamide	60 and 120 mg/kg	-	-	Schmid et al., 1999
Omeprazole	150 and 300 mg/kg	-	-	Adler et al., 2001
Taxol	50 mg/kg	-	-	Adler et al., 2001
Genistein	250 and 500 mg/kg	-	-	Ruthatz, unpublished
Colchicine	1.5 mg/kg	-	-	Schmid et al., 1999
	3 mg/kg	+	-	Attia et al., 2002
Trichlorfon	200, 300 and 405 mg/kg	+	-	Sun et al., 2000
Nocodazole	18 mg/kg	-	-	Attia, unpublished
	35 and 50 mg/kg	+	-	
Albendazole	1000 mg/kg	+	-	Ruthatz, unpublished
	2500 mg/kg	-	-	
Carbendazim	500 mg/kg	-	+	Adler et al., 2001
	1000 mg/kg	-	-	
Thiabendazole	100 mg/kg	-	-	Schmid et al., 1999
	300 mg/kg	-	+	
Diazepam	3 and 75 mg/kg	-	-	Schmid et al., 1999
	150 mg/kg	-	+	
	300 mg/kg	+	+	
Griseofulvin	500 mg/kg	+	-	Shi et al., 1999
	1000 and 2000 mg/kg	+	+	
VP-16	12.5 mg/kg	-	+	Attia et al., 2002
	25 and 50 mg/kg	+	+	
MER	15 mg/kg	-	-	Attia et al., 2002
	30 mg/kg	-	+	
	60 mg/kg	+	+	
Amsacrine	5 mg/kg	-	-	Attia, unpublished
	10 mg/kg	+	-	
	15 and 20 mg/kg	+	+	
Vinblastine	0.5 mg/kg	-	-	Schmid et al., 2001b
	1 mg/kg	+/-	-	
	2 mg/kg	+	-	

3 Bone-Marrow Micronucleus-Studies

The MN test (Schmid, 1973) was developed for evaluating the ability of test agents to induce structural and/or numerical chromosomal damage. In most testing situations, the test agent is administered acutely (generally once) to mice or rats, and the frequency of MNPCE determined in slides prepared from bone marrow harvested 24 and 48 h after treatment. In some experimental situations, peripheral blood can be sampled instead of bone marrow and mature erythrocytes (NCE) scored in addition to or instead of PCE for the presence of MN. We used the conventional bone marrow MN test complemented with the fluorescent *in situ*

hybridization with the mouse minor satellite DNA probe to investigate the mechanisms of induction of MN in mice treated with the topo II inhibitors VP-16 and MER. This assessment is particularly important for chemical clastogens, which may also have aneugenic potential. In order to determine the reliability of the methods, two-model mutagens, MMC and COL, known to be predominantly clastogenic and aneugenic, respectively, were used as positive control substances.

In the two control groups, the average of 0.34% MNPCE (Table 9) is higher than in earlier studies of our laboratory (Kliesch et al., 1981; Schriever-Schwemmer and Adler, 1994; Schriever-Schwemmer et al., 1997). However, the mean value for control groups is at the upper limit of the range regarded as acceptable for negative controls (3.4/1,000; Salamone and Marvournin, 1994). The control frequencies of MN with a fluorescent signal for the minor DNA probe with 44.3% and 43.1% in the present study as well as the distribution of signals per MN were consistent with 49.5% and 46.7% reported earlier (Schriever-Schwemmer and Adler, 1994; Schriever-Schwemmer et al., 1997). For 1 mg/kg of MMC, the frequency of 2.02% MNPCE observed in the present study, is again slightly higher than the 1.45% reported by Schriever-Schwemmer and Adler, (1994). The frequencies of MN with a fluorescent signal of the minor probe compare well in the two studies, i.e. 23.8% in the present study and 19% in the published study (Schriever-Schwemmer and Adler, 1994).

COL was previously tested at a dose of 1 mg/kg and 1.23% and 1.05% MNPCE, respectively, were found 24 h after treatment in two different studies (Adler et al., 1991; Schriever-Schwemmer and Adler, 1994). The present result of 1.14% MNPCE obtained with 3 mg/kg of COL was in the same range as those of the earlier studies. The frequencies of MN with fluorescent signals for the minor DNA probe were 74.1% in the present study and 75% reported earlier (Schriever-Schwemmer and Adler, 1994). Thus, the data with the solvent controls and the positive controls demonstrated that the FISH methodology used in the present experiments was efficient and reliable.

3.1 The Topo II Poison Etoposide (VP-16)

In the mouse bone marrow MN test, VP-16 caused a dose-dependent increase in MN induction up to 1 mg/kg (Ashby et al., 1994). In the tested dose range of 0.01 to 10.0 mg/kg, the lowest effective dose was 0.1 mg/kg. Turner et al. (2001) confirmed the sensitivity of the mouse bone marrow MN test to VP-16. An inverse dose response was found between 1 and 15 mg/kg (Ashby et al., 1994). The decline of the MN yields with increasing doses was accompanied by a significant reduction of PCE frequencies so that at low PCE rates hardly any MNPCE could be seen. However, the authors conclude that the depression in erythropoiesis alone cannot explain the inverse dose-response relationship. Neither one of these *in vivo* MN studies determined the origin of the VP-16-induced MN.

In agreement with the above-cited report, the present experiment showed that exposure to 1 mg/kg VP-16 yielded 3.44% MNPCE. A total of 208 MN were analysed by FISH and the frequency of signal-negative MN, derived from acentric chromosome fragments was 60.1% and 39.9% MN was signal-positive MN, produced by chromosome lagging. Thus, of the total of 3.44% MN observed, 2.07% contained an acentric fragment and 1.37% contained lagging chromosomes (Table 11 and Fig. 27). Both values are significantly different from the concurrent solvent control and indicate that VP-16 is clastogenic as well as aneugenic.

This result is similar to the one obtained by CREST-staining of VP-16-induced MN in Chinese hamster cells (Hermine et al., 1997) and contrasts the data in neonatal human lymphocytes (Slavotinek et al., 1993) and the human lymphoblastoid cell line TK6 (Wang and Eastmond, 2002). The differences between the three *in vitro* results cannot be attributed to different drug concentrations in the cell cultures since Slavotinek et al. and Wang and Eastmond both used 0.2 μM as their lowest concentration and Slavotinek et al. and Hermine et al. both used concentrations above 1 μM. Also, the repair capacities of the various cell types used cannot explain the discrepancies. Hermine et al. (1997) found no significant differences in the responses to VP-16 between repair-competent V79 cells and repair-deficient V79 cell mutants. Thus, other technical features of the test procedures may have produced the low frequencies of CREST-positive MN in two of the studies.

On the other hand, Kallio and Lähdetie, (1996) determined the presence of centromeres in MN in early mouse spermatids after treatment of male mice with different doses of VP-16 during preleptotene, S phase and diplotene-diakinesis as well as the meiotic divisions using FISH with the minor probe and, in parallel, immunolabelling of kinetochore proteins with CREST antibodies. They found with the FISH technique that VP-16-induced

MN showed significantly higher signal-positive MN compared with the controls. Similarly, after CREST labelling, the majority of MN induced by VP-16 showed kinetochore signals when meiotic S phase and diplotene-diakinesis were treated. This would suggest that most induced MN were due to lagging of whole chromosome(s). However, they found that more than 80% of the small MN observed was signal-positive and a large pool of minute MN almost exclusively (92%) contained kinetochore or centromere-DNA signals. The authors concluded that clastogenic events in the centromeric region of the acrocentric mouse chromosomes were responsible for these observations. They suggested that VP-16 caused clastogenic and aneugenic events in male mouse meiosis in vivo in at least two ways. First, inhibition of topo II-mediated strand religation in the decatenation process of centromeric DNA during meiosis I results in DNA fragmentation. Second, lagging of bivalents (two homologous chromosomes joined by chiasmata) during anaphase of meiosis I involves VP-16-mediated inhibition of the resolution of chiasmata, which are formed between homologous chromosomes by crossing-over events.

These conclusions are in agreement with the results of the cytogenetic studies with VP-16 in mouse oocytes by Mailhes et al. (1994). They reported increases in hyperploidy as well as chromatid breaks at the centromere region of c-banded metaphase II chromosomes after VP-16 treatment of female mice. Later on, Mailhes et al. (1996) confirmed that VP-16 is both aneugenic and clastogenic in mouse one-cell zygotes when administered to female mice during the pre-ovulation phase of meiosis. However, c-banding is not able to show whether a break occurred in the pericentric heterochromatin or at the actual centromere. Using the mouse minor satellite DNA probe can make this distinction because it hybridizes closely to the centromeric DNA (Wong and Rattner, 1988).

Of the VP-16-induced signal-positive MN in the present study, 59.0% had one signal, 31.3% contained two signals and 9.6% had ≥3 signals (Table 10). This distribution shows that more than half of the signal-positive MN were formed by a single lagging chromosome and about 30% of the signal-positive MN contained entire lagging chromosomes, i.e. non-disjoined chromatids. This can be deducted because the minor probe shows a hybridization signal on each of the two chromatids in mitotic metaphases (Fig. 21 and Schriever-Schwemmer and Adler, 1993). Since we did not differentiate MN by size, we cannot exclude that MN with multiple signals (>3) contained fragmented centromeric DNA as observed by Kallio and Laehdetie, (1996) for meiotic MN. However, from all the published evidence discussed here, there is no doubt that VP-16 has clastogenic as well as aneugenic activity.

3.2 The Catalytic Topo II Inhibitor Merbarone (MER)

The present study showed that the topo II-inhibitor MER increased the MN frequencies dose-dependently as described by Wang and Eastmond, (2002). However, the lowest positive dose in the present study was determined to be 15 mg/kg ($p< 0.05$, Mann-Whithey *U*-test) as opposed to 40 mg/kg by Wang and Eastmond, (2002). Exposure to 60 mg/kg MER yielded 4.26% MNPCE. Furthermore, MER caused a dose-dependent depression of erythroblast proliferation indicating an inhibition of erythroblast proliferation most likely by mitotic arrest (Table 9 and Fig. 26). A total of 199 MN from the 60 mg/kg MER-treatment group were analyzed by FISH and 92 MN (46.2%) were signal-positive. In contrast, Wang and Eastmond, (2002) found no significant increase in the frequency of CREST-positive MN in bone marrow of mice treated with the same dose of MER. Comparing the two centromere-labelling procedures used in these studies (Chen et al., 1994; Schriever-Schwemmer and Adler, 1994) it is unlikely that the difference is due to the modes of centromere detection, i.e. CREST versus FISH with the minor probe. Additionally, it is somewhat inexplicable that CREST-negative MN frequencies in mouse spermatid MN were as low as 14.7 to 21.7% after MER-exposure of male mice, indicating very low incidences of clastogenicity during meiosis (Kallio and Laehdetie, 1997).

Kallio and Laehdetie, (1997) found that 80 mg/kg MER did not induce minute MN and most of the large, medium, and small MN contained one or more CREST-signals (78.3-85.5%), indicating loss of bivalents or chromosomes during the preceding meiotic divisions. In parallel cytogenetic studies of meiotic chromosomes, the authors found increased frequencies of meiotic univalents (in meiosis I) and hypoploidies (in meiosis II) and no significant induction of structural chromosome damage. It was concluded that MER impaired homologous chromosome separation and/or sister-chromatid separation by inhibiting the decatenation activity of top II during both meiotic divisions. In our sperm-FISH study with MER in germ cells of male mice both drugs induced aneuploidy during meiosis that resulted in disomic sperm. They also caused meiotic arrest that resulted in diploid sperm.

These previous reports indicate that VP-16 is clastogenic and anuegenic during mitotic as well as meiotic divisions. In contrast, in vivo exposure to MER caused only clasogenic events during mitosis and only aneugenic events during meiosis (Kallio and Laehdetie, 1997; Wang and Eastmond, 2002). The data of the present experiments demonstrate that this conclusion is not correct. In our MN studies, MER showed significantly increased frequencies of signal-positive and -negative MN.

Of the MER-induced signal-positive MN, 44.6% had one signal and 44.6% contained two signals, while 10.9% had ≥3 signals with the minor satellite probe (Table 10). Since this probe labeled mitotic chromosomes at both chromatids (Fig. 21), it can be concluded that about equal frequencies of MN contained single chromatids (chromosome loss) and biarmed chromosomes (non-disjunction).

4 Conclusion

By using the BrdU-incorporation assay it could be shown that topo II poison VP-16 induced a meiotic delay of 24 h while the catalytic topo II inhibitor MER did not prolong the meiotic divisions. Thus, the differences in the mechanisms of action between VP-16 and MER were reflected in their different effects on cell cycle progression in spermatocytes of mice. Quantitation of sperm with fluorescent signals by the LSC saved time and improved throughput. These data confirm that cell cycle delay and aneuploidy induction are closely if not causally related as discussed by Chen et al. (1981) and Miller and Adler, (1992), also support the conclusion of Schmid and his colleagues, (2001a), which suggested that the effect of a chemical on the meiotic cell cycle progression is determined first in order to chose the appropriate sperm sampling time to detect aneuploidy induction.

By using the sperm-FISH analysis, it could be shown that the topo II-inhibitor VP-16 and MER induce aneuploidy during meiosis that results in disomic sperm and caused meiotic arrest that results in diploid sperm. The dose-response curves for both endpoints were linear. The positive result was expected for VP-16 based on the studies already published on its aneugenic effects in somatic and germinal cells. In respect to their aneugenic effects in spermatocytes detected by the sperm-FISH assay, both chemicals were similarly effective despite their different mechanisms of inhibiting topo II activity as discussed the Introduction.

Male cancer patients receiving chemotherapy with these drugs may stand a higher risk of siring chromosomally abnormal offspring, i.e. with Down, Klinefelter's or Turner Syndromes. The chemotherapy with these drugs may also reduce the fertility of the patients. Genetic counselling of cancer patients before having a child should take these results into consideration. However, the sensitive period of spermatogenesis is meiosis. Therefore, the increased genetic risk is limited to 3-4 months after the end of chemotherapy.

By using FISH analysis with the minor mouse-satellite DNA-probe for erythrocyte MN it could be shown that VP-16 and MER are aneugens as well as clastogens in somatic cells *in vivo* despite their different modes of topo II inhibition. While the mechanism of

clastogenicity of the topo II poison VP-16 is understood as an accumulation of enzyme-mediated DNA cleavages, the clastogenicity of the catalytic inhibitor MER may be a secondary effect of the inhibition of decatenation. Both the aneugenic and the clastogenic potential of VP-16 and MER in somatic cells can give rise to secondary cancer in patients treated with drug regimens that include these drugs. The presently shown aneugenic effect in mouse bone marrow cells contradicts the impression that the aneugenic activity of MER is restricted to meiotic cells. The formation of MN is a good example of drug induced chromosomal alterations that do not kill the cell. In this study we have shown that topo II inhibitors from different chemical classes are able to induce MN in concentrations of low toxicity, which are in the clinically relevant dose range. MN might be induced without apoptosis under certain conditions. If somatic cells containing MN survive and divide they may be precursors of malignant cells (Stopper et al., 1994). Even if they are not precursors themselves they are an indication of the amount of genotoxic damage induced in healthy tissue by topo II therapy.

Many chemicals may have clastogenic and/or aneugenic potential. The bone marrow MN test complemented by FISH with a pancentromeric DNA probe is able to detect the contribution of chromosome breakage and chromosome loss to the induction of genotoxic effects. The present mouse bone marrow MN studies showed that exposure to 1 mg/kg VP-16 yielded 3.44% MNPCE. However, in the sperm FISH assay, we found that the lowest positive dose, which caused disomic sperm, was 25 mg/kg of VP-16. Similarly, 15 mg/kg of MER was the lowest positive dose that caused MN in bone marrow while, in sperm FISH assay, the lowest positive dose was 60 mg/kg of MER.

These observations suggest that MN in bone marrow are induced at lower doses than disomies in sperm, hence the bone marrow is the more sensitive tissue. It must of course be noted that the assays measure different end-points. Chromosome loss and breakage is measured in the MN test, and non-disjunction is detected in the sperm-FISH assay. Therefore, the present data confirm the general paradime of hazard assessment, that the positive outcome of the bone marrow MN test is an indicator of the genotoxic potential of a compound in germ cells. However, to quantify aneuploidy induced in germ cells is important for risk assessment purposes.

Taking into account the fundamental differences between the meiotic process and the mitotic process, e.g. requirement for chromosome pairing, formation of chiasmata to allow recombination, prolonged duration of meiosis in oocytes, it will always be necessary to confirm the aneugenic potential of a chemical detected in *in vitro* by studies in somatic and in

germinal cells. Theoretically, the differences in cell biology may give rise to qualitative differences in response of germ cells and somatic cells to aneugens and the possibility of unique germ cell aneugens should not be neglected.

Other pharmaceuticals, dietary components (such as flavones, tannins and catechins), agricultural and industrial agents that interact with topo II must be tested because they may exhibit the same potential. Delineating the mechanisms of both their benefit and their hazard will provide a fruitful area for future research.

Awareness of the genetic hazard from aneugens has always been present, however, generally accepted and validated methods to test for this genetic endpoint have not been available. The main reason is that the targets for gene mutations and chromosomal aberrations are DNA and chromatin while aneuploidy-induction has multiple targets, i.e. tubulin, spindle fibres, centrioles, centromeres, teleomeres, motor proteins, and cell-cycle checkpoint proteins. The present data contribute to the assessment of aneuploidy induction by topo II inhibitors. Other classes of aneugens have been tested successfully with the assays used in the present study. Therefore, it is conclusion that aneuploidy tests such as the sperm-FISH assay and MN test complemented by FISH with pancentromeric DNA probes are now validated and should be included in the testing of chemicals for adverse genetic effects. To protect humans from exposure to aneugenic chemicals, the aneuploidy tests evaluated in the EU-Project PEPFAC should be included in the International Guidelines for Testing of Chemicals (EEC and OECD).

E. SUMMARY

Topo II inhibitors were selected as a group of suspected aneugens for testing in a coordinated research program on chemically induced aneuploidy funded by the EU-Project PEPFAC (Protection of the European Population from Aneugenic Chemicals). Of these, VP-16 and MER were chosen for the present experiments. VP-16 is used routinely to treat a variety of cancers in patients of all ages. However, evidence is accumulating that VP-16 is genotoxic, and that it can induce secondary tumours and impair fertility. Another topo II inhibitor MER that is in clinical trials as an anticancer agent has been reported to produce significant chromosomal alterations in cells *in vitro* and *in vivo*.

In the present study, we used a mouse model to asses the ability of the two mechanistically different topo-II inhibitors, VP-16 and MER, to induce aneuploidy in mouse germ cells and bone marrow cells. Three types of experimental studies were applied, the BrdU-incorporation assay to test if the chemical treatment altered the duration of the meiotic divisions, the sperm-FISH assay for aneuploidy induction during male meiosis and the bone marrow MN test complemented by FISH with a pancentromeric DNA probe to determine the clastogenic or aneugenic origin of MN.

Experimental Design and Results

(i) BrdU-incorporation assay for meiotic delay

In order to determine the optimum sampling time for sperm from the epididymis in the germ-cell aneuploidy-assay, it was tested if the chemicals under study could cause meiotic delay. The progression from meiotic divisions to epididymal sperm was determined by injecting male mice with the thymidine analogue BrdU to label the last S-phase during preleptotene of meiosis. The animals were injected with the test chemicals VP16 or MER, 13 days later when the labeled cells undergo the first and second meiotic division. At 20-24 days after treatment with the test chemicals, BrdU-containing sperm were identified with a fluorescein-labelled anti-BrdU antibody and green fluorescent sperm were scored automatically with a LSC.

It was found that VP-16 (50 mg/kg) induced a meiotic delay of about 24 hours. A significant reduction of BrdU-labelled sperm was observed at 22 days. On days 23 and 24, there were no significant differences between the VP-16 and the control groups. Thus, the optimal sampling time of sperm for the sperm-FISH assay was 23 or 24 days after VP-16 treatment. MER (80 mg/kg) treatment did not cause meiotic delay and the frequencies of

BrdU labelled sperm in treated and in control groups were similar on all days. Therefore, the 22 days sampling time was chosen for the sperm FISH assay according to the timing of spermatogenesis.

(ii) Sperm-FISH assay to determine aneuploidy induction during meiosis

For the mouse sperm-FISH assay, the animals were treated with different doses of VP-16 (12.5, 25 and 50 mg/kg), MER (15, 30 and 60 mg/kg), the model aneugen COL (3 mg/kg) or with DMSO as solvent control. Males were sacrificed 24 days after administration of VP-16 or COL and 22 days after administration of MER and sperm were collected from the *Caudae epididymes* 24 days after VP-16 or COL or 22 days after MER treatment. The frequencies of disomic and diploid sperm were determined by FISH with DNA probes specific for mouse chromosomes 8, X and Y, each labelled with a different colour. At least 10,000 sperm from each of 5 animals per dose-group were analysed under the fluorescence microscope for the number of colour signals present. Disomic sperm were characterized by three signals (XX8, YY8, XY8, X88 or Y88), diploid sperm contained four signals (XX88, YY88 or XY88).

The frequency of disomic sperm was significantly increased after treatment with the positive aneugen COL. Diploid sperm were not induced by COL treatment, indicating that no complete meiotic arrest occurred. In animals treated with different doses of VP-16, the frequency of disomic sperm increased significantly after treatment with 25 and 50 mg/kg VP-16. Furthermore, VP-16 caused a dose-dependent significant increase in the frequencies of diploid sperm at all three doses tested. The dose responses for VP-16-induced disomic and diploid sperm can be described by linear equations. The prevalence of autodiploid (XX88, YY88) sperm and disomic XX8 or YY8 sperm indicate that the second meiotic division was more sensitive to VP-16 than the first meiotic division. To determine if subacute treatment would have an effect because prophase stages would be included in VP-16 treatment, doses of 1, 2 and 4 mg/kg were injected on 13 consecutive days and sperm were sampled 23 days after the last treatment. We found that, the highest dose group, which received a total of 52 mg/kg, gave the only significantly positive results. These data suggest that earlier prophase stages contribute relatively less to VP-16-induced aneuploidy in male germ cells. In animals treated with different doses of MER, the frequency of disomic sperm was increased significantly after treatment with 60 mg/kg. Furthermore, MER caused significant increases in the frequencies of diploid sperm at 30 and 60 mg/kg. The dose-response curves for both endpoints were linear. As with VP-16, the second meiotic division was more sensitive to MER treatment than the first meiotic division.

(iii) Bone marrow MN test using FISH with a pancentromeric DNA probe

Two experiments were performed with the mouse bone marrow MN test. In the first experiment, animals were treated with 1 mg/kg VP-16, 3 mg/kg of COL (positive control aneugen) or 1% DMSO as solvent control I. In the second experiment, the animals were treated with 7.5, 15, 30 or 60 mg/kg MER, 1 mg/kg of MMC (positive control clastogen) or 15% DMSO as solvent control II. The animals were sacrificed 24 hours after treatment and femoral marrow cells were collected. At least four slides were made for each animal and two slides per animal were stained with May-Gruenwald/Giemsa solutions for conventional assessment of the MN frequencies. The remaining unstained slides from the animals treated with VP-16, COL, 60 mg/kg MER, MMC as well as from the two solvent controls were used for the FISH analysis with the mouse minor satellite DNA probe to discriminate MN of clastogenic and aneugenic origin. At least 2000 PCE from each of five animals per dose were scored for the presence of MN in the conventional MN assay. After FISH-labelling, between 100 and 200 MN were analyzed under the fluorescence microscope for the presence of centromeric signals.

The frequencies of MNPCE in control I (1% DMSO) and in control II (15% DMSO) were not significantly different (0.37 and 0.31%). About 45% of the MNPCE in control I, and 43% of the MNPCE in control II were centromere-positive. COL and MMC showed the expected significant increases in the frequencies of MNPCE (1.14% and 2.02%, respectively). For COL, the majority of the induced MN showed centromeric signals, reflecting the expected aneugenic effect. For MMC, the majority of induced MN were centromere-negative, confirming the clastogenic effects of MMC.

A dose of 1 mg/kg VP-16 induced about 3.5% MNPCE and about 60% of them were centromere-positive, indicating that they were formed by acentric fragments and reflecting the clastogeic activity of VP-16. Correspondingly, about 40% of induced MN were centromere-positive, reflecting an aneugenic activity of VP-16. In animals treated with different doses of MER, the frequencies of MNPCE increased significantly in a dose-dependent manner with 15 mg/kg being the lowest effective dose. Furthermore, MER caused a dose-dependent depression of erythroblast proliferation indicated by a decrease in PCE/NCE+PCE ratio. At the highest dose of 60 mg/kg MER, a total of 4.5% MNPCE were found and about 54% of them were centromere-negative, indicating their formation by acentric fragments and reflecting the clastogeic activity of MER. Correspondingly, about 46% of induced MN were centromere-positive, representing the aneugenic activity of MER.

The present study demonstrated that the topo-II inhibitors VP-16 and MER induced aneuploidy during meiosis that resulted in disomic sperm and caused meiotic arrest that resulted in diploid sperm. By using the FISH analysis with minor satellite DNA-probe it could be shown that VP-16 and MER are aneugens as well as clastogens in somatic cells *in vivo* despite their different modes of topo-II inhibition. Therefore, pharmaceuticals, dietary components (such as flvones, tannins and catechind), agricultural and industrial agents that interact with topo II raise general concern that they may have the same potential. Topo II inhibitors thus have the potential for both, useful effects as new therapeutic agents, and harmful effects as mutagens. Delineating the mechanisms of both will provide a fruitful area for future research.

Finally, it is concluded that aneuploidy tests such as the sperm-FISH assay and FISH with pancentromeric DNA probes to determine the clastogenic or aneugenic origin of MN are now available to be included in the testing of chemicals for adverse genetic effects. To protect humans from exposure to aneugenic chemicals, the aneuploidy tests evaluated in the EU-Project PEPFAC should be included in the International Guidelines for Testing of Chemicals (EEC and OECD).

F. ZUSAMMENFASSUNG

Aneuploidien oder numerische Chromosomenaberrationen sind charakterisiert durch eine Abweichung des normalen diploiden (somatische Zellen) oder haploiden (Keimzellen), Chromosomensatzes und zählen zu den häufigsten chromosomalen Aberrationen in der menschlichen Population; um die 50 % aller spontanen Aborte sind aneuploid, die Mehrzahl darunter Trisomien. Nur wenige Aneuploidien überleben bis zur Geburt und sind meist mit kongenitalen Missbildungen wie körperliche und mentale Retardierung verknüpft. Ebenso hat Aneuploidie in somatischen Zellen ernsthafte Konsequenzen. Als Folge sind neben dem Zelltod die Entwicklung von Tumoren zu nennen.

Die Topoisomerase II-Inhibitoren Etoposid (VP-16) und Merbaron (MER) wurden darauf hin untersucht, ob sie in somatischen Zellen und in männlichen Keimzellen der Maus zu Chromosomenfehlverteilungen (Aneuploidien) führen können. Es konnte gezeigt werden, dass VP-16 zu einer Verzögerung im Ablauf der meiotischen Teilungen in Spermatozyten der Maus führt, MER jedoch nicht. Das wichtigste Ergebnis war, dass beide Substanzen in männlichen Keimzellen und im Knochenmark zu Aneuploidien führen. Darüber hinaus wurde im Knochenmark eine chromosomenbrechende Wirkung nachgewiesen. Diese Ergebnisse zeigen, dass für Krebspatienten nach Behandlung mit diesen Substanzen zwei Risiken bestehen: 1. Ein Kind mit einer chromosomalen Störung zu zeugen (z.B. Trisomie 21), und 2. einen sekundären Tumor zu entwickeln.

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H. APPENDIX

1 Chemicals, Kits, Solutions and Buffers

Absolute ethanol (Merck, Deutschland):

70% ethanol (70 ml ethanol + 30 ml dH_20)

90% ethanol (90 ml ethanol + 10 ml dH_20).

Acetic acid (Merck, Deutschland).

Agar (Sigma, Deutschland).

Agarose 1% (Ultra pure agarose; GibcoBRL, Life Technologies, Deutschland):

1 gm agarose was melted in 100 ml of 1 x TAE buffer using a microwave oven. Gel typically contain 1% agarose is appropriate for separating DNA fragments of 500-10,000 bp size.

Ampicillin (GibcoBRL, Life Technologies, Deutschland):

Each vial contains 200 mg ampicillin sodium and rehydrated with 20 ml sterile dH_2O.

Anti-Bromodeoxuridine (Anti-BrdU; Diagnostic International, Deutschland):

Each vial contains 0.5 mg/ml Anti-BrdU monoclonal antibody made in rat. The working solution 50 µg/ml was prepared by dilution 1: 10 with PNBR buffer shortly before use.

Anti-Digoxigenin-Fluorescein, Fab Fragments (Anti-DIG-FITC; Boehringer, Deutschland):

Each vial contains 200 µg Lyophilized Anti-DIG-FITC made in sheep, the stock solutions were prepared by reconstituting the vial by 1 ml dH_20 and stored in aliquots at -20°C. The working solution 50 µg/ml was prepared by dilution 1: 4 with PNBR buffer before use.

Anti-Rat-FITC (Goat $F(ab')_2$ Anti-Rat IgG FITC conjugates; Caltag, Lab., USA):

Each vial contains 700 µg/ml Anti-Rat-FITC. The working solution 70 µg/ml was prepared by dilution 1: 10 with PNBR buffer shortly before use.

Biotin-16-dUTP (Biotin-16-2´-deoxy-uridine-5´-triphosphate; Roche Diagnostics, Deutschland).

Biotinylated Anti-Streptavidin (Vector Laboratories, USA):

Each vial contains 0.5 mg Lyophilized Biotinylated Anti-Streptavidin made in goat, the stock solutions were prepared by reconstituting the vial by 1 ml dH_20 and stored in aliquots at -20°C. The working solution 10 µg/ml was prepared by dilution 1: 50 with PNBR buffer shortly before use.

BrdU (5-Bromo-2`-Deoxyuridine; Sigma, Deutschland):

100 mg/kg BrdU was prepared by dissolving 150 mg in 15 ml 0.9% NaCl.

Cy3-Streptavidin (Dianova, Deutschland):

The contains of each vial (1 mg/0.6 ml Cy3-Streptavidin) were stored in aliquots at -20°C and the working solution 6.6 µg/ml was prepared by dilution 1: 250 with PNBR buffer shortly before use.

DAPI (4`,6-Diamidin-2`-phenylindol-dihydrochloride; Roche Diagnostics, Deutschland):

Each vial contains 10 mg/ml DAPI; the stock solution 1 mg/ml was prepared by dilution 1: 10 by dH_20 and stored at -20°C in Eppendorf cups.

Digoxigenin-11-dUTP (Digoxigenin-11-2´-deoxy-uridine-5´-triphosphate; Roche Diagnostics, Deutschland).

DMSO (Dimethylsulphoxide; Merck, Deutschland).

dNTP-Mix (minus dTTP) (GibcoBRL, Life Technologies, Deutschland):

Each vial contains 0.2 mM each of dATP, dCTP, dGTP, 500 mM Tris-HCl (pH 7.8), 50 mM Mg Cl_2 and 100 mM 2-mercaptoethanol.

DTT (Dithiothreitol, Sigma, Deutschland):

10 mM DTT (0.1542 g) was dissolved in 0.1 M Tris-HCl (100 ml) pH 8.

dTTP (Deoxynucleoside Triphosphate Set; Boehringer, Deutschland):

The working solution (1 mM) was prepared by diluting one µl of dTTP with 99 µl dH_20.

***Eco* R1** (*Escherichia coli* R; Biolabs, New England): Sequence cut is GAATTC.

***Eco* R1 buffer** (Biolabs, New England).

Ethidium bromide (Sigma, Deutschland).

Eukitt (Kindler GmbH & CO, Deutschland).

Fetal Calf Serum (FCS; GibcoBRL, Life Technologies, Deutschland).

Fluorescein -Anti Sheep IgG (RAS; Vector Laboratories, USA):

Each vial contains 1.5 mg/1.5 ml active conjugate made in rabbit. The working solution 10 µg/ml was prepared by dilution 1: 100 with PNBR buffer.

Formamide (FA; Carl Roth, Deutschland):

70 % FA was prepared by mixing 70 ml FA, 10 ml 20 x SSC and 15 ml dH_20.

50 % FA was prepared by mixing 50 ml FA, 10 ml 20 x SSC and 35 ml dH_20.

The pH 7.0 was adjusted by 50 % HCL and 1 N NaOH then completed to 100 ml with dH_20, stored at 4°C and used during 1-2 months.

Giemsa's solution (Merck, Deutschland).

Glycogen (Boehringer, Deutschland).

HBSS (Hanks balanced salt solution with Phenolred and free from Mg^{2+} and Ca^{2+}, Sigma, Deutschland).

Isopropanol (Fluka, Deutschland).

0.56% KCl (Potassium chloride; Sigma, Deutschland): 0.56 gm / 100 ml dH_2O.

LB (Luria-Broth-Medium):

10 g Trypton (Difco), 5 g yeast extract and 5 g NaCl in 1000 ml dH_2O.

LIS (Lithium-3, 5-diiodosalicylic acid, Sigma, Deutschland):

4 mM LIS (0.1583 g) were dissolved in 0.1 M Tris-HCl (100 ml) pH 8.

10 x loading buffer (GibcoBRL, Life Technologies, Deutschland).

May-Grünwald's solution (Merck, Deutschland).

MM 1.0 (Master Mix):

5 ml formamide, 1 g dextran sulfate (Fluka, Deutschland) and 0.5 ml 2 x SSC were heated to 70°C for several hours to dissolve the dextran sulfate, cooled, pH adjusted to 7.0 and the volume brought to 7 ml with dH_2O then stored at -20°C.

MM 2.1 (Master Mix):

5.5 ml formamide, 1 g dextran sulfate and 0.5 ml 2 x SSC. MM 2.1 solutions were prepared as MM 1.0.

2 N HCl (Hydrochloric Acid; Merck, Deutschland):

23 ml of HCl 32% diluted with 77 ml dH_20.

Paraformaldehyde (PA; 4% and 1% PA; Sigma, Deutschland):

4 gm or 1 gm PA powder were dissolved in 50 ml of 100 mM $MgCl_2$ solution (1.017 gm $MgCl_2$/50 ml dH_2O), ≈17 drops of 1 N NaOH were added and heated to 70°C to speed up this process until clear solution was produced. The solution cooled until 35°C then filtered and diluted with 50 ml 2 x PBS (pH 7.4).

PBS buffer (Phosphate buffered saline):

16.4 g NaCl, 0.8 g KCl, 3.45 g Na_2HPO_4, and 0.6 g KH_2PO_4 were dissolved well in 800 ml dH_2O and the pH was adjusted to 7.4. The volume was completed to one litre with dH_2O then sterilized by autoclaving.

2 x PBS buffer (For PA solutions):

16.0 g NaCl, 0.4 g KCl, 2.88 g Na_2HPO_4, and 3.0 g KH_2PO_4 were dissolved well in 800 ml dH_2O and the pH was adjusted to 7.4. The volume was completed to one litre with dH_2O then sterilized by autoclaving.

PI (Propidium Iodide; Sigma, Deutschland).

Each vial contains 10 mg PI powder. The working solution (2.0 μg/ml) was prepared by diluting 200 μl of 10 μg/ml with 800 μl dH_2O.

PN Buffer (Phosphate-Nonidet P-40-buffer):

80.055 g Na_2HPO_4. H_2O were dissolved in 4.5 L dH_2O and the pH 8.0 was adjusted by the addition of suitable volume of NaH_2PO_4. H_2O (13.8 g were dissolved in 500 ml dH_2O), then 5 ml Nonidet P-40 (Boehringer, Deutschland) were added and sterilized by autoclaving.

PNBR-Buffer:

5 g blocking reagent (BR; Boehringer, Deutschland) were dissolved in 100 ml PN buffer at 70°C using magnetic stirrer then 20 mg Na Azide (bacterial free) were added. The solution leaved for about 30-60 min for cooling then sterilized by filtration and stored in 1.5 ml Eppendorf cups at -20°C.

Rubber Cement (Marabuwerke GmbH & Co., Deutschland).

Salmon Sperm DNA (10 mg/ml; GibcoBRL, Life Technologies, Deutschland).

20 x SSC (Saline Sodium Citrate buffer):

3 M NaCl (175.32 g) and 0.3 M $C_6H_5Na_3O_7.2H_2O$ (84.22 g) were dissolved in 1000 ml dH_2O. Then, the pH was adjusted to 7.0 with 1 N HCl and 1N NaOH and sterilized by autoclaving.

For 2 x SSC buffer, 20 x SSC was diluted to 1:10 with dH_2O.

50 x TAE buffer (Tris-Acetat-EDTA):

121.1 g of Tris base, 28,5 ml 100% acetate acid and 50 mM Na_2EDTA 2 H_2O dissolved in 450 ml dH_2O then the pH was adjusted to 8.0. The resulted volume was completed to 500 ml with dH_2O and sterilized by autoclaving.

For 1 x TAE buffer, 50 x TAE was diluted to 1:50 with dH_2O.

TE-Buffer (Tris/EDTA):

1 mM EDTA (37.22 mg) were dissolved in 20 ml of 10 mM Tris-HCl buffer (pH 7.4), then completed to 100 ml with dH_2O and sterilized by autoclaving.

Tris-HCl buffer (0.1 M, pH 8.0):

60.55 g $C_4H_{11}NO_3$ (AppliChim, Deutschland) were dissolved in 4500 ml dH_2O then the pH was adjusted to 8.0. The resulted volume was completed to 5000 ml with dH_2O and sterilized by autoclaving.

Triton X-100 (Sigma, Deutschland):

The working solution 0.1% was prepared by diluting 100 µl of Triton X-100 with 10 ml 2 x SSC.

Vectashield mounting medium (Vector Labs., USA).

2 Equipments

BACHOFER GmbH (Germany): Speed Vac Centrifuge

Compucyte, Cambridge (USA): Laser Scanning Cytometer

HANNA Instruments (Germany): pH-Meter HI98113

Kottermann (Germany): Incubators

Leitz Wetzlar (Germany): Light Microscope

MEDAX Nagel GmbH (Germany): Hotplate

MetaSystems (Germany): Computer Program ISIS3

Scientific Industries (USA): Vortex-Genie 2

Sigma (Germany): Centrifuges

Tomy Seiko Co. (Japan): Microcentrifuge

Zeiss: Fluorescence Microscope