POSSIBLE EFFECTS OF HORMONAL CONTRACEPTIVES ON HUMAN AND BABOON MITOTIC CHROMOSOMES

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DECLARATION

I declare that this dissertation is my own unaided work, except for the statistical analysis of the data, which was kindly done by the staff of the Institute for Biostatistics (Transvaal Branch). This thesis is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg, and has not been submitted for a degree in any other University.

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To my parents Amélia and Benjamim

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ABSTRACT

Hormonal contraceptives - 'the pill' as it is known to the general public - are among the most universally used drugs in our contemporary world. Daily, millions of healthy young women are taking the 'pill' for a purpose other than the control of disease. The value and efficacy of these compounds in the face of an ever increasing population explosion cannot be ignored or contested; however, there is no single drug devoid of ill effects, and hormonal contraceptives are no exception to this rule. Some of the adverse effects have already been recognized, some are at present merely suspected and possibly the most important of such effects still elude us. In this last category are the possible ill effects on the genetic material.

The aim of this study was therefore to try to contribute in the elucidation of the possible harmful effects of hormonal contraceptives on the genetic material using chromosome breakage and sister chromatid exchanges as test systems for indicating genetic damage.

Chromosome breakage was assessed in peripheral blood lymphocytes of fifty women using different types of hormonal contraceptives for various periods of time, as well as on fifty matched controls. Sister chromatid exchanges (SCEs) were assessed in five additional cases using hormonal contraceptives and five matched controls.

The selection of individuals for this study was preceded by the completion of a questionnaire, to avoid as far as possible, all cases subjected to known clastogenic agents. The questionnaire included information on age, race, number of pregnancies and children,

health status, occupational and therapeutic exposure to X-rays and other radiation, recent exposure to drugs and medicines, recent viral diseases and immunizations.

Experimental studies were done on two female Chacma baboons *(Papio ursinus)* using an injectable hormonal contraceptive for one year. Chromosome breakage was assessed on peripheral blood and bone marrow metaphases before and after the treatment on these two animals.

Cultures were established by standard techniques and every 'study' individual and her own matched 'control' were simultaneously and similarly processed except for the animal studies.

The use of hormonal contraceptives was found to be associated with a highly significant increase of chromosome aberrations in the human studies.

Sister chromatid exchanges were also increased in the hormonal contraceptive group

The pilot study on two baboons to compare breakage in two different cell lines, suggested an increase in chromosome abnormalities both in bone marrow and peripheral blood, after one year of treatment with an injectable hormonal contraceptive. These preliminary results warrant an expanded study.

The likelihood that the association found is a causal one, i.e. that hormonal contraceptives are capable of producing chromosome breakage and therefore cause damage to the genetic material, is postulated. If this assumption is correct, the genetic damage could be phenotypically expressed either as malignancy or in the

form of abnormalities in the offspring.

A review of the literature was carried out and some of the documented possible ill effects of hormonal contraceptives are tentatively interpreted in this thesis as either direct or indirect manifestations of genetic damage.

Speculation was also directed to the theoretically possible long-term ill effects of hormonal contraceptives on the genetic material which we are unable to accurately assess at this stage, because hormonal contraceptives have not been in use for a sufficient length of time.

Our knowledge of chemical carcinogenicity, mutagenicity and teratogenicity is at present relatively scanty and in many instances controversial. The same applies to hormonal contraceptives; little is known about all their systemic effects, and their possible role in the aetiology of malignancy and/or congenital and inherited abnormalities.

The undeniable need for contraceptives in this day and age cannot be ignored or neglected. However, at the same time one must realize the potential hazards if one continues the use of a drug with inadequate knowledge of its side effects. This is particularly relevant in the case of a drug which is continuously administered for twenty-one of twenty-eight days, year in and year out, during a major portion of a woman's reproductive life.

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CHAPTER 1

1. INTRODUCTION

1.1 History of contraception

The desire for control of conception is as old as man. Contraceptive measures have been used since man associated sex with subsequent pregnancy. Various practices have been used over the centuries; many of them magical and ineffective, but some rational and effective such as coitus interruptus. When there was a failure of these practices unwanted fertility was controlled by abortion and infanticide.

It is interesting to note that in ancient medical history we can find an antecedent for almost all the modern methods of contraception in use today.

Coitus interruptus is the earliest recorded effective method of contraception. It was used in ancient societies and has been epomymized in the biblical story of Onan (Genesis 38, vv. 7-10).

Condoms have also been used for centuries. The first written description of the condom (then a linen sheath) was given by an Italian anatomist Fallopius in his treatise published in 1564. The earliest sheaths were used for protection against venereal diseases, and only later in the eighteenth century the contraceptive value of the condom was appreciated. Both de Sade and Casanova (Kerr and Parboosingh, 1974) refer to the condom in lavish terms and Casanova acknowledges its contraceptive value.

The history of contraceptive cervical occlusive devices is very bizarre. In primitive societies the earliest barrier techniques

included roots, grass, seed pods, honey and gum among others. The Egyptians favoured crocodile dung, and there was even a controversy as to whether Egyptian crocodile dung or Indian elephant dung was the more effective!

Although the majority of chemical substances used as cervical occlusive devices were designed to act as a mechanical barrier, some of them possibly, also had a spermicidal effect; the basis of modern local spermicidal chemical contraception.

Modern use of the diaphragm as a mechanical method of contraception has probably its primitive counterpart in the squeezed half lemon used in olden days to cover the cervix for prevention of pregnancy.

Intrauterine devices (IUD) were only introduced about a century ago; but we can consider that the first IUD was a stone placed in the uterus of a female camel prior to the long trips across the Sahara desert.

Even the oral contraceptives had their prototype in ancient societies; the ingestion of a wide range of plants and metals was common practice, but unfortunately some of these compounds proved to be lethal not only to the gamete but also to the unfortunate woman! Furthermore, the collagen sponge, which is still on trial at the present moment, has its predecessor in the sponge used in Egypt more than 3 000 years ago and later used as the only contraceptive method in ancient Israel.

1.2 History of hormonal contraception

Beard (1897) first postulated that progesterone secreted by the corpus

luteum of the ovary was responsible for the inhibition of ovulation during pregnancy. In 1934 progesterone was isolated; this was followed by the synthesis of a multitude of steroids by several pharmaceutical companies.

Experimental work on rabbits (Makepeace *et al.*, 1937) and in rats (Astwood and Fevod, 1939) confirmed Beard's theory of a progesterone inhibitory effect on ovulation. By 1939 and the early 1940's oestrogens were already being regularly used for the treatment of dysmenorrhoea by inhibition of ovulation. It was however found that this inhibition gradually decreased over successive cycles and was therefore not consistently effective. In 1945, after World War II, the attention of the world (free from the war) focused on the problem of population growth, and the subject of population explosion made newspaper headlines. At the same time massive migration from rural to urban areas created intensive population pressures in many cities and societies.

These two major developments created the ideal background for the realization of the imperative need to develop new and more efficient contraceptive methods.

One of the more attractive possibilities was the development of an 'easy to use' active oral contraceptive. After a short time it was found that two 19-norsteroid compounds, norethynodrel and norethindrone, when orally administered, were the most effective to prevent pregnancy in animals (Chang, 1978). These animal studies were followed by the first clinical trial of the compounds in humans in Puerto Rico (Chang, 1978).

In 1956, the two trials on animals and humans were published

concurrently in Science (Pincus *et al.*, 1956; Rock *et al.*, 1956).

The Puerto Ricon study however, showed a high frequency of 'spotting' and bleeding. This side effect led to the introduction of an oestrogen into the progestational compounds to aid in maintaining the endometrium and therefore prevention of break through bleeding. This combination of drugs proved to be even more effective for contraception (Garcia *et al.*, 1958). By 1959 the use of oral contraceptives was well established and in June 1960 they were licensed for sale in the United States. This revolutionary innovation took a mere nine years from conception to its release for general clinical use!

The first oral contraceptive was commercialized in the USA by the trade name of ENOVID (G.D. Searle) and each 10 mg tablet contained norethynodrel in combination with 1,5% mestranol (Drill, 1977). Later this drug became known as 'the pill', a name that became synonymous in the eyes of the general public for all types of oral contraceptives.

The introduction of oral contraceptives in the early 1960's had an unprecedented impact on the field of contraception. The high efficacy and convenience of this method of contraception led to its worldwide and popular acceptance. Furthermore, it also contributed to the acceptance of family planning as a socially acceptable procedure.

There is no doubt that one of the most significant developments of our century has been the discovery of the oral contraceptives. As stated before: "Posterity may decide that the greatest contribution of the 20th century to the health of mankind, was not a vaccine, an

antibiotic or a transplant, but the contraceptive pill" (Editorial, Lancet, 1974).

1.3 Population growth - the need for contraception

When we think in terms of global population growth, a vivid picture emerges: it took from the beginning of time to the year 1830 for the world's population to reach one billion; the second billion was reached in a further one hundred years; and only thirty more years for the third billion! Prospective estimations for the year 2 000 are a world population of between five and six billion people! Without going into lengthy discussions on economic, medical, social and political considerations, the necessity for effective fertility control for the survival of mankind becomes quite obvious.

To ignore the value and contribution of oral contraceptives, faced with our planet's population explosion, would be quite unrealistic. One can imagine the impact on our society if oral contraceptives were no longer available!

One must however never forget that no single drug is devoid of side effects, and hormonal contraceptives are no exception.

1.4 Ill effects

Soon after the pill began to be widely used, a WHO committee (1966), commented: "The administration of biologically active substances to human beings must always be accompanied by some element of risk that cannot be avoided by most careful and exhaustive scientific study of the drug before it is introduced." Although this statement was mainly directed at the time to vaccines, it can be applied to

any drug in general and to 'the pill' in particular.

Sir Alan Parkes (Peel and Potts, 1969) summarises the problem facing decision-makers more directly: "It is always difficult to prove a negative, and impossible to do so in advance. In fact we face the dilemma that no woman should be kept on the 'pill' for twenty years until, in fact, a substantial number have been kept on the 'pill' for twenty years."

We are at present about twenty-one years into the modern era of contraception. It is calculated that more than fifty-five million healthy young women, worldwide, are taking 'the pill'! We are therefore faced with the unique situation that never had so many people, taken such potent drugs, voluntarily, over such long periods of time, for a purpose other than the control of disease.

For the above reasons, contraceptive steroids have been the subject of more scientific publications than any other drug in history. Yet, the controversy for and against them grows with the passage of time.

Some of the adverse effects of the hormonal contraceptives are already known; some are still merely suspected; and possibly the potentially most important ones, still elude us. (A more detailed review of some of the side effects and risks of hormonal contraceptives are presented in Section 2.6.2.)

We are unfortunately still a long way from knowing all the risks of systemically active contraceptives!

1.4.1 Ill effects on genetic material

One important aspect in the investigation of the ill effects of any

environmental agent, be it chemical, physical or biological, is the possible ill effect on the genetic material. Several human or nonhuman, direct or indirect test systems are available for the study of this problem. In practical terms the main tests for the detection of genetic damage fall into two main categories: tests directed to individual genes, and tests directed at the whole genome. Chromosome aberrations (in spite of the fact that they are subject to a number of limitations) are among the best tests available at present for studying the whole genome.

1.5 Chromosome aberrations as indicators of genetic damage

The study of chromosome aberrations as an indication of genetic damage, by environmental agents, first emerged in 1927. In a classic paper entitled "Artificial transmutation of the gene" (Muller, 1927), it was demonstrated that X-rays produced chromosome aberrations and mutations.

The prediction that drugs and chemicals could also produce chromosome aberrations was then made. This prediction proved to be true during World War II, when it was shown that chromosome aberrations were induced by nitrogen mustard.

These observations created considerable scientific interest, but did not cause overmuch concern to the public or medical profession as mustard and related compounds had a very restricted use, viz. in warfare and cancer therapy.

In recent years however, reports of chromosome aberrations induced by a variety of chemicals, used by a large number of people,

have caused a great deal of concern.

Perhaps the paper that focused most attention on this problem was the report of an increase in chromosome aberrations in 'hippie' populations taking LSD (Cohen *et al.*, 1967). Cyclamates and pesticides, two other major products in general use, also cause alarm because of their capacity for inducing chromosome aberrations.

Since then a multitude of compounds in man's modern environment have been tested for their capacity to break chromosomes, i.e. their clastogenic effect (the word clastogen was first suggested by Shaw (1970) and comes from the Greek word 'clast' that means break, fracture or fragment).

Long lists now exist of the different chemical, physical and biological agents capable of producing chromosome breakage.

Contraceptive steroids are among the compounds previously tested for their possible clastogenic effects, but in spite of the fact that the literature for hormonal contraceptives is so vast, their effects on chromosomes are scantily documented and controversial.

It therefore appeared worthwhile to the author to perform chromosome studies on a sample of carefully selected subjects using hormonal contraceptives.

Thus the present study was conceived.

CHAPTER 2

2. MATERIALS

2.1 Human mitotic chromosomes

For the study of hormonal contraceptives and their possible effects on human mitotic chromosomes, venous peripheral blood was used from women on different types of hormonal contraceptives. The women had been taking the preparations for varying periods of time.

Suitable female controls were also used. A sample comprising 100 cases was studied; fifty 'study group' (women on hormonal contraception) and fifty 'control group'. However, only eighty-eight cases were used for the statistical analysis (see Section 4.7). Ten extra blood samples, five obtained from women on hormonal contraceptives and five obtained from controls were used in the study of sister chromatid exchanges (SCE).

2.2 Source of materials

The peripheral blood samples from the women of the 'study group' were obtained from females attending Family Planning Clinics. The clinic for the White population sampled was situated at the Queen Victoria Maternity Hospital and the clinic for the Black population sampled was situated at the Johannesburg General Hospital (Non-European Section), both being in the Johannesburg area (about two kilometres apart).

'Control group' samples were obtained from women who attended the Family Planning Clinic but who had never used any form of hormonal contraception and from female medical students who also had not used hormonal contraceptives.

2.3 Preliminary questionnaire

The choice of both 'study group' and 'control group' was preceded by completion of a detailed questionnaire, with the aim of avoiding all cases that may have been subjected to factors known to be harmful to chromosomes, and to match each pair as closely as possible.

2.3.1 Race

Both Negroid (twenty-three cases) and Caucasoid (sixty-five cases) women were used in the study (Figure 2.1). Each pair ('study' case and respective 'control') were matched by race with the exception of five pairs.

2.3.2 Age

Age limits were initially empirically established between twenty and thirty-five years of age. Later the lower limit of twenty was changed to sixteen years (this will be discussed later in this chapter). The age distribution for both control and study groups is shown in Figures 2.2 and 2.3.

2.3.3 Health status

Only healthy women, perfectly well at the time of the interview (i.e. not suffering from 'flu', 'cold', upper respiratory tract infections, diarrhoea, discharge, etc. for at least one month prior to the interview) were selected. History of past diseases was elicited.

2.3.4 Parity and condition of offspring

The number of pregnancies, miscarriages, stillbirths and the condition

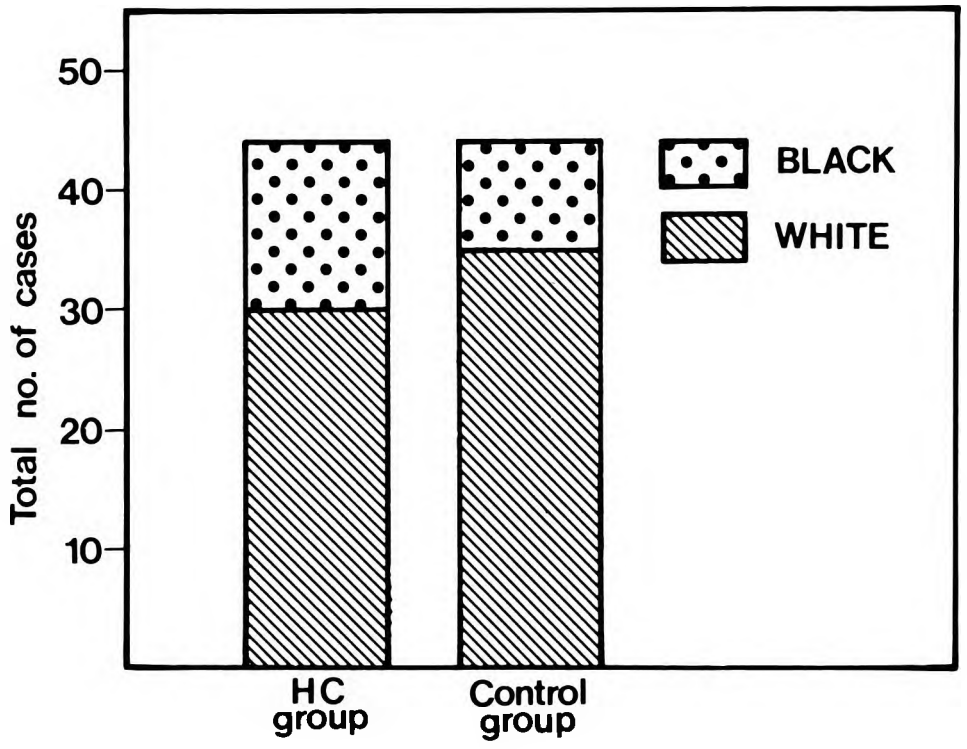


Figure 2.1. Race distribution of both study and control groups.

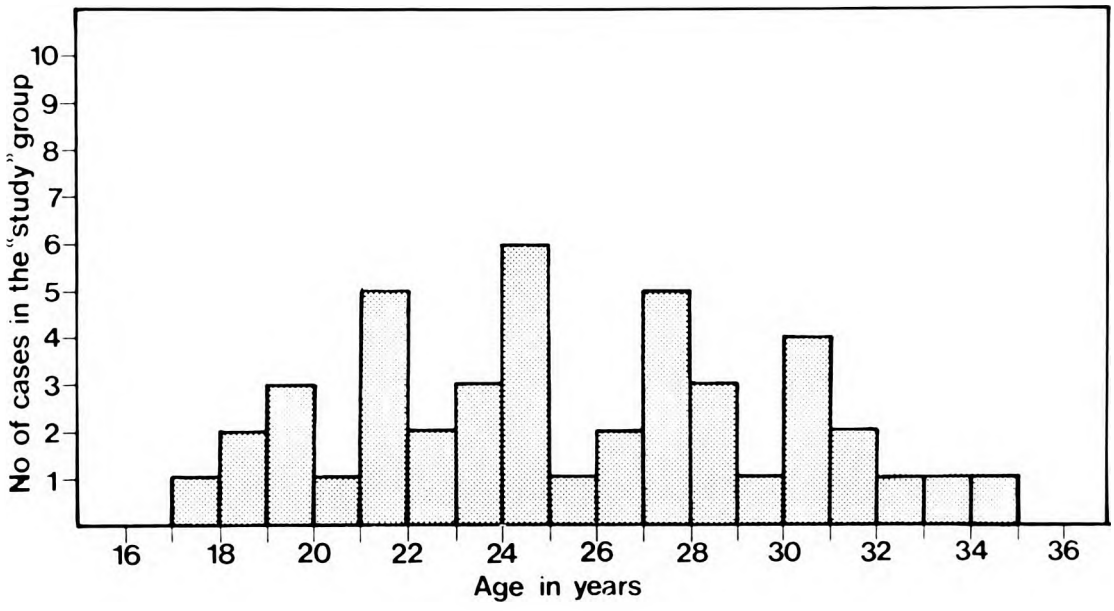


Figure 2.2. Age distribution of the study group.

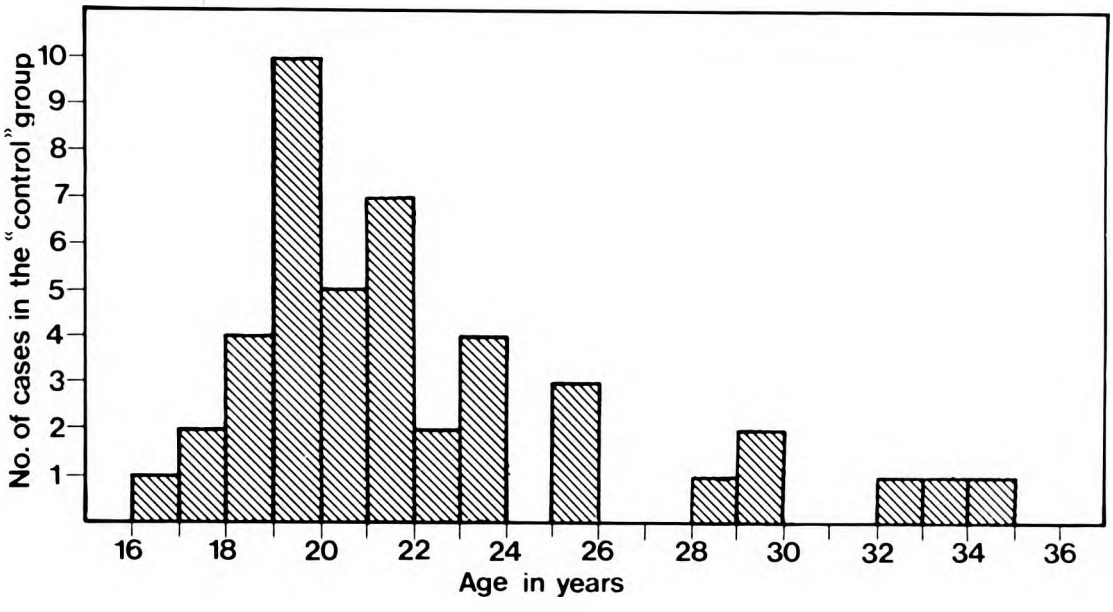


Figure 2.3. Age distribution of the control group.

of live children were noted.

There were no cases of multiple miscarriages, stillbirths or abnormal offspring. The number of children born to the subjects varied from zero to five (Figures 2.4 and 2.5).

2.3.5 Occupation

All women were questioned as to exposure during work to dangerous industrial chemicals (i.e. benzene, vinyl chloride, arsenic, etc.) and to radiation in the medical or industrial fields. Any woman with a history of exposure to any of these agents was excluded from the study.

2.3.6 History of exposure to X-rays or other types of radiation (i.e. neutrons, radioisotopes)

Information in this regard was collected. All cases with an history of recent exposure to radiation, i.e. less than one month prior to the interview, and all cases with an history of exposure to radiation other than routine chest or teeth X-rays, were excluded.

2.3.7 Drugs

Certain drugs have been incriminated as to their capacity to induce chromosome aberrations (see Section 2.13), so the question, "What drugs are you taking?" was also included in the questionnaire. Any subject taking a drug which is thought to induce chromosome breakages was excluded from the study. An exception was the use of antibiotics when taken in usual dosages and for the usual periods of time (usual dosages and usual periods of treatment were arbitrarily established as ± 2 000 mg of antibiotic/day for ± 10-14 days). Such individuals were accepted into the study, provided that the course of antibiotic

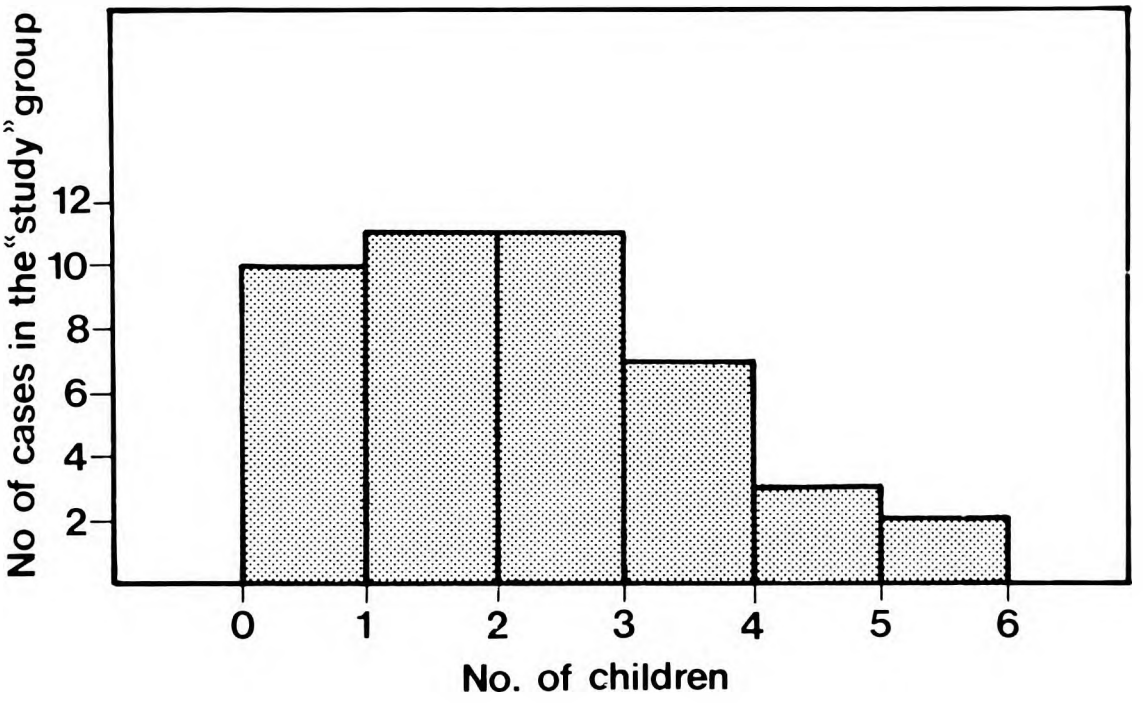


Figure 2.4. Distribution of the number of children born to women of the study group.

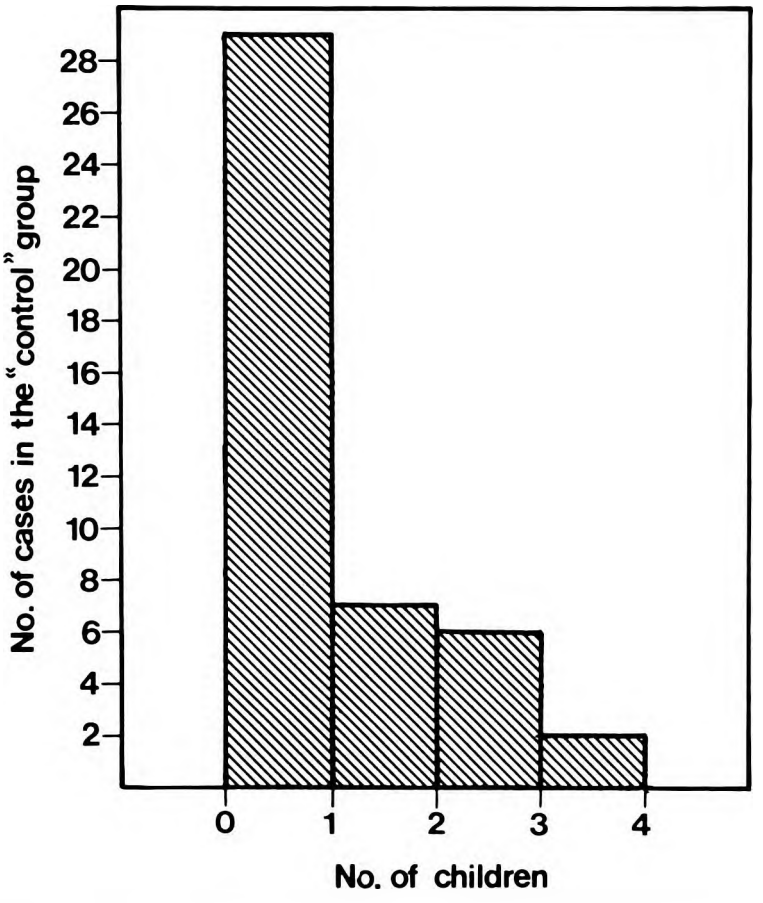


Figure 2.5. Distribution of the number of children born to women of the control group.

therapy had been completed at least one month prior to the collection of the blood sample for the present study.

This interval of time was established on a purely empirical basis and will be discussed later (see Section 2.16).

2.3.8 Immunizations

Women submitted to vaccinations within one month (again established on an empirical basis) prior to the interview were excluded from this study.

2.3.9 Smoking habits

It was decided to assess the smoking habits only after the study had been underway for some time (see Figure 2.6).

All the information listed above, with the exception of smoking habits, was essential before deciding on the suitability of a woman as part of the present study and there was, as a result, great difficulty in finding adequate numbers of subjects.

2.4 'Control group'

An essential requirement in every member of the control group was that hormonal contraceptives had never been administered in the past. Several cases were rejected even when the hormonal contraceptives were taken for only a few days, or taken a long time previously for treatment of irregular menstrual periods or dysmenorrhoea.

2.5 'Study group'

In subjects judged to be suitable to form part of the 'study' group

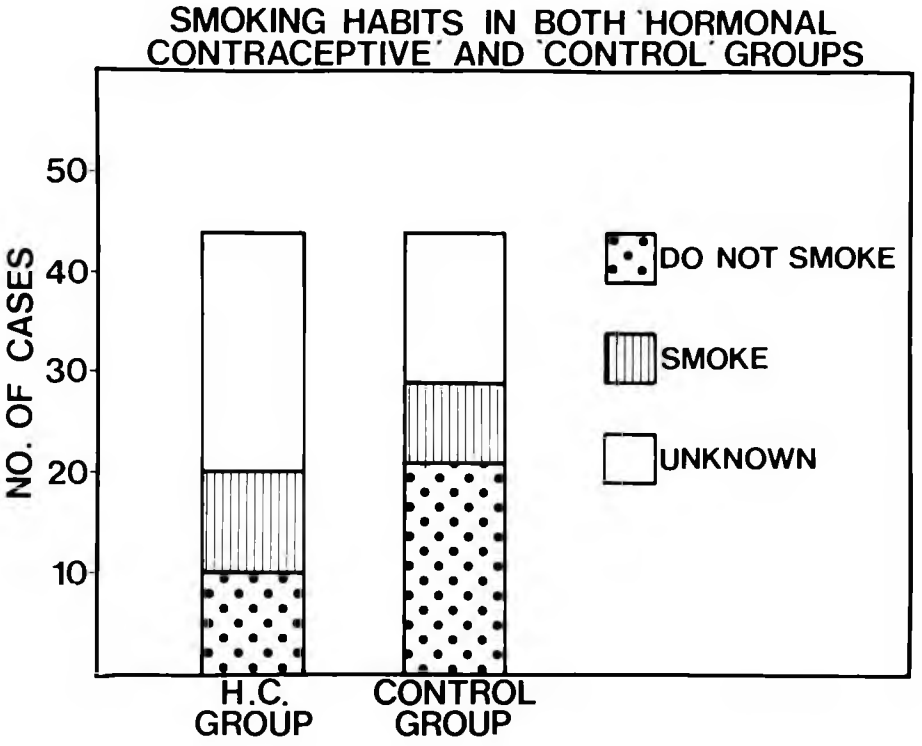


Figure 2.6. Smoking habits of the hormonal contraceptive group compared to those of the control group.

the following questions were asked:-

1. How long had hormonal contraception been used?
2. What was the name or names (where more than one had been utilized) of the hormonal contraceptives used?
3. Were there any breaks in administration during the use of such hormonal contraceptives and what was the duration of such breaks?

2.5.1 Duration of usage of hormonal contraceptives

The duration of the use of hormonal contraceptives ranged between seven months and ninety-eight months with a mean of 38,1 months (see Figure 2.7).

2.5.2 Intervals between the usage of hormonal contraceptives

In fifteen cases, intervals when the hormonal contraceptives were discontinued were reported, varied from one to sixty months. Pregnancies and resulting discontinuation of the use of hormonal contraceptives were reported in four cases.

2.5.3 Types of hormonal contraceptives involved in the present study

It was always difficult to obtain suitable subjects in this study and time was an important factor in the preparation of this thesis. Thus it was not possible to concentrate on women taking any specific hormonal contraceptive, but use had to be made of what was currently available.

Several types of hormonal contraceptives had been used by the

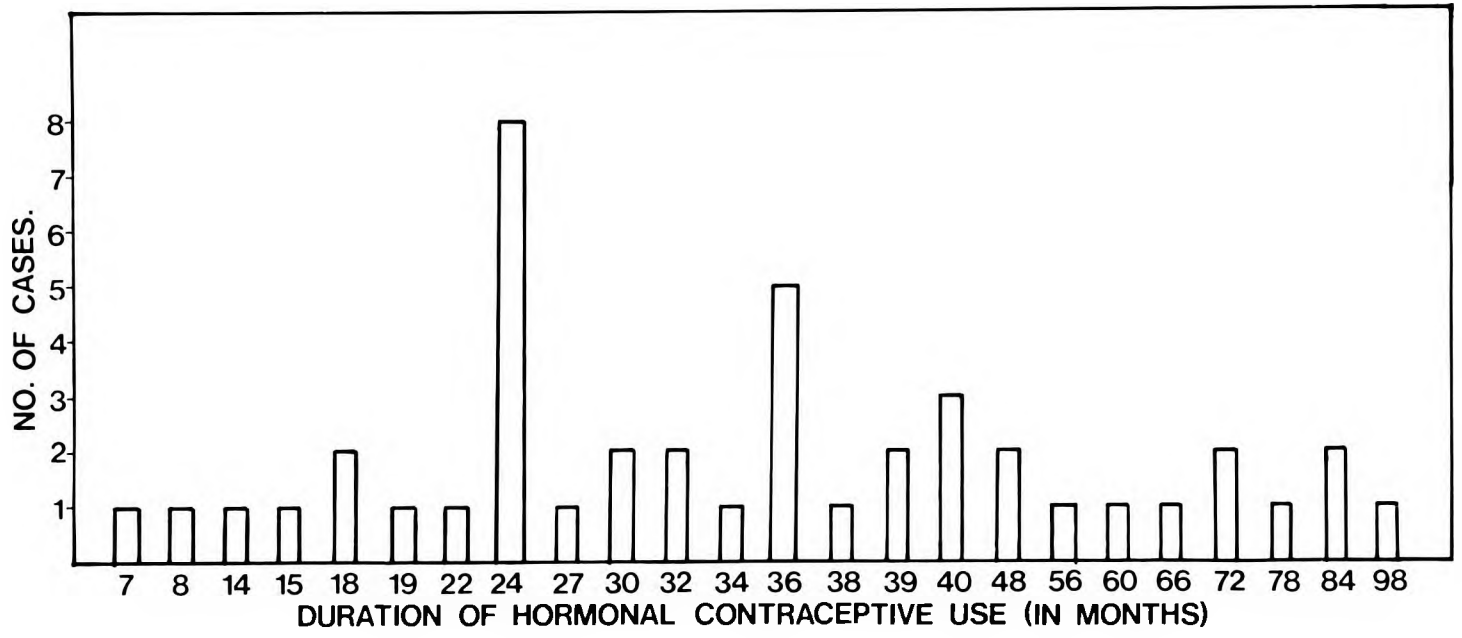


Figure 2.7. Distribution of study group cases according to the duration of hormonal contraceptive usage.

women participating in the present study. They were:

Minovlar ED,
Nordiol,
Depo-Provera,
Serial,
Ovulen,
Nordette,
Unigynon,
Gysilon,
Micro-Novum,
Ovral,
Normovlar,
Lyndiol 2.5,
Gynovlar,
Norinyl.

Table 2.1 lists the hormonal contraceptives used by the commercial name, as well as other relevant data, including chemical composition and pharmaceutical company. The frequency of use of each drug or combination of drugs are shown in Figure 2.8.

2.6 Hormonal contraceptives

2.6.1 Introduction

Although much is still unknown about contraceptive steroids, certain facts are clear. They are not natural substances and should not be identified as such.

Whether administered singly or in combination, regardless of dosage, route or frequency of administration, it must be remembered that they are synthetic compounds with multiple actions. Therefore their effects cannot be equated with pregnancy or with phases of

TABLE 2.1. Information on relevant data on the hormonal contraceptives encountered in the present study

Commercial name	Chemical composition			Route of administration	Pharmaceutical Company
Minovlar ED	Norethisterone acetate Ethinyl oestradiol	1 mg 0,05 mg		Oral	Schering A.G. Berlin/Bergkamen
Nordiol	d-Norgestrel Ethinyl oestradiol	250 mcg 50 mcg		Oral	Wyeth
Depo-Provera	Medroxyprogesterone acetate	150 mg/ml		Intramuscular	Upjohn
Serial	Ethinyl oestradiol Ethinyl oestradiol plus megestrol acetate	0,1 mg 0,1 mg 1 mg	(16 tablets) (5 tablets)	Oral	Allen and Hanburys
Ovulen	Ethynodiol diacetate Mestranol	1 mg 0,1 mg		Oral	Searle
Nordette	d-Norgestrel Ethinyl oestradiol	150 mcg 30 mcg		Oral	Wyeth
Unigynon	Untraceable			Oral	Untraceable
Gysilon	Untraceable			Oral	Untraceable
Micro-Novum	Noretherone	0,35 mg		Oral	Ethnor
Ovral	Norgestrel Ethinyl oestradiol	0.5 mg 0,05 mg		Oral	Wyeth
Normovlar	Levonorgestrel Ethinyl oestradiol Levonorgestrel Ethinyl oestradiol	0,05 mg) 0,05 mg) 0,125 mg) 0,05 mg)	(11 tablets) (10 tablets)	Oral	Schering A.G. Berlin/Bergkamen
Lyndiol 2.5	Lynestrenol Ethinyl oestradiol	2,5 mg 0,05 mg		Oral	Organon
Gynovlar	Norethisterone acetate Ethinyl oestradiol	3 mg 0,05 mg		Oral	Schering A.G. Berlin/Bergkamen
Norinyl	Norethisterone Mestranol	1 mg 0,05 mg		Oral	Syntex Pharmaceuticals

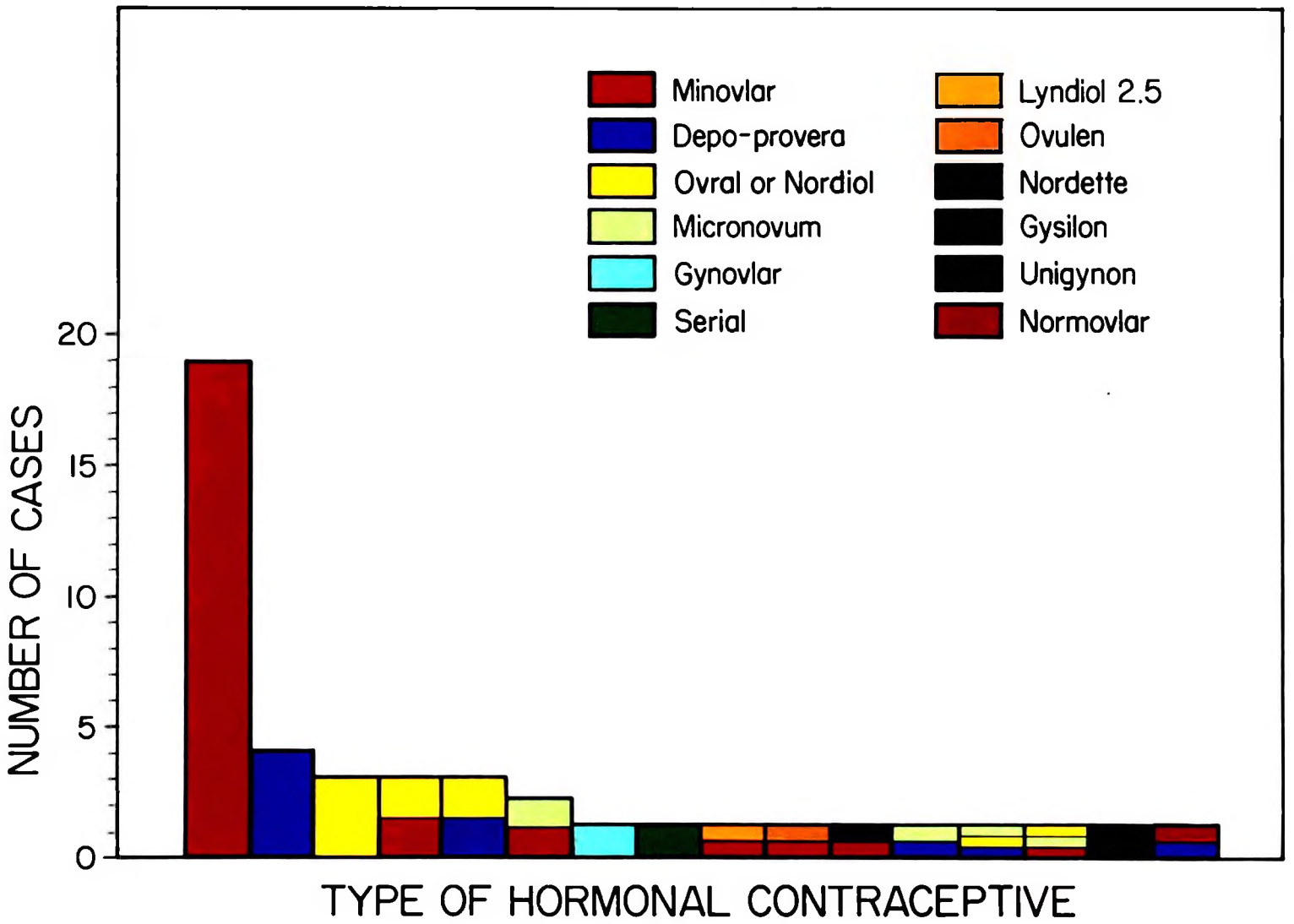


Figure 2.8. Frequency of usage of the different types of hormonal contraceptives either singly or in combination.

the menstrual cycle. Contraceptive steroids induce a pharmacologic state, not a physiologic one (Speroff, Glass and Kase, 1978).

There were originally three main categories of steroid contraceptives: combination, sequential and daily gestagen types (Briggs, 1978; Mishell, 1978).

The combination type, the most widely used and most effective, consists of a combination of an oestrogen and a gestagen given continuously for three weeks.

The sequential type, not so much in use at present and taken off the market in certain countries, consists of oestrogen alone for two weeks followed by a combination of oestrogen and gestagen for another week. The third type consists of a small dose of gestagen ingested every day without oestrogen. More recently the so-called long-acting depot products such as medroxyprogesterone acetate or norethindrone enanthate have appeared on the market.

The oestrogen compounds present in all types of contraceptive pills are either ethinyl oestradiol or mestranol (ethinyl oestradiol-3-methyl-ether).

The gestagen or progestin compounds present in contraceptive steroids are either derivatives of 19-nortestosterone or derivatives of 17-α-acetoxy progesterone. Singly or in combinations these various compounds provide the basis of all the different formulations of hormonal contraceptives at present available.

2.6.2 Mechanism of action

The main mechanism of action of the combination pill is a blocking

of the normal hypothalamo-hypophyseal secretions and therefore a prevention of ovulation.

The progestational compound suppresses LH secretion by a negative feedback action on the hypothalamus while the oestrogen component suppresses FSH secretion by a similar mechanism. The effects of the progestational agent always takes precedence over oestrogen; apart from this LH inhibitor effect it also exerts an action on the endometrium leading to atrophy and a nonreceptive endometrium for egg implantation (Speroff, 1976; Briggs, 1978).

The cervical mucus under the influence of the progestational agent also becomes thicker in consistency and scanty, thus retarding sperm penetration. It is also possible that the progestational agent alters the mobility of the muscle of the uterus and oviduct and therefore influences the egg and sperm movements.

On the other hand, the oestrogen compound of the pill serves two other important purposes apart from FSH inhibition. It provides stability to the endometrium preventing break-through bleeding, and potentiates the action of the progestational compound.

2.6.3 Metabolic effects

As a systemic drug reaching all the organs in the body, hormonal contraceptives have many other actions apart from preventing ovulation. These effects can be classified, for convenience, into three main categories:

1. Effects on female reproductive organs.
2. General metabolic effects.
3. Effects on other organ systems.

2.6.3.1 Effects on female reproductive organs

In this category are included the stromal fibrosis of the ovary, endometrial atrophy, polypoid hyperplasia of endocervical glands and increase in vaginal infections. Breast tenderness and reduction in milk production are also included in this category (Mishell, 1978).

2.6.3.2 General metabolic effects

As far as general metabolic effects are concerned 'the pill' can induce some degree of insulin resistance giving an impaired oral glucose tolerance test in about forty per cent of women (Speroff, Glass and Kase, 1978). There are also possible alterations in the metabolism of lipids (increase in serum triglyceride and cholesterol levels) (Briggs, 1978) and serum proteins (decrease in blood amino acid levels) (Briggs, 1978; Mishell, 1978). An increase in globulins, blood coagulation factors, carrier proteins and cortisol has also been noted (Mishell, 1978). The changes in protein have been shown to be responsible for thromboembolic disease and hypertension known to be associated with the use of hormonal contraceptives (Mishell, 1978).

Changes in water and electrolyte metabolism are responsible for the oedema and associated weight gain in certain women (Mishell, 1978). Alterations in tryptophan metabolism are responsible for the neuropsychiatric symptoms noted in oral contraceptive users (Mishell, 1978). Vitamin and mineral alterations have also been noticed in oral contraceptive users (Briggs, 1978).

2.6.3.3. Effects on other organ systems

In this category are included the effects on liver which range from asymptomatic abnormal liver function tests and jaundice, to benign or malignant tumours (Nissen *et al.*, 1978); Mishell, 1978). Central nervous system effects include nausea, vomiting and migraine headaches, to cerebral vascular accidents (Mishell, 1978). Skin pigmentation problems, adverse ophthalmological effects, possible increase in incidence of mesenteric thrombosis and ulcerative colitis have also been described (Mishell, 1978). Gall bladder disease is also associated with the use of oral contraceptives (Editorial, Br Med J, 1981).

A high incidence of genitourinary infections and viral infections (Mishell, 1978; Swan, 1981) can be related to the alterations of the immune mechanism noticed in hormonal contraceptive users (Mishell, 1978).

2.7 Discussion of materials

2.7.1 Number of subjects included in the study

Criticism may be levelled at the apparently small number of individuals from whom specimens were collected over a period of twenty-two months. When the project was planned, no difficulty was envisaged in recruiting large numbers of women on hormonal contraceptives and normal matched controls into the study. The author was led to believe that many subjects attended the two Family Planning Clinics each week (see Section 2.2). However both clinics only functioned on a part-time basis with identical time tables, which precluded the collection of samples from both clinics on the same day. Furthermore the chromosome culture time requirements (see Section 3.2.3) and specified criteria

for immediate processing of samples (see Section 3.2.1), only allowed for collection of specimens once a week.

Further difficulty in recruiting subjects was encountered when individuals were interviewed; it was soon realized that only a small proportion of women could be accepted into the study because the majority did not satisfy the stringent criteria for their inclusion (see Section 2.3). These selection criteria were deemed to be one of the most important aspects of the study. A certain number of women also refused to give blood for research purposes.

In summary, collection of material for this study turned out to be a difficult task; there were weeks when not a single blood sample could be collected and the maximum number of study and control specimens collected in a single week was only four!

2.7.2 Race and chromosomes

In a multiracial society it would not be realistic to study a sample from only a single population group. This was the reason why two different races, i.e. Caucasoid and Negroid were sampled.

As a general rule it is a fact that in South Africa the socio-economic conditions of the Negroid population are lower than those of the Caucasoid population. Poorer socio-economic conditions have irrevocably led to less adequate nutrition and have created a susceptibility to and a more frequent history of viral and infectious diseases.

(continued on page 26)

On the other hand, a more sophisticated White population would be more exposed to the advantages or disadvantages of a modern society. These imply a greater exposure to drugs and X-rays and a higher rate of smoking.

The question was: Is there any difference in chromosome breakage and/or DNA repair in these two population groups? This question will be considered later in the light of the results obtained (see Section 7.15).

2.7.3 Age and chromosomes

Variations in karyotype in phenotypically normal persons with increase of age are known (Ford, 1973). The change usually consists of a loss of a single chromosome. In women, the process starts generally in the sixth decade of life and, eventually, up to seven per cent of cells may have only forty-five chromosomes. The missing chromosome is almost certainly the inactive X chromosome of the cell, since experience has shown that cells which are monosomic for an autosome do not usually survive (Ford, 1973).

The knowledge of these age-dependent chromosome aberrations, together with early menopause hormonal variations (Speroff, Glass and Kase, 1978) that certain women present led to the establishment of an upper limit of 35 years of age for this study. This was to avoid such age dependent variations.

The lower limit of twenty years was set in the initial belief that it would be difficult to locate women younger than twenty years of age taking hormonal contraceptives. This belief was soon shown to be erroneous! It was not, at first, realized how difficult it

would be to find controls above the age of twenty! The age limit was then lowered to sixteen years of age. All the cases below this limit were rejected because of the possible hormonal imbalance sometimes present in the first years after the onset of puberty (Speroff, Glass and Kase, 1978).

2.7.4 Diseases and chromosomes

In this regard, careful questions were devoted to viral diseases, as they are considered to be mutagenic agents producing chromosome aberrations. The subject is reviewed by Harnden (1974) and Makino (1975).

Among other diseases, meningitis, smallpox, chickenpox, measles and German measles are said to produce chromosome aberrations that generally do not persist beyond the acute phase of the disease (Harnden, 1974; Makino, 1975). In the group of viral diseases, hepatitis seems to produce the worst virus-induced chromosome aberrations, and these can persist for several months - up to six months (Mella, 1968). For this reason cases with a previous history of hepatitis were excluded.

Patients who had suffered from measles, chickenpox, mumps, German measles (the commonest childrens' viral diseases) were included in the study, provided that the disease was contracted at least some years previously, generally in childhood, to ensure a complete DNA repair of the possible chromosomal abnormalities produced by the aetiological virus.

Subjects suffering from chronic diseases (i.e. heart, kidney or endocrine diseases, rheumatic fever, chronic arthritis, etc.)

were excluded from this study on the basis of the long-term treatment many were receiving or had received, and not on the basis of the disease itself. A few cases were rejected on these grounds, namely, patients with rheumatic fever taking antibiotics prophylactically, patients with hypothyroidism and several patients suffering from severe acne on long-term antibiotic therapy.

2.7.5 Parity and chromosomes

The writer's daily work in a cytogenetics laboratory of a Human Genetics Department shows that frequently multiple miscarriages, stillbirths and abnormal children, single or in association, are indicative of a balanced carrier parent for a chromosome translocation abnormality or of a carrier state for a chromosome breakage syndrome, i.e. Fanconi's anaemia. For these reasons questions on pregnancies, parity and condition of offspring were included in the questionnaire but no cases had to be rejected on any of these grounds.

2.7.6 Occupation and chromosomes

The reports of chromosomal abnormalities in chemically-exposed workers are quite recent. The first studies were carried out on benzene-exposed workers by Pollini and Colombi (1964a and b).

Their work, although done in the very early days of cytogenetics, showed numerical and structural chromosome abnormalities in peripheral blood of six patients with aplastic anaemia induced by benzol, and in bone marrow samples from patients with severe marrow aplasia due to benzol. Three further articles published by Forni *et al.* (1966, 1967, 1971) confirmed the results of Pollini and Colombi. In the first work (1966) peripheral blood and occasionally bone marrows were

studied chromosomally in three groups of workers: (1) workers who had suffered from benzene-induced anaemia, (2) workers chronically exposed to inhalation of benzene without any signs of anaemia and (3) controls. Both groups (1) and (2) showed increased stable and unstable chromosome abnormalities which persisted for years after the exposure to benzene had ceased. The second study in 1967 was performed on a case of benzene anaemia evolving into acute myeloblastic leukaemia on which serial cytogenetic studies were carried out and multiple chromosome aberrations found. The possible mechanism of induction of leukaemia by benzene was suggested to be the multiple chromosome aberrations. The third study in 1971 was done on ten workers exposed to benzene and toluene and twenty-four exposed to only toluene. A careful choice of thirty-four controls matched for age and sex was done and the proportion of unstable and stable abnormaltities were significantly higher in the benzene group.

Further work by Tough *et al.* (1965, 1970) confirmed the excess of chromosome aberrations found by previous workers, and stressed the importance of a correct selection of controls due to the multitude of confounding factors. Surveys have also been carried out in industrial populations exposed to other chemicals. It is reported by Ducatman *et al.* (1975) that vinyl chloride induces chromosome aberrations, particularly of the unstable type. In a study conducted on eleven vinyl chloride polymerization workers, the number of chromosome abnormalities found was significantly higher than those found in ten healthy male controls. In the same year, Funes Cravioto *et al.* (1975), reported the same findings in a limited study of ten subjects (seven males exposed to vinyl chloride and three controls). But the largest series on the effects of vinyl chloride on chromosomes

came from a well conducted study by Purchase *et al.* (1975) in which fifty-six workers and twenty-four controls were cytogenetically investigated. The results obtained confirmed the previously reported findings of the clastogenic effects of vinyl chloride. Picciano *et al.* (1977) did not confirm these findings however. In a study of 209 workers exposed to vinyl chloride for various periods of time, they found that when the cytogenetic data obtained from the groups of exposed workers were compared with the data from controls, there were no significant differences. The authors stressed, however, that their 'study' group had been exposed to relatively low levels of vinyl chloride, and that may have a bearing on the results. Epichlrohydin (ECHH), an alkylating agent widely used in industry as a solvent for resins, in high occupational doses, has also been incriminated as an inducer of chromosome aberrations, particularly chromatid and chromosome breaks. This study was conducted by Kučerová and Zhurkov (1977) in thirty-five workers occupationally exposed to high doses of ECHH; blood for cytogenetic studies was collected before exposure (controls) and after one year and two years of exposure.

Arsenic has also been reported to be a clastogenic agent in a few studies. Petres *et al.* (1970) found an increased frequency of chromosome aberrations in wine growers exposed to arsenic as a pesticide and also on psoriatic patients treated with arsenic. Beckman *et al.* (1979) have carried out cytogenetic studies on a series of workers from Rönnskär smelter in Sweden who had been exposed to arsenic in various occupational and environmental situations. When the cytogenetic data from the exposed population were compared with controls from a different area, it was shown that a significant

increase in chromosome abnormalities was present, although no correlation could be made between the frequency of abnormalities and degree of arsenic exposure. In this study it seemed that there may be an interaction between cigarette smoking and arsenic exposure.

In the radiation field adequate protection exists to provide safety measures for workers subjected to occupational radiation exposure. There are internationally accepted maximum permissible levels to which such workers may be exposed. Evans *et al.* (1979) have nonetheless recently found a significant increase in chromosome damage in nuclear dockyard workers who had been exposed to doses well below the internationally agreed maximum levels. They found, in a population of 197 men who were studied cytogenetically at the time of the 'preclassification' examination (controls) and afterwards annually for a period of ten years, that the frequency of the aberrations was a linear function of dose, and was influenced by age and time of blood sampling after exposure.

Other studies (Evans *et al.*, 1967) on chromosomes of lymphocytes from people exposed to ionizing radiation have also revealed increased aberration yields after *in vivo* exposure to relatively low doses of X-rays, γ-rays or fast neutrons. A study conducted on workers at a UK Atomic Energy Establishment showed that the workers exposed to mixed neutron-γ radiation at levels below the safety standards had a significant excess of dicentric and ring chromosomes in comparison with on-site controls (Evans *et al.*, 1967). However, in this study no quantitative dose-effect relationship was evident, possibly due to inadequacy of control data, a varying pattern of dose accumulation between individuals, too small a number of subjects or too few cells scored (Evans *et al.*, 1979).

These same considerations are probably very valid in trying to explain the poor agreement between the results reported by different workers when testing for the clastogenic effects of a specific chemical or physical agent.

2.7.7 Radiation and chromosomes

The damaging effects of radiation on chromosomes are well documented (Makino, 1975; Bauchinger, 1978), and to such an extent that chromosome aberrations have been suggested as a biological indicator and monitoring method of the effects of radiation (Komarov, 1973).

It was Bender and Gooch in 1962 who suggested, for the first time, that one effective way of determining whether an individual has been exposed to ionizing radiation was to look for an increase in chromosome abnormalities in his lymphocytes (Bauchinger, 1978).

Studies from different laboratories have shown that the aberration yield in human lymphocytes can serve as a quantitative indicator of the radiation dose, and also that the behaviour of the irradiated lymphocyte *in vitro* and *in vivo* are quite similar. These two abovementioned findings led to the standardization of 'dose response curves' - 'calibration curves' which make it possible to determine the dose received based on the frequency of the chromosome abnormalities found (Bauchinger, 1978). These curves have, however, one disadvantage in that the dose estimation is based on the yield of certain kinds of chromosome aberrations, e.g. dicentrics and rings which are particularly increased at high doses of radiation. This poses a problem for the low dose ranges, particularly in occupational exposures, in that a significant increase in the yield of dicentrics

and rings may not be detected. Meanwhile, more studies have been recommended before the use of chromosome aberration yield as a biological dosimeter can be accepted without question (Dolphin, 1978; Bauchinger, 1978). In particular, the need to record other types of chromosome abnormalities, apart from dicentrics and rings, was stressed, and also the need for further studies between radiosensitivity and response to PHA stimulation.

The fact that radiation has been identified as a confounding factor that can increase chromosome aberration levels influenced the writer in selecting subjects who have not been exposed to diagnostic (for exceptions, see Section 2.3.6), therapeutic or occupational radiation.

2.7.8 Drugs and chromosomes

Sulphur mustard [Bis(2-chlormethyl)sulphide] was one of the first drugs to be found to produce chromosome aberrations (Fishbein *et al.*, 1970). This compound, first synthesized in 1822, was the first of the alkylating agents to be used for therapeutic purposes, and this was largely during World War I. Since then, considerable scientific and medical interest has arisen concerning other therapeutic drugs and their possible capacity to produce chromosome damage.

Antibiotic and antineoplastic agents have been, among other drugs, those on which more extensive investigation has been carried out. A possible explanation for this choice is probably the fact that the action of many of these drugs is directed through the DNA and RNA and thus likely to cause chromosome damage (Balson and Roberts, 1977).

Mitomycin C (Cohen and Shaw, 1964; Nowell, 1964), streptonigrin (Cohen, 1963) and phleomycin (Jacobs *et al.*, 1969) have all been

found to produce breaks *in vitro* even at low doses. Apart from causing breakage, both mitomycin C and phleomycin (Jacobs *et al.*, 1969) were shown to cause mitotic inhibition as well. In Nowell's (1964) work with mitomycin C it was shown that the number of chromosome aberrations was dependent on the length of time the cells were incubated with mitomycin C.

In Jacobs' work (1969) on phleomycin, both mitotic inhibition and chromosome breakage increased as the phleomycin concentration increased.

Daunomycin, an antibiotic also used in leukaemias and certain solid tumours was proved to be capable of producing chromosomal aberrations *in vitro*. Vig *et al.* (1968) treated cultured human lymphocytes with different concentrations of daunomycin incorporated at different times of the cell cycle. In this work the aberration production was apparently dependent on the concentration of daunomycin used rather than on the duration of exposure. Further *in vitro* work done by the same authors (Vig *et al.*, 1969) confirmed the previous findings and, furthermore, concluded that G_1 is the sensitive phase of the cell cycle to daunomycin. Theologides *et al.* (1968) also quotes daunomycin as an inhibitor of cell growth and a clastogenic agent on both normal and tumour cells growing *in vitro*. Other antibiotics such as streptomycin, chloramphenicol and tetracyclines have been reported as not having a chromosome breaking effect on human chromosomes (Shaw, 1970).

However, chloramphenicol at concentrations of 10-40 µg/mℓ has been reported to cause chromosome aberrations *in vitro* by Mitus and Coleman (1970). These results were, however, disputed by Jensen

(1972) who did not find any *in vitro* effect on human lymphocytes nor *in vivo* effect on human bone marrow cells.

In the field of antitumour drugs, clastogenic effects have been proved in the case of certain of these. Cyclophosphamide has been shown by Hampel *et al*. (1969) to produce nonrandom chromosome aberrations in *in vitro* studies. *In vivo* studies done on children with nephrosis (Fisher *et al*., 1978) who received cyclophosphamide at doses of 75 mgm^{-2} for one and one half to six months, showed a small but insignificant increase in the number of chromatid aberrations. Myleran (Gebhart, 1969) *in vitro* is capable of producing gap, chromatid and chromosome type aberrations which are nonrandom and dose related.

Bleomycin has been studied by Bornstein *et al*. (1971) *in vivo* in four patients with cancer. Bone marrow chromosome studies were done prior, during and after treatment with bleomycin. An increase of chromosome breakage was noted after the treatment and this persisted for about one month.

Methotrexate (amethopterin), a chemotherapeutic agent, used in many neoplasms has been the subject of dispute as to its capacity to induce chromosome aberrations in different human tissues. Hampel *et al*. (1966) in a study of the *in vitro* effects of several cytostatic drugs on human lymphocytes, reported negative effects when methotrexate was added directly to the cultures. This negative effect on chromosomes was confirmed by Fisher *et al*. (1978) in their study of the cytogenetic effects of chemotherapy and chemotherapy combined with partial body irradiation on peripheral blood lymphocytes of children with leukaemia.

However, Jansen (1967) reported clastogenic effects on bone marrow cells from a small sample of four patients treated with methotrexate for psoriasis. Bone marrow metaphases were analyzed before and after the treatment and a significant increase of chromatid and chromosome breaks as well as acentric fragments were found.

Anticonvulsant drugs have also been investigated for chromosome damaging effect. Brøgger (1970) has not found an increase of chromosome abnormalities in patients treated with phenytoin and ethotoin when compared with controls. These results were not confirmed by Roman and Caratzali (1971) in a study done on rats, where the clastogenic effect of phenytoin was demonstrated in bone marrow cells of rats treated with the above-mentioned drug. However, these chromosome aberrations were not found in the same rats seven days after treatment had stopped. Psychotrophic drugs, especially lysergic acid diethylamide (LSD) have also been submitted to cytogenetic studies. After the initial report of Cohen *et al.* (1967), where increased chromosome breakage was attributed to LSD, several additional reports were published on chromosome breakage and the use of LSD. Some of these reports gave positive results and some negative results as to the clastogenic effect of LSD. It soon became apparent that these conflicting results were partially due to, amongst other factors, the fact that the LSD users are usually on multiple drugs, and because the LSD consumed was obtained from illicit sources and therefore the purity and dosage was very difficult to control. In addition, the LSD users were often malnourished and had a high incidence of viral infections. Further studies done *in vivo*, where LSD was administered under controlled conditions and medical supervision (Hungerford *et al.*, 1968; Tjio *et al.*, 1969), still gave contradictory results,

but the difference between the amount of chromosome abnormalities found in users and control groups was much smaller than initially reported.

Diazepam, one of the more commonly used tranquilizers was found by Staiger (1969) and Stenchever and Frankel (1969) not to have any damaging effects on human chromosomes in an *in vitro* study where dosage and varying time exposure were varied.

Aspirin, the most widely used analgesic drug worldwide was investigated by Mauer *et al.* (1970) who found no increase in chromosome damage in an *in vitro* study performed on human lymphocytes where different doses of acetylsalicylic acid were added to the cultures. Loughman (1970) however, found a significant increase of chromatid gaps when acetylsalicylic acid was added to the cultures at a concentration of 6,5 µg/mℓ.

Long term fibroblast cultures were also subjected to different doses of aspirin in work done by Meisner and Inhorn (1972). In this work chromosome rearrangements were seen when aspirin was added at high concentrations of 100 and 250 µg/mℓ. In the selection of subjects for this study, many had taken occasional tablets of aspirin, but none were taking this substance for long periods of time or in heavy doses.

Although alcoholism is a problem of our society and there are reports on chromosome damage in chronic alcohol users (Obe and Herha, 1975), alcohol intake was not queried as it is very difficult to obtain a true answer on such a delicate question, especially in females. In any case, the study conducted by Obe and Herha was done on excessive alcohol drinkers, patients of a psychiatric clinic undergoing treatment for their alcoholism which could contribute to

the increase of chromosome damage found.

Finally, mention must be made of caffeine, a drug that is in almost universal use, in the medical field as well as in our daily life as an additive to beverages. Weinstein *et al.* (1972) showed no significant increase in chromosome damage in human lymphocytes from volunteers after a regimen of 800 mg of caffeine per day, taken orally for one month. In this *in vivo* study, the highest level of caffeine in the plasma was 30 µg/mℓ. It is interesting to note that in this same study, *in vitro* exposure of human lymphocytes showed chromosome damage when caffeine was added to the cultures at doses of 250-750 µg/mℓ.

No attempt was made in the present study to quantify the caffeine intake of different subjects. To the author it seemed an impossible task. In view of the multitude of commercially available beverages with various concentrations of caffeine, personal tastes and so on, it would be quite impossible to arrive at a reasonably accurate estimate of caffeine intake in any individual.

To the author's knowledge, no other study in which the effects of certain chemical, physical or viral agents on chromosomes were tested, took into account the caffeine habits of the individuals under study. One exception, logically, would be when the caffeine itself is the substance tested.

2.7.9 Immunizations and chromosomes

A great number of immunizations, i.e. rubella, measles, yellow fever and smallpox vaccines contain live atenuated virus. It has been proved that live virus vaccines can cause chromosome breakage *in vivo*

and *in vitro* (Knuutila *et al.*, 1976). Increased breakages have been found in patients during the period of viraemia after rubella vaccination (Knuutila *et al.*, 1976) and an increase in chromosome aberrations in human lymphocytes was also detected with measles and yellow fever vaccines (Makino, 1975).

Thus the information on recent vaccinations was included in the selection questionnaire.

2.7.10 Smoking habits and chromosomes

Purchase (1978) has identified cigarette smoking as a confounding factor in the cytogenetic study of a population exposed to a chemical agent. In his study on a population exposed to vinyl chloride, there was an increase of abnormalities associated with cigarette smoking which was not present in the control group.

This synergistic effect of smoking on chromosome breakage produced by chemicals has not been confirmed by Funes-Cravioto *et al.* (1978) in their study on laboratory and factory workers dealing with several chemical agents. On the other hand, chromosome aberrations in individuals who smoke heavily (40-60 cigarettes per day) have been described by Obe and Herha (1978). In their work, chromosome studies were performed on twenty smokers and the results were compared with controls. An impressively higher incidence of dicentrics, rings and chromatid interchanges were found in the group who smoked heavily.

These reports led the author to contact the subjects of this study in order to determine their smoking habits. Unfortunately information could not be obtained in all cases, as many of the women could not be contacted, either through the Family Planning Clinic or

at their home addresses.

2.7.11 General

After excluding all the unsuitable cases on the basis of the protocol presented, as dicussed in this chapter, the author was confronted with problems such as the common cold, flu or diarrhoea, trivial vaginal discharges, teeth and chest X-rays and the abused antibiotic therapy so common nowadays. To the author's knowledge no data on chromosome abnormalities induced by the latter agents has been reported, the only exception being certain antibiotics as discussed in Section 2.13.

In the face of these problems it seemed reasonable to the author to establish a 'safe period' of one month, free from disease and from exposure to chemical, physical or biological agents, prior to the blood collection.

Why one month and not one day, one week or one year? It must be emphasized that the 'safe period' of one month was established on a purely arbitrary basis, although the opinion of other cytogeneticists was consulted (Nelson, 1977; Bobrow, 1980). It was also based on the known fact that in a normal healthy person, the greater amount of damage that occurs in the genetic material can be successfully repaired in a matter of a few hours by the DNA repair mechanisms (Editorial, Lancet, 1977).

HUMAN STUDIES

CHAPTER 3

3. METHODS

3.1 Short-term peripheral blood cultures

Specimens of venous blood were collected from a 'study' subject and a matching 'control' on the same day.

Initially, macroperipheral blood culture (see Section 3.3) was used (ten cases), but due to reluctance on the part of certain subjects to donate what they considered too large an amount of blood for purely research purposes, microperipheral blood techniques (see Section 3.2) were introduced. The latter technique required less blood and was more acceptable to the subjects. It must be emphasized that exactly the same methods were used for matching study and control subjects whether macro- or microtechniques were used.

The principle of peripheral blood lymphocyte culture is based on the incubation of whole blood or buffy white cell layer in a suitable growth medium in the presence of a mitogenic agent (Priest, 1977). The mitogen acts by producing a lymphocyte 'blastoid' reaction with a subsequent short-lived burst of mitotic activity (Priest, 1977).

3.2 Microperipheral blood technique

3.2.1 Collection of peripheral blood

The skin was thoroughly cleansed with seventy per cent alcohol and allowed to dry properly. One to one and an half mℓ of venous blood was drawn into a sterile disposable plastic syringe containing 0.025 mℓ of 1 000 units per mℓ heparin (Boots Company). The syringe containing the blood and heparin was then gently shaken to ensure a perfect mixing for

effective anticoagulant action. The blood specimen was transported to the laboratory in the syringe, with the needle protected by the plastic needle cover to prevent contamination. The blood sample was processed on the day of collection.

3.2.2 Establishment of cultures

The processing of samples for culture was undertaken in an ultraviolet light box and all the materials used were sterile. Sterile disposable plastic syringes and needles were used throughout the processing of the bloods for culture.

Cultures were established in sterile glass McCartney bottles (30 mℓ in size). These were cleaned and sterilized by washing for several hours in Gill (antibacterial liquid soap), rinsing in running tap water, finally in distilled water and then heat sterilized at 180°C for thirty minutes.

At least two cultures were set up from each blood specimen. 0,3 Millilitres of heparinized blood was added to each of two sterile culture bottles, already containing 5 mℓ of culture medium M150 (modified medium TC199), 1 mℓ of foetal calf serum (Wellcome) and 0,02 mℓ of phytohaem-agglutinin P (Difco). The bottle tops were tightly sealed on the bottles and cultures were incubated at 37°C.

3.2.3 Incubation

The cultures were incubated at 37°C and were gently shaken once a day during the forty-eight hour incubation period.

3.2.4 Colchicine pretreatment

After forty-six hours incubation, colchicine, diluted in sterile water to give a final concentration of 0,04 µg/mℓ was added. One of each duplicate culture was incubated for a further two hours after addition of colchicine and the other for one and an half hours.

3.2.5 Harvesting procedure

After incubation with colchicine, the sedimented cells were suspended in the supernatant medium by means of shaking.

Two major steps were involved in the harvesting procedure:

I. Hypotonic pretreatment

The suspended cells were transferred to a glass centrifuge tube and centrifuged at ± 800 rpm for seven minutes. After centrifugation, the supernatant fluid was decanted, taking care that the cell button was not disturbed, and 10 mℓ of hypotonic solution (0,075 M KCl) at 37°C was slowly added.

The cells were gently resuspended in hypotonic solution and incubated at 37°C for ± twelve minutes. The suspension was centrifuged again for five minutes at 800 rpm (i.e. total time of seventeen minutes in hypotonic solution).

II. Fixation

The hypotonic supernatant was carefully decanted, leaving ± 0,5 mℓ of supernatant covering the cell suspension. Great care was taken so that the cells were not disturbed. Ten mℓ of freshly prepared fixative (consisting of three parts of

absolute methanol to one part of glacial acetic acid) was added drop by drop, with continuous agitation to keep the cells suspended for the first ± 2 mℓ. The cells were then left undisturbed in this first fixative at room temperature for fifteen minutes. This was followed by further centrifugation and decanting of the supernatant. Fixative was changed until the supernatant was clear and colourless. This needed either two or three further changes of fixative. The final amount of fixative varied from 1 to 2 mℓ, depending on the size of the cell button. A larger cell button required a correspondingly larger amount of fixative. The aim was to achieve a cell suspension that was neither too thick nor too thin, but of 'milky' consistency.

The final suspension was left undisturbed in the deep-freeze (± -4°C) for fifteen minutes.

3.2.6 Spreading of chromosomes

Meticulously clean slides are essential for good spreading. Slides were precleaned as follows:-

1. Prepacked 'water cleaned' new glass slides were placed in pure methanol overnight.
2. Slides were washed in running tap water, followed by washing in distilled water. The slides were then boiled in distilled water for two to three hours.
3. Slides were carefully dried with a clean cloth and placed in the deepfreeze (± -4°C) for at least half an hour and removed shortly before needed.

Four to five drops of cold cell suspension were dropped onto a clean, chilled slide, by means of a disposable Pasteur pipette (size 225 mm) held vertically from a standard height of 30 cm.

Two different techniques for the drying of slides were used:

1. Flame drying for a few seconds over a Bunsen burner flame intended for standard Giemsa stained slides.
2. Slow air-drying in a 60°C oven, overnight, intended for certain banding techniques.

3.2.7 Staining of chromosomes

Standard Giemsa stain.

Slides were stained for ten to fifteen minutes in commercial Giemsa (Gurr R66) stock solution, diluted to ten per cent in deionized distilled water, pH 7. The slides were rinsed in tap water and allowed to dry.

Different staining techniques were used in the various banding procedures:

a. After trypsin pretreatment (see Section 3.4.1) slides were stained in four per cent Giemsa in phosphate buffer, pH 6,8, for approximately five to ten minutes.
b. After urea pretreatment (see Section 3.4.2) slides were stained in four per cent Giemsa stain in phosphate buffer, pH 7, for eight to ten minutes.
c. After hydrolysis pretreatment (see Section 3.4.3) slides were stained in ten per cent Giemsa in phosphate buffer, pH 6,8, for about ten to fifteen minutes.

3.3 Macroperipheral blood technique

3.3.1 Collection of peripheral blood

After the same skin cleansing procedure used for the microperipheral blood technique (see Section 3.2) 10 mℓ of venous blood was drawn into a sterile disposable plastic syringe containing 0,25 mℓ of heparin (1 000 units/mℓ).

3.3.2 Establishment of cultures

Cultures were established in McCartney bottles containing 5 mℓ of culture medium M150 + 0,02 mℓ phytohaemagglutinin (no foetal calf serum was used), and 1 mℓ of buffy layer was added to each bottle. The buffy layer was obtained by centrifugation in a sterile tube, of the 10 mℓ heparinized blood sample at 800 rpm for ten minutes.

3.3.3 Incubation

The same as for microtechnique (see Section 3.2.3).

3.3.4 Colchicine pretreatment

The same as for microtechnique (see Section 3.2.4).

3.3.5 Harvesting procedure

The same as for microtechnique (see Section 3.2.5).

3.3.6 Spreading of chromosomes

The same as for microtechnique (see Section 3.2.6).

3.3.7 Staining of chromosomes

The same as for microtechnique (see Section 3.2.7).

3.4 Banding techniques

Three G-banding techniques were used:

1. Trypsin-Giemsa.
2. Urea.
3. Phosphate buffer hydrolysis.

1. Trypsin-Giemsa banding

This technique proved to be the most consistently reliable, producing chromosome banding of reasonably good quality. The technique used is based on modifications of Seabright's original method (Seabright, 1971), and Priest's modification (Priest, 1977) of Seabright's method.

Slides one day old or at most two days' old containing chromosome spreads obtained by slow air drying (see Section 3.2.6.2) were immersed in a 0,05% Trypsin solution in phosphate buffered saline, pH 6,8, at 37°C. The slides were kept in the prewarmed Trypsin solution for a period of time which varied between five to fifteen seconds. The slides were then rinsed thoroughly in a Coplin jar, containing 50 mℓ of phosphate buffered saline, pH 6,8, plus 2,5 mℓ of foetal calf serum (Wellcome or Gibco) and then given a second rinse in phosphate buffer saline, pH 6,8, without serum. Slides were then stained as described previously (see Section 3.2.7a).

2. Urea treatment technique for G-banding (Shiraishi and Yosida, 1972)

This technique gave less consistently successful results.

Air dried slides, aged seven to fourteen days, were incubated at 60°C overnight. Slides were then immersed in a mixture of three volumes of stock urea (8M) and one volume of Sörenson's buffer solution (M/15), pH 6,8, at 37°C for about ten minutes. Afterwards, slides were rinsed in tap water and dipped into the stain prepared as described previously (see Section 3.2.7b).

The main advantage of this method is that the same slide may be used over and over again if it is undertreated, by simply reimmersing directly in the urea without destaining.

3. G-banding by phosphate buffer hydrolysis (Chaudhuri *et al.*, 1971)

Air dried chromosome preparations were placed in Sörensen's phosphate buffer, pH 7,7, at 62°C for twenty-two hours. Slides were then stained as described previously (see Section 3.2.7c).

3.5 Special techniques

3.5.1 Sister chromatid exchanges (SCE)

A technique to demonstrate SCE was performed in ten cases, to compare the frequency of SCE between study and control groups. The mechanism of visualization of SCE will be discussed later (see Section 3.9.11).

The technique is based on the incorporation of BrdU (5-bromodeoxy-uridine) into replicating DNA through two cell cycles.

A 68 hour peripheral blood culture was established (6 mℓ of M150 + 3 mℓ of AB serum + 0,4 mℓ of PHA + 0,5 mℓ of whole blood), and BrdU in sterile distilled water at a final concentration of 30 µg/ml of culture, was added at the time of establishment of culture. The culture bottles were kept in the dark (wrapped in aluminium foil) during the incubation period and harvested in the usual manner (see Section 3.2.5) and flame dried slides were made as described previously (see Section 3.2.6.1). A fresh slide preparation, preferably less than one day old was stained for ten minutes in acridine-orange stain (AO) at 4°C - concentration 400 mgs per cent AO in phosphate buffer, pH 6,8. The slide was rinsed with tap water and then placed in phosphate buffer, pH 6,8, for ten minutes to remove the excess stain. The stained slide was mounted in the same buffer and the edges of the coverslip were sealed with clean nail varnish.

After screening and photography, the coverslip was carefully removed and the slide was stained in ten per cent Giemsa in phosphate buffer, pH 6,8, for ten minutes.

3.6 Photography of chromosomes

Metaphases were photographed with a Wild Photo automatic 35 mm camera attached either to a Reichert Zetopan or a Leitz Wetzler Dialux microscope, using a green filter or a combination of green and blue filters. Metaphases were photographed at a magnification of X1 000 (10X eyepiece and 100X oil immersion objective).

High contrast film (Kodak) at ASA 10 or Panatomic X (Kodak) at ASA 40, were used for photography of unbanded metaphases and SCEs

(Giemsa stained), but only High contrast film at ASA 10 was used for Giemsa banded metaphases.

Acridine-orange stained metaphases (SCE) were photographed with Panatomic X film at ASA 270.

The films were developed with suitable developers, enlarged and printed on photographic paper No.3 or No.4 depending on the degree of contrast required.

3.7 Recording of results

A 'blind' study was performed using coded slides only.

3.7.1 Screening of unbanded slides

Metaphases that appeared reasonably well-spread and complete under low magnification were selected for further study. Under X1 000 magnification the chromosome number was determined and only complete metaphases were recorded for the predetermined minimum number of thirty unbanded metaphases to be analyzed in each case. There were a few cases where less than thirty metaphases were available for analysis (see Sections 3.9.8 and 4.1).

The criteria for a complete metaphase were based on a modal number of forty-six, excluding chromosomal material without a centromere, i.e. acentric fragments. Rearranged chromosomes with more than one centromere were still scored as one chromosome regardless of their configuration. Therefore dicentrics and triradial or quadriradial rearrangements were still scored as one chromosome. The principle of only considering metaphases with forty-six chromosomes was not adhered

to if a chromosomal abnormality was detected during the initial chromosome counting. For instance, cells with the above rearrangements would, by definition have contained less than forty-six chromosomes (see Section 3.9.9).

After chromosome counting, chromosomes were grouped and then carefully screened for abnormalities. All discontinuities in a chromatid or chromosome, their size, their localization, alignment or nonalignment, deletions, acentric fragments, dicentrics, rings and rearrangements, were recorded. Photographs of abnormalities were

(continued on page 51)

taken only where identification of the aberration was uncertain.

3.7.2 Screening of banded slides

Where possible, ten complete G-banded metaphases, were photographed and karyotyped.

The only criteria used for selection of banded metaphases were completeness and reasonable banding quality.

3.7.3 Screening of slides for SCE frequency

In the ten cases where the frequency of SCEs was studied, ten complete metaphases with approximately the same degree of chromosome coiling were photographed; the number of SCEs were scored from the photographic prints. Sister chromatid exchanges in the short and long arms of the same chromosome were counted separately. Sister chromatid exchanges at the centromeric region were carefully analyzed, bearing in mind the morphology of the chromosome, as SCEs at that level were sometimes an artifact due to twisting of the chromatids at the centromere.

3.8 Statistical methods

The statistical analysis of the data was performed by the staff of the Institute for Biostatistics (Transvaal Branch).

The paired t-test was used to test two different parameters:

1. the number of *abnormalities per cell* between the members of each pair.
2. the number of *abnormal cells* between the members of each pair.

In both the first and second analyses, three independent calculations

were performed: a) on the combined data derived from cells with a modal number of 46 chromosomes and under b) using only those cells with a modal number of 46 and c) on data from only aneuploid cells.

When considering the number of abnormalities per cell, these were subdivided into 'technical' and 'nontechnical' (see Section 4.3). The paired t-test procedure was again used to compare the number of 'technical' and 'nontechnical' abnormalities per cell between the members of each pair according to the following format (see Table 3.1).

The use of the paired t-test was preceded by the application of Hotelling's test (see Section 3.9.12).

An analysis of variance using Bonferroni's procedure, was performed on the data relating to abnormalities per cell to determine: 1) if there was any specific type of hormonal contraceptive which would cause more abnormalities than another and 2) to compare each specific hormonal contraceptive group with their matched controls.

The chi-square test on a 2 x 2 table was applied to assess any significant relationships between: 1) the duration of the hormonal contraceptive use and the amount of chromosome abnormalities; 2) smoking habits and the amount of chromosome abnormalities; 3) age and amount of chromosome abnormalities; 4) race and amount of chromosome abnormalities. The chi-square test was also applied to analyze combined data obtained from the banded studies performed in both hormonal contraceptive and control groups.

3.9 Discussion of methods

3.9.1 Type of technique for peripheral blood culture

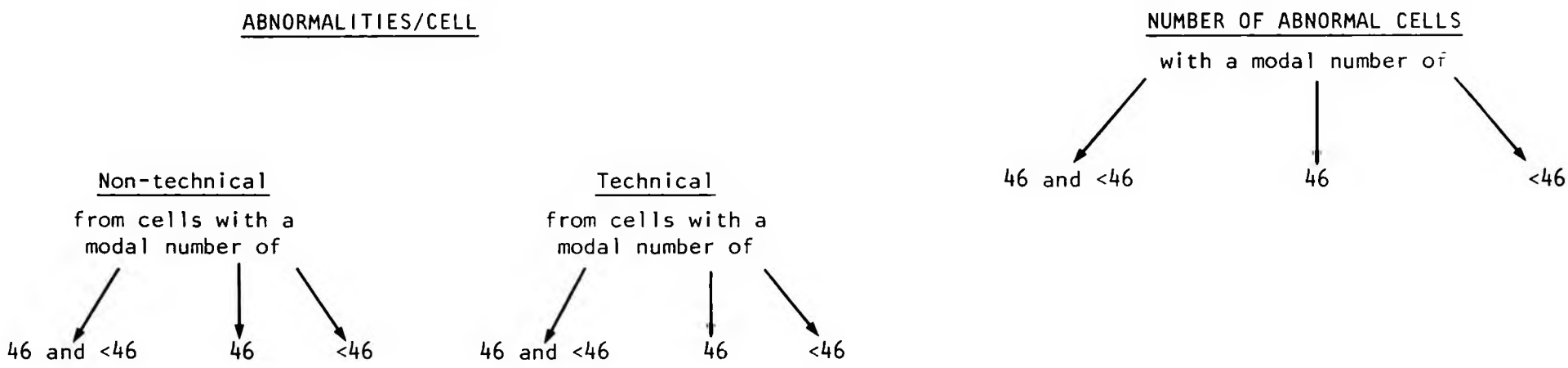
The decision influencing the use of the microtechnique for peripheral blood culture has already been referred to (see Section 3.1). Another factor in favour of this technique is that it gives less chromosome aberrations than the macrotechnique (Buckton and Evans, 1973).

3.9.2 Culture period

Although the author is aware that seventy-two hour cultures yield more metaphases than forty-eight hour cultures, a forty-eight hour

(continued on page 53)

TABLE 3.1. Different statistical analyses performed on the unbanded data using Student's t-test



culture period was chosen because only cells cultured for forty-eight hours may show chromosome damage attributable to occurrences in the G_1 stage of *in vivo* lymphocytes (Ford, 1973).

3.9.3 Establishment of cultures

An unbreakable rule in the present study, was the establishment of control and study group cultures, using the same batches of media, foetal calf serum, heparin and phytohaemagglutinin on the same day and at the same time, to ensure the same culture conditions (e.g. the same fluctuations in temperature). This is essential, because any study on chromosome breakage, as a measure of chromosome damage, should have the most rigid controls possible, as breakages are sometimes due to cultural and technical conditions (Ford, 1973). No blood specimen was ever stored overnight in a refrigerator. The reason for this were reports on the use of cold treatment for accentuation of heterochromatic regions in many plant species (Darlington and La Cour, 1938, 1940), and in some animals (Callan, 1942; Koller, 1946). Hampel and Levan (1964) also found that chromosome breaks could be induced by low temperatures in human cell lines.

More recently, Shiraishi (1970) exposed human lymphocyte cultures to 0-3°C for twenty-four hours. A segmental pattern of darkly stained and unstained areas was obtained. These unstained areas appeared as secondary constrictions or gaps.

It was therefore decided to 'play safe' by avoiding low temperatures to prevent accentuation of secondary constrictions which could be mistakenly classified as breaks or gaps. Interestingly,

Purchase (1978) mentions that undue delay in culturing blood after sample collection could influence the results when assessing chromosome breakage, but he does not explain the mechanism.

3.9.4 Colchicine pretreatment

Colchicine, an invaluable innovation in chromosome analysis, interferes with the assembly of microtubules for spindle formation (Sharma and Talukda, 1974; Priest, 1977). The destruction of microtubules leads to accumulation of dividing cells at metaphase, and furthermore, the chromosomes are not entangled in the spindle, which facilitates better spreading. The normal ranges quoted for colchicine concentration vary from 0,5 µg/mℓ to 0,04 µg/mℓ, and duration of action recommended also varies from one to six hours (Priest, 1977). Higher concentrations and longer time periods will cause greater contraction of metaphase chromosomes, therefore obscuring chromosome morphology and may even lead to polyploidy and endoreduplications, thus introducing errors not present in the original tissue (Sharma and Talukda, 1974; Priest, 1977).

To avoid these problems, a low dose of colchicine was used, 0,04 µg/mℓ, and the duration of treatment was two hours for conventional unbanded analysis and half an hour less in the cultures established for banding, which requires even less condensed chromosomes for satisfactory band visualization (Priest, 1977).

3.9.5 Hypotonic treatment

Progress in chromosome methodology since 1952 is partially due to the use of hypotonic solutions to swell the cells in order to disperse the chromosomes. Several solutions are used in different schedules,

with varying periods of application and temperature (Priest, 1977).

Potassium chloride (0,075 M) was the only hypotonic solution used in the present study, and the spreading obtained was satisfactory.

3.9.6 Fixation

Fixatives are used to preserve the content of cells as closely to their living state as possible, and to render them resistant to further changes caused by subsequent processing. The properties desired in an ideal fixative are those of rapid chromatin precipitation, checking the autolysis of proteins and enhancing the basophilic staining of chromosomes (Sharma and Talukder, 1974).

A very wide range of fixatives for chromosome preparation are available, but the most commonly used fixative and the one used in the present study, was a mixture of glacial acetic acid and absolute methanol.

3.9.7 Harvesting procedure

During the harvesting procedure, special care and attention were given to certain details that are not generally considered to be important in a routine cytogenetic laboratory. Thus, because they theoretically enhance the technical breakage rate, they deserve greater care in the context of a study where breakage is used as a basis of measuring chromosomal damage. Therefore, the number, speed and length of centrifugations, and number of changes and length of exposure to fixatives were noted. The size of Pasteur pipettes used, the force exerted when cells were pipetted, as well as the height from which the cell suspension was dropped on to the slide,

were also standardized as far as possible. Any minor variation introduced where needed, was applied to both study and control groups in exactly the same way.

3.9.8 Number of cells scored

The predetermined number of 30 metaphases scored in this study, was based on a pilot study performed in twenty cases (ten study subjects and ten control subjects). Thirty scored cells per case in the pilot study, showed a significant difference in the frequency of chromosome abnormalities between the two groups (a chi-square test was used). Expert statistical advice was then sought and the pilot study was also discussed with the head of the department and all agreed that thirty cells would be sufficient in a much larger study sample.

From the start it was realized that if a significant difference between the study and control groups was *not* found, the number of cells in both groups would have to be increased (Bobrow, personal communication). Fortunately there was a highly significant difference.

In those cases where fewer than thirty metaphases were scored, this obviously reflected a poorer culture quality (either in number of metaphases, spreading or random aneuploidy). Thus in these cases, only those cells that strictly fullfilled the criteria described (see Section 3.7.1) were included. The target number of thirty cells could have been more easily met if the author had not been so strict in the criteria laid down for their selection (see Section 3.7.1).

Another factor which reduced the number of available metaphases for this study was that they were all derived from forty-eight hour

cultures, i.e. after only one cycle of *in vitro* division, which *always* yield a much lower mitotic index than the seventy-two hour or synchronized blood culture techniques standardly used in routine cytogenetics laboratories.

3.9.9. Criteria for selection of metaphases

Metaphases that appeared reasonably well-spread and complete under low magnification were selected for further study, as recommended by WHO (1973).

The arbitrary decision to include metaphases with less than forty-six chromosomes only if they showed an abnormality, was based on the following reasoning:

A dramatic difference in the frequency of chromosome abnormalities was not expected in this study (as opposed to radiation studies or chromosome breakage syndromes). Therefore if systematic screening of apparently complete metaphases under low magnification, was adhered to, there would obviously be some metaphases with one or more missing chromosomes. Theoretically the same degree of random aneuploidy would be expected in both hormonal contraceptive and control groups. However, it has been shown that certain drugs eg. 6-mercaptopurine and folic acid antagonists (Jensen, 1967) can cause an increase in aneuploidy.

Whether this occurs with hormonal contraceptives is unknown. If hormonal contraceptives do in fact induce increased aneuploidy, the number of missing chromosomes in the predetermined 30

metaphases selected for analysis, would be greater in the hormonal contraceptive group than in the control group. Consequently the data would have been biased, as the missing chromosomes could have been abnormal ones. However if an abnormality was found in a cell with less than forty-six chromosomes, the likelihood of the missing chromosomes having shown aberrations as well would be diminished. This was based on the rationale that very few abnormalities were found in previous studies of a similar nature (Goh, 1968, Badr *et al*, 1971, McQuarrie *et al*, 1970, Littlefield *et al*, 1971 and 1975). Subsequent analysis of the data confirmed this supposition, in that very few cells showed more than one aberration (see appendix Table 4.9).

Even if this reasoning was not valid the *control group was also subjected to the same selection criteria.* The data obtained from both complete and incomplete cells were separately statistically analyzed as well as in combination (see Section 4.3).

3.9.10 Banding techniques

A new 'era' in cytogenetics was introduced in 1969 when Zech described the brilliant fluorescence of the human Y chromosome. From that time, the study of human chromosomes was revolutionized by the description of techniques which enabled the accurate identification of every chromosome of the human karyotype. This identification is possible by means of the characteristic and specific banding patterns of individual chromosomes (Paris Conference, 1971).

Many banding techniques have been described which differentially stain specific areas of chromosomes. The main techniques are G-banding

(Seabright, 1971), R-banding (Dutrillaux and Lejeune, 1971; Bobrow *et al.*, 1972), C-banding (Arrighi and Hsu, 1971) and Q-banding (Caspersson *et al.*, 1970).

For the purpose of this study, only Giemsa banding (G-banding) was employed because it is one of the easiest, most reproducible and one of the most informative techniques. (C-banding demonstrates only heterochromatic regions; Q-banding requires special fluorescence equipment, and band analysis can only be done by means of photographic prints, and is mainly used for the Y chromosome and chromosome variants; R-banding gives as much information as G-banding, but good

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banding is technically more difficult to achieve.)

The multitude of Giemsa banding techniques reported in the literature are partially due to the variability of the quality of bands obtained. This has led to many modifications of the original techniques described, i.e. Giemsa ASG banding (Sumner *et al.*, 1971), Trypsin-Giemsa banding (Seabright, 1971), and Pronase-Giemsa banding (Dutrillaux *et al.*, 1971).

Schnedl (1974) emphasized the variability of banding quality from one laboratory to another, and also within the same laboratory under identical working conditions. This variability often leads to small modifications of a standaridized technique in order to adapt to the prevailing conditions.

Several modifications were necessary in the early stages of this study before the author could establish a suitable Giemsa banding technique which produced consistently acceptable results.

3.9.11 The value of banding techniques in detection of clastogenic effects

The importance of banding techniques is unquestionable. These techniques assist in the detection of chromosome aberrations by means of the particular specific banding pattern of each chromosome, especially where the size and position of the centromere remains unaltered, i.e. paracentric and some pericentric inversions and equal reciprocal translocations, would not be detected on unbanded preparations.

The use of banded chromosomes does not assist, however, in the

detection of the so-called unstable abnormalities, i.e. dicentrics, acentrics, rearrangements, breaks or gaps (Buckton *et al.*, 1978). In fact, fewer dicentrics may be seen in banded preparations than in unbanded preparations (Buckton *et al.*, 1878). The reasons for this discrepancy are probably: 1) the tendency to select better quality cells for banded analysis than is necessary on a conventionally Giemsa stained preparation; 2) the centromere is less obvious on a banded chromosome.

The same applies to breaks or gaps with no obvious displacement of the broken ends, which can easily be missed on banded chromosomes if they occur in a negatively stained region. Such breaks and gaps in unbanded preparations should be clearly visible.

Without doubt, banding techniques are capable of giving more information in interpreting any chromosome abnormalities detected, but unfortunately this extra information entails a considerable time increase in the already time-consuming procedure of scoring chromosome abnormalities. The extra time spent on analysis of banded preparations is due to the more demanding scoring criteria; spreading and banding must be reasonably good in the majority of cells to assure a random selection. Banded karyotype analysis is also more time consuming than analysis of unbanded karyotypes (Bauchinger, 1978). These are the reasons why banded methods have not been routinely used in studies of clastogenic effects (Brøgger, 1979).

One may question whether the effort and time consumed on banded techniques in this particular type of study is worth the extra information achieved. Lawler and Walden (1978) suggested that it is

logical to assume that when the frequency of unstable abnormal cells is increased, that of cells with stable abnormalities may also rise.

This is in fact exemplified in the case of patients with polycythaemia vera treated with ^{32}P, who were found to have clones of cells with stable rearrangements in their marrow chromosomes and unstable abnormalities in peripheral blood chromosomes (Kay *et al.*, 1966).

The limitations of chromosome studies as indicators of genetic damage have to be realized. We are never likely to ascertain all the damage in every cell (Bobrow, 1978). In any event we are only attempting to compare classes of damage between treated and untreated cells; and as long as the type of scoring is consistent between the two groups, a valid comparison should be achieved (Bobrow, 1978).

3.9.12 Special techniques

3.9.12.1 Sister chromatid exchanges

The use of autoradiography for detecting sister chromatid exchanges by Taylor (1958) and others has been replaced by new methods in which the sister chromatids are made to stain differently. The break-through came with the publication of Zakharov and Egolina's (1972) work in which the Chinese hamster chromosomes were treated with BrdU (bromodeoxy-uridine) for two rounds of replication and subsequently stained with Giemsa - the two sister chromatids stained differently. The treatment with BrdU for two cell cycles leads to one of the chromatids being unifilarly substituted (i.e. substituted in only one polynucleotide chain) with BrdU due to the semiconser-

vative mechanism of DNA replication, whereas the other sister chromatid is bifilarly substituted, i.e. both polynucleotide chains have been substituted by BrdU (Figure 3.1). The unifilarly substituted chromatid stained darkly and the bifilarly substituted one stained lighter, enabling the visualization of SCE (Figure 3.2). Several reasons have been postulated to explain the differential staining of the sister chromatids, but no definitive answer has yet been found.

Latt (1973) has suggested that the difference in colour between the two chromatids is caused by a differential quenching of fluorescence by BrdU incorporated in either one or two strands of the DNA. Zakharov and Egolina (1972) noted that after Giemsa staining the pale chromatid was usually longer than its sister, and they implicated the protein synthesis that affects chromosome condensation and spiralization which was delayed by the substitution of thymine by BrdU.

Ikushima and Wolff (1974) have postulated that differences in the binding of protein to BrdU containing chromatids is the cause of the differential staining.

More recent electron microscopic work by Korenberg and Ris (1977) with Chinese hamster ovary (CHO) chromosomes had also attributed the effect to nonhistone proteins affecting the condensation of chromosomes.

Fluorescent dyes, acridine orange and Hoechst 33258 (Latt, 1973: Dutrillaux *et al.*, 1974), Giemsa stain (Zakharov and Egolina, 1972), or a combination of both (Perry and Wolff, 1974), can be used to demonstrate SCEs.

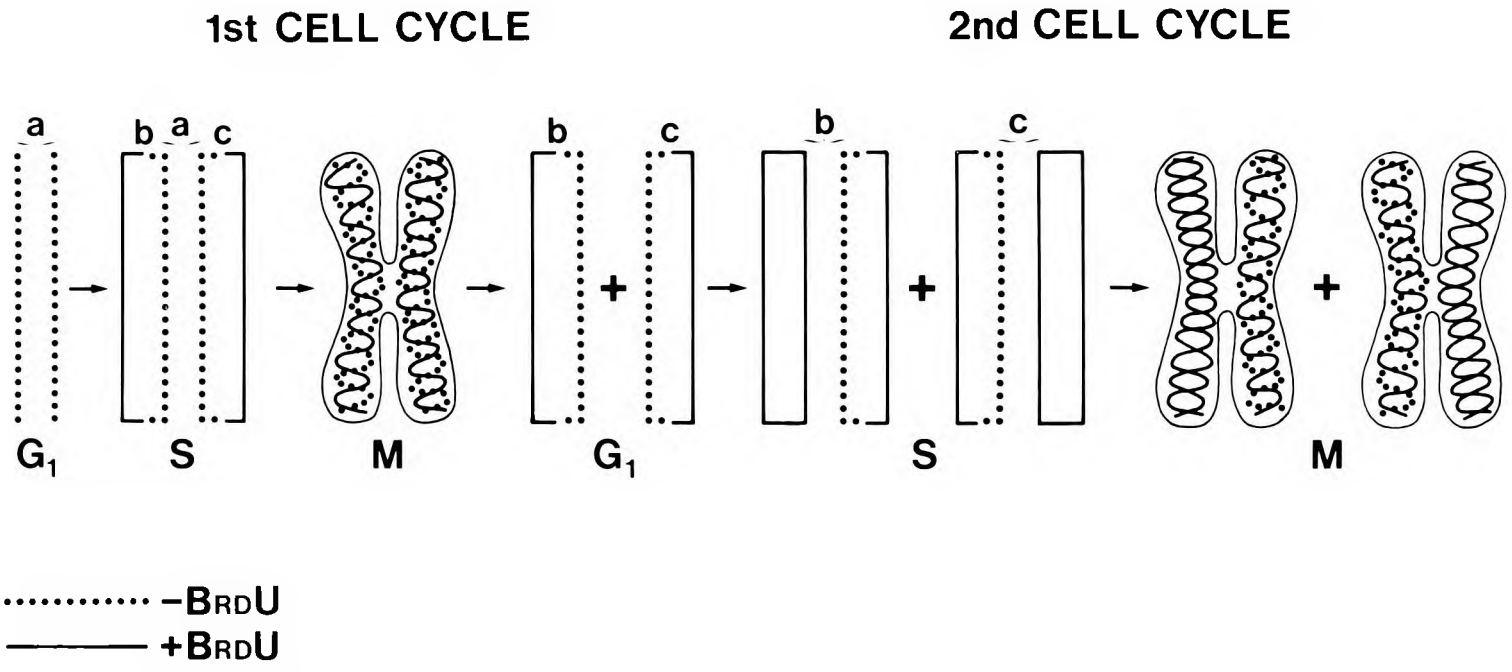

Figure 3.1. Diagrammatic representation of the mechanism leading to the visualization of sister chromatid exchanges (SCEs).

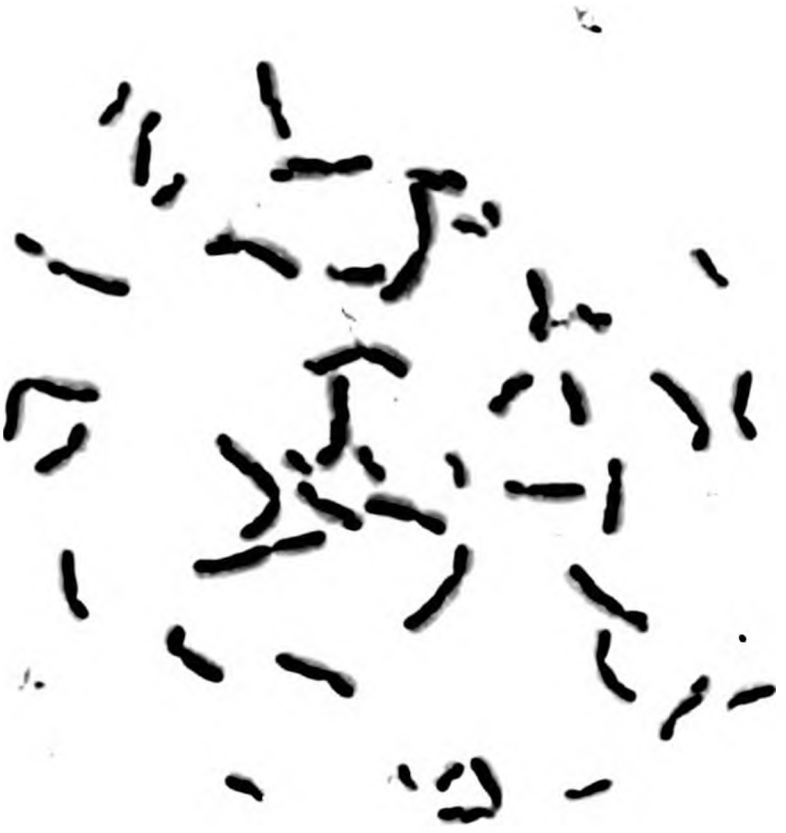

Figure 3.2. Sister chromatid exchanges (SCEs) after Giemsa staining. The BrdU unifilarly substituted chromatid is stained more darkly than the bifilarly substituted one.

The combined technique - FPG (fluorescence-plus-Giemsa) (Perry and Wolff, 1974) presents all the advantages of the fluorescent techniques with the additional advantages of producing nonfading permanent preparations that do not require fluorescence microscopy or photography (Perry and Wolff, 1974).

In the present study the use of acridine-orange for fluorescence staining proved to be very satisfactory and no pretreatment was required (e.g. use of 33258 Hoechst). However, the technique was not always consistent, and sometimes the slides had to be washed and restained several times before satisfactory results were achieved. Subsequent Giemsa staining gave results, even in metaphases that did not show good differentiation between the two chromatids with the fluorescent staining.

Logically only complete metaphases were included in the analysis, as hypodiploid cells could have presented several SCEs in the missing chromosomes with impossible accurate count.

Uncoiled chromosomes, longer than the coiled ones, gave a better resulution of SCEs, particularly when short areas of the chromosomes are involved in the exchanges in nearby regions or at the telomeres. Thus as far as possible, very short or very long chromosomes were ignored.

3.9.13 Statistical methods

Hotelling's test was the first statistical test used to determine whether the use of hormonal contraceptives was associated with a significant difference in the number of chromosome abnormalities when compared with controls. This procedure was used to test all variables

simultaneously for a difference between control and study groups. The difference found was significant and therefore the paired t-test could then be applied. The paired t-test is considered one of the most efficient statistical procedures when applicable. In this study the paired t-test was considered valid since there were forty-four observations (forty-four subjects from each group) which had a reasonable range of values and thus the central limit theorem came into effect.

The use of an analysis of variance to assess any significant correlation between the type of hormonal contraceptive and the amount of chromosome abnormalities may not be quite valid, because the data was not normally distributed and some of the sample sizes were very small. Because there were four groups to be compared to each other Bonferroni's procedure was used. This is a multiple comparison procedure that can be applied when more than two groups are compared with each other. It tests the groups pair-wise keeping the overall significance level at 0,05.

The question whether the number of chromosome abnormalities varied with age, race and smoking habits in both control and study groups was approached by means of χ^2 tests.

The chi-square test was applicable in those instances dealing with 'discrete' or 'discontinuous' variables, i.e. numerical values attached to a finding that stands on its own and cannot take intermediate values. Dealing with counts the assumption could not be made that the variables were normally distributed and therefore the ordinary parametric procedures were not applicable.

CHAPTER 4

4. RESULTS

4.1 General

From the fifty original pairs (a pair consisting of a study of individual and respective control) only forty-four pairs were submitted to statistical analysis and are therefore reported (see Section 4.7). Of the eighty-eight individuals studied chromosomally, a total of 2 541 unbanded metaphases were obtained; 1 286 from the study group with a mean of 29,2 metaphases per study individual and 1 255 from the control group with a mean of 28,5 metaphases per control individual.

Although thirty unbanded metaphases per case was the number aimed at, there were seventeen out of eighty-eight cases where the limit established was not attained due to poor quality of the metaphases (see Section 3.9.8). Of these seventeen cases, six cases belong to the study group and the remaining eleven cases belong to the control group.

The criteria for selection of unbanded metaphases have already been defined in Chapter 3 (see Sections 3.7.1 and 3.9.9). Abnormalities in cells with less than forty-six chromosomes were included in the total number of abnormalities and also in a separate group for statistical purposes (see Section 4.3).

Giemsa-banding was performed in fifty out of the eighty-eight cases; twenty-five banded cases (fifty per cent) belonged to the study group and the other twenty-five cases (fifty per cent) to the control group. There was a total of 360 banded complete metaphases; 188 from the study group with a mean of 7,52 banded metaphases per study case and 172 banded complete metaphases from the control group with a mean of 6,88 banded metaphases per control case.

There are a number of nomenclature systems for the classification of chromosome aberrations (Buckton and Evans, 1973; Kilian and Picciano, 1976; Report, Mammalian Chromosomes News Letter, 1971). At this point it is necessary to define the criteria the author used in this study for the classification of the different types of chromosome abnormalities found. These criteria were based on a composite of several of the above classifications of chromosome aberrations.

Dicentric

Chromosome with two centromeres resulting from the malunion of centric broken ends of two chromosomes (see Figure 4.1).

Acentric fragment

Paired chromatids that lie parallel to one another without a centromere, resulting from terminal or interstitial deletions (see Figure 4.2).

Ring

Paired chromatids in the shape of a ring with or without centromeres resulting from the malunion of two broken opposite ends of a chromosome (see Figure 4.3).

Rearrangement (triradial, quadriradial)

Aberrant chromosome figure with distortion of the usual chromatid pattern, resulting from chromatid interchanges involving two homologous or nonhomologous chromosomes (see Figures 4.4, 4.5 and 4.6).

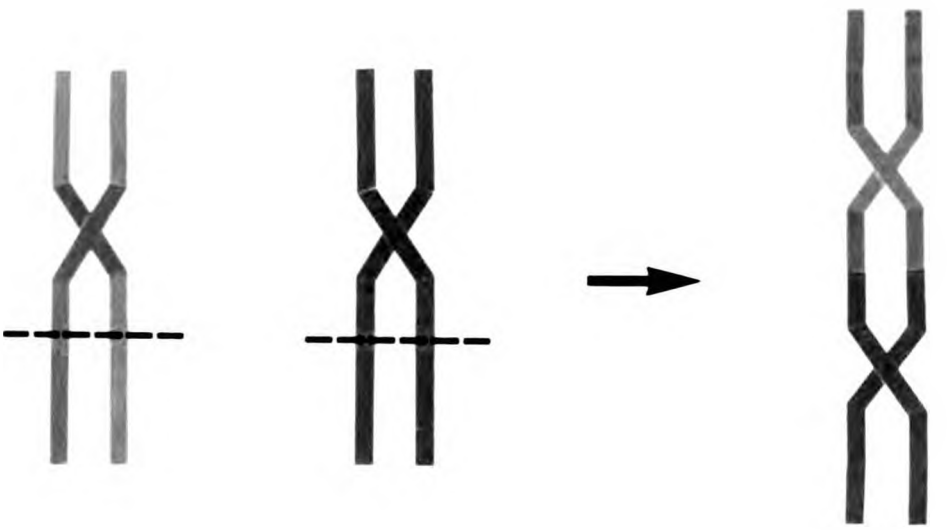

Figure 4.1. Diagrammatic representation of the formation of a dicentric chromosome.

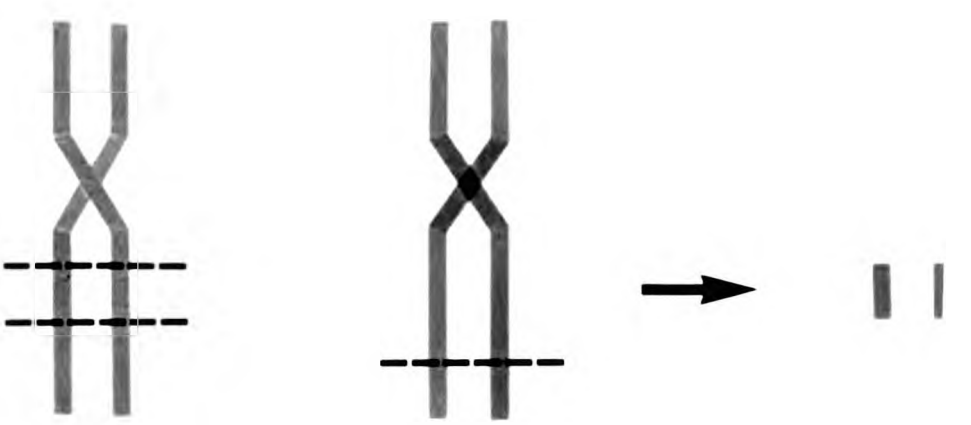

Figure 4.2. Diagrammatic representation of acentric fragment formation.

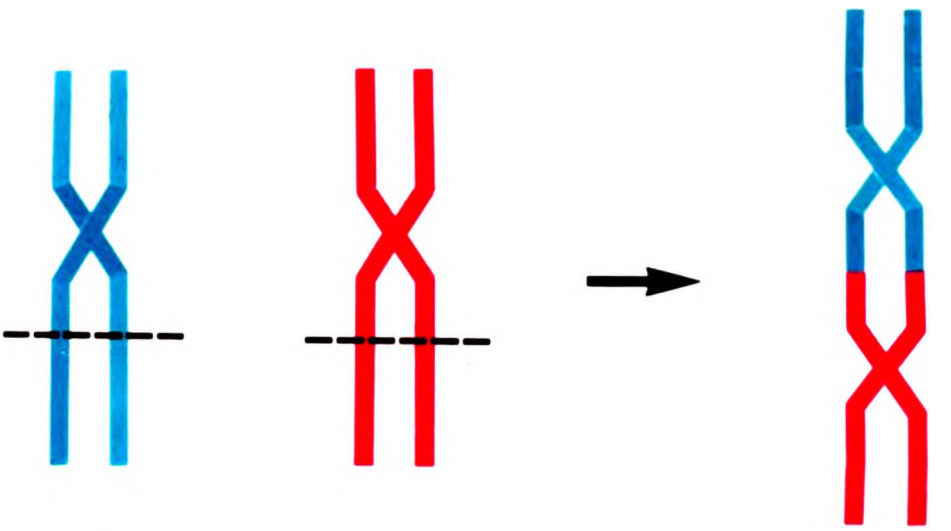

Figure 4.1. Diagrammatic representation of the formation of a dicentric chromosome.

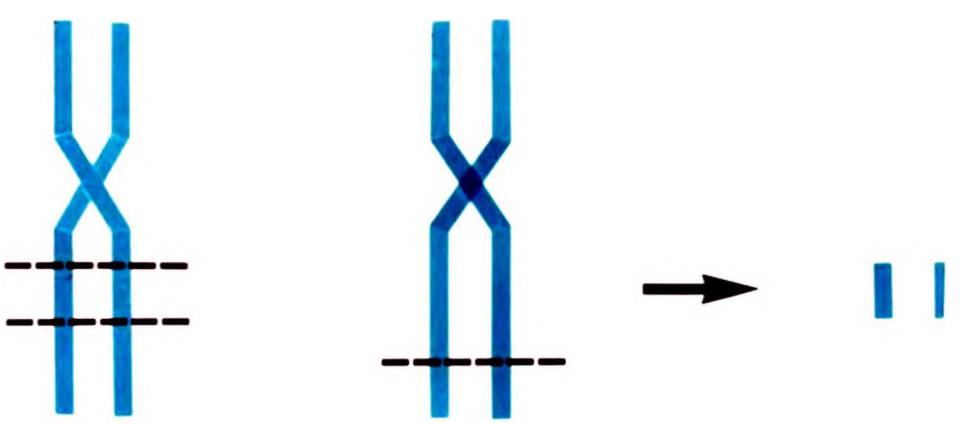

Figure 4.2. Diagrammatic representation of acentric fragment formation.

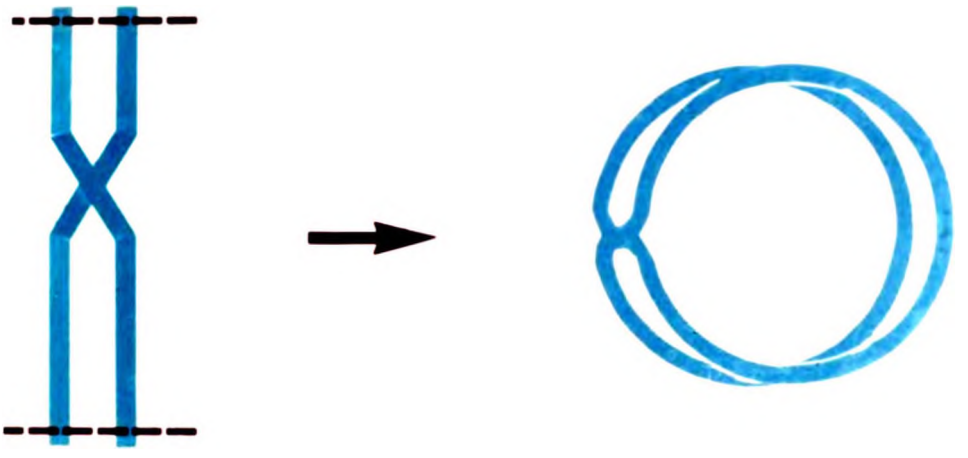

Figure 4.3. Diagrammatic representation of ring formation.

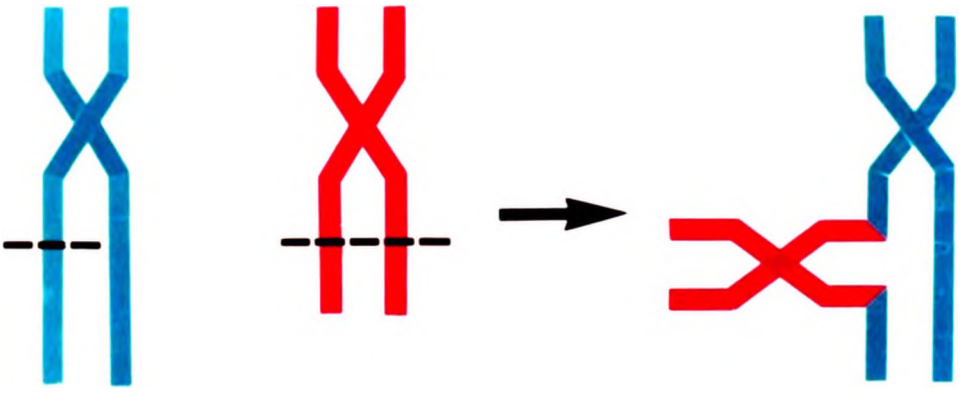

Figure 4.4. Diagrammatic representation of the formation of a triradial figure.

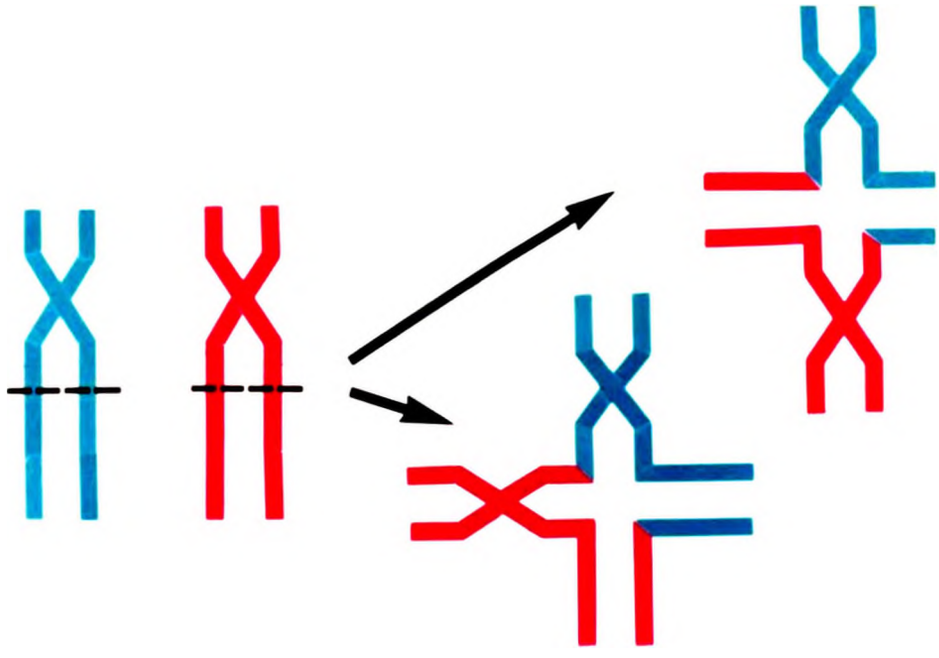

Figure 4.5. Diagrammatic representation of quadriradial interchange between two homologous chromosomes.

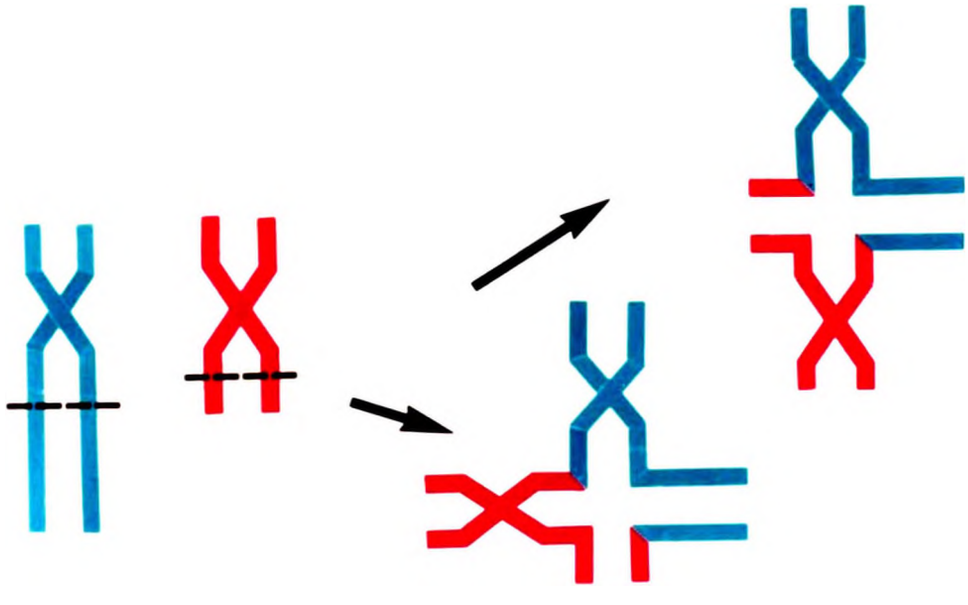

Figure 4.6. Diagrammatic representation of quadriradial interchange between two non-homologous chromosomes.

Chromosome deletion

Loss of chromosome material from both chromatids of a chromosome resulting either from one break (terminal deletion) or two breaks (interstitial deletion) in each chromatid (see Figure 4.7).

Chromatid deletion

Loss of chromosome material in a single chromatid of a chromosome resulting either from one break (terminal deletion) or two breaks (interstitial deletion) in a single chromatid (see Figure 4.8).

Chromosome break

Aligned discontinuity present in both chromatids of a chromosome, which is equal or larger than the width of the chromatid, or any nonaligned discontinuity affecting both chromatids of a chromosome.

In both cases the discontinuity should be in close association with an identifiable piece of chromosome material (see Figure 4.9).

Chromatid break

Aligned discontinuity present in a single chromatid of a chromosome, which is equal or larger than the width of the chromatid, or any nonaligned discontinuity in a chromatid of a chromosome.

In both cases the discontinuity should be in association with an identifiable piece of chromosome material (see Figure 4.10).

Chromosome gap

Aligned discontinuity in both chromatids of a chromosome, smaller than the width of a chromatid (see Figure 4.11).

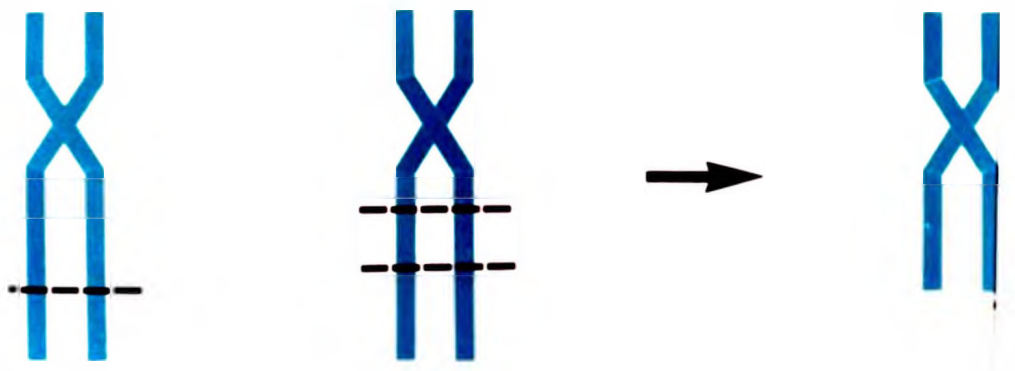

Figure 4.7. Diagrammatic representation of chromosome deletion.

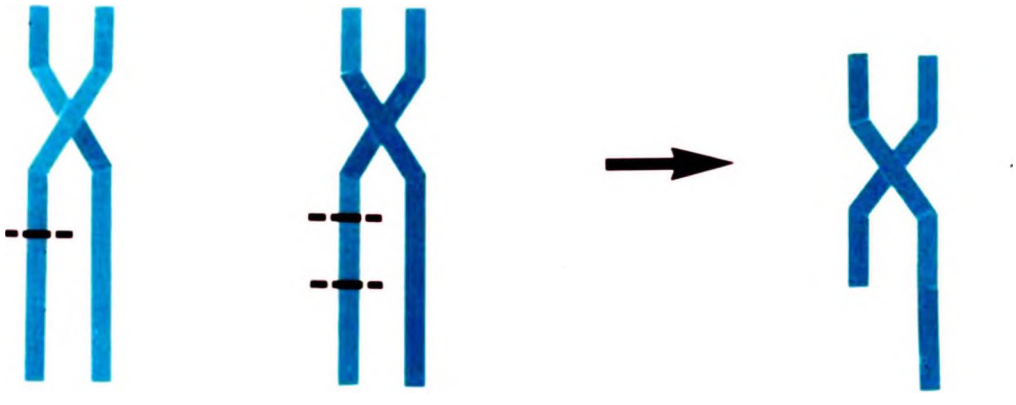

Figure 4.8. Diagrammatic representation of chromatid deletion.

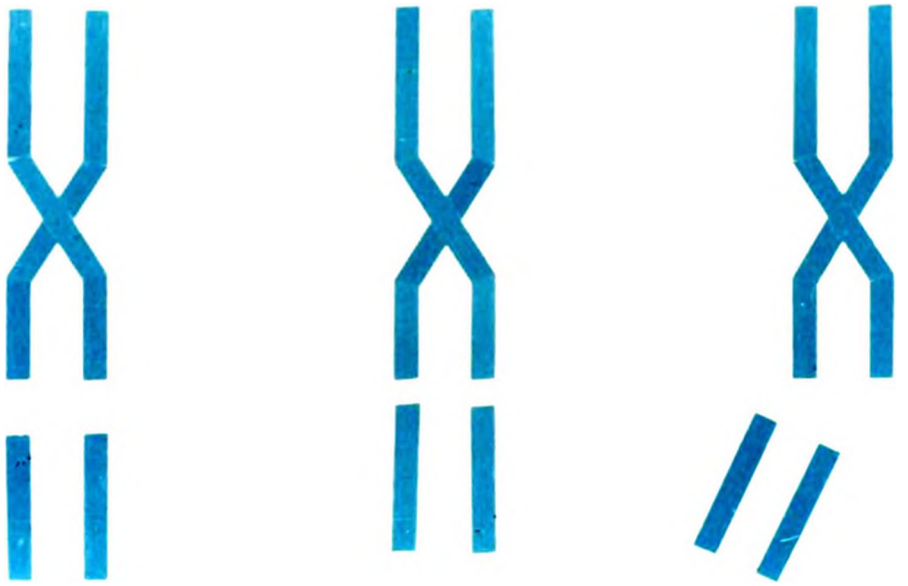

Figure 4.9. Diagrammatic representation of the different types of chromosome breaks.

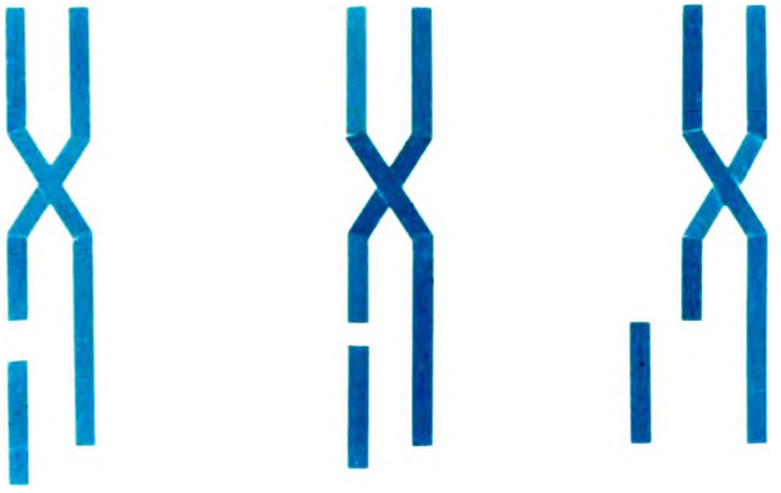

Figure 4.10. Diagrammatic representation of the different types of chromatid breaks.

Chromatid gap

Aligned discontinuity affecting one single chromatid of a chromosome, smaller than the width of a chromatid (see Figure 4.12).

Marker chromosome

Chromosome that by virtue of its size, position of centromere or banding pattern, cannot be identified as part of the normal karyotype.

4.1.1 Stable and unstable abnormalities

The majority of the abnormalities found in this study belonged to the group of so-called 'unstable' abnormalities, in view of their tendency to disappear from the circulation due to difficulties of division at mitosis. However, dicentric chromosomes can survive for at least a few cell divisions, rings can go through mitosis easily and deletions can be considered a stable abnormality, i.e. they can remain in the circulation and are capable of replication and division.

The above defined abnormalities were the ones found in this study. The complete data on the chromosome abnormalities found in the eighty-eight subjects are presented in Table 4.1 (see Appendix). It is also presented as a table which includes the clinical and chromosomal data in a coded form for computer analysis (Table 4.2) (see Appendix). The key of the table appears on pages 238 and 239.

Results in both study and control groups, unbanded and banded, will be subdivided according to the different types of abnormalities found and comments will follow when appropriate.

4.2 Unbanded studies

(Study group n = 1 286 cells; control group = 1 255 cells; see

Figure 4.11. Diagrammatic representation of a chromosome gap (see text).

Figure 4.12. Diagrammatic representation of a chromatid gap (see text).

Section 4.3 for statistical analysis.)

4.2.1 Dicentric chromosomes

Study group

In this group a total of five dicentric chromosomes were found in unbanded preparations. None of the dicentrics had an accompanying acentric fragment. These five dicentrics were derived from four different cases, i.e. there was one case with two dicentrics.

The mean value of dicentric chromosomes in the study group was 0,00388/cell.

Control group

In this group two dicentric chromosomes were found in unbanded preparations. They belonged to two different cases (one case 2/1 a student, and the other case 43/1 a housewife) and both were detected in cells with less than forty-six chromosomes. No accompanying acentric fragment was found associated with either of these two dicentrics.

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The mean value of dicentric chromosomes in the control group was 0,00159/cell.

4.2.2 Acentric fragments

Study group

In the study group a total of thirty acentric fragments were found. These thirty acentric fragments were detected in nineteen different cases as follows:-

One case (case 22/2 - a woman on Normovlar and Depo-Provera for a total period of twenty-two months) with four acentric fragments, one case (case 19/2 - a woman on Micro-Novum and Minovlar for thirty-eight months) with three acentric fragments, six cases with two acentric fragments each, and the other remaining eleven cases with a single acentric fragment each.

Ten of the thirty acentric fragments detected were found in cells with less than forty-six chromosomes.

The mean value of acentric fragments in the study group was 0,023/cell.

Control group

In this group two acentric fragments were found in unbanded preparations. The two acentric fragments detected belonged to two different controls; one of them was found in a cell with forty-six chromosomes and the other in a cell with less than forty-six chromosomes.

The mean value of acentric fragments in the control groups was 0,00159/cell.

4.2.3 Ring chromosomes

Study group

Only two ring chromosomes were found in the unbanded preparations of the study group. They belonged to two different cases and both were found in cells with forty-six chromosomes.

The mean value of ring chromosomes in the study group was 0,00155/cell.

Control group

Two ring chromosomes were found in the control group. They belonged to two different cases (both students) and both rings were present in cells with forty-six chromosomes.

The mean value of ring chromosomes in the control group was 0,00159/cell.

4.2.4 Rearrangements

Study group

A total of seven rearrangements were detected in this group. Two of these seven rearrangements belonged to the same case (case 9/2 - a woman on Minovlar ED for thirty-six months). It is of interest to note that this woman also had a dicentric chromosome.

There were two other cases (case 14/2 and 31/2) in whom two rearrangements were found in the same individual, but in both these two cases one rearrangement was found in the banded preparation and the other on an unbanded one. Case 31/2 had been on hormonal contraceptives (Ovulen and Minovlar) for the longest time in this study - ninety-eight months. Case 14/2 had been on oral contraceptives

for eight months and then on Depo-Provera for thirty months.

All the rearrangements were found in hypodiploid cells as expected because of the involvement of two or more chromosomes in the formation of the aberrant chromosome.

The mean value of rearrangements in the study group was 0,00544/cell.

Control group

Only one rearrangement was found in the control group. It was found in a hypodiploid cell from a student.

The mean value of rearrangements in the control group was 0,00079/cell.

4.2.5 Chromosome deletions

Study group

Two chromosome deletions were detected. They belonged to two different cases and both were found in cells with forty-six chromosomes.

The mean value of chromosome deletions in the study group was 0,00155/cell.

Control group

No chromosome deletions were detected in the control group.

4.2.6 Chromatid deletions

Study group

In this group twenty chromatid deletions were found in unbanded preparations. The maximum number of chromatid deletions present in

a single case were found in case 6/2, a woman on Minovlar ED for twenty-four months in whom a dicentric chromosome was also found.

Of the twenty chromatid deletions detected, thirteen were found in cells with forty-six chromosomes and the remainder in hypodiploid cells.

The mean value of chromatid deletions in the study group was 0,015/cell.

Control group

Only one chromatid deletion was detected in the control group and this was found in a diploid cell.

The mean value of chromatid deletions in the control group was 0,00079/cell.

4.2.7 Chromosome breaks

Study group

Sixty-nine chromosome breaks were detected in the unbanded metaphases of this group. The maximum number of chromosome breaks present in a single case was eight (case 31/2), who also had two rearrangements (one banded and one unbanded) in the cells screened.

Fifty of the sixty-nine chromosome breaks were found in cells with forty-six chromosomes and the remaining nineteen in cells with less than forty-six chromosomes.

The mean value of the chromosome breaks in the study group was 0,053/cell.

Control group

In this group fourteen chromosome breaks were found.

The maximum number of chromosome breaks found in one single case was two, and there were two such cases.

Of the fourteen chromosome breaks detected, ten were in complete metaphases and the other four were in cells with less than forty-six chromosomes.

The mean value of chromosome breaks in the control group was 0,011/cell.

4.2.8 Chromatid breaks

Study group

Three-hundred and forty-one chromatid breaks were found. The maximum number of chromatid breaks present in a single case was fifteen; there were three such cases (cases 34/2, 42/2 and 43/2). It is interesting to note that both cases 34/2 and 42/2 had used the same types of hormonal contraceptive (Minovlar ED and Ovral), although the exposure time was eighteen months in one and forty months in the other. Case 43/2 had used hormonal contraceptives for seventy-two months. Two-hundred and sixty-three of the 341 chromatid breaks were found in cells with less than forty-six chromosomes.

The mean value of chromatid breaks in the study group was 0,26/cell.

Control group

There were 193 chromatid breaks in the control group. The maximum number found in a single individual was eleven chromatid breaks. Of 193 chromatid breaks, 150 were detected in complete cells and the remaining forty-three in incomplete cells.

The mean value of chromatid breaks in the control group was 0,15/cell.

4.2.9 Chromosome gaps

Study group

Three chromosome gaps were found in the study group, all in different cases and all in cells with forty-six chromosomes.

The mean value of chromosome gaps in the study group was 0,0023/cell.

Control group

Only one chromosome gap was found and this was in a cell with forty-six chromosomes.

The mean value of chromosome gaps in the control group was 0,00079/cell.

4.2.10 Chromatid gaps

Study group

Thirty-eight chromatid gaps were detected in this group; of these, twenty-nine were found in complete cells and the remaining nine in cells with less than forty-six chromosomes.

The maximum number of chromatid gaps found in one case was four and there were two such cases.

The mean value of chromatid gaps in the study group was 0,029/cell.

Control group

There were fifty-three chromatid gaps in this group. Thirty-nine

of them were found in cells with forty-six chromosomes and fourteen in cells with less than forty-six chromosomes. The maximum number of chromatid gaps found in a single individual was six; there were two such cases.

The mean value of chromatid gaps in the control group was 0.042/cell.

Examples of some of the unbanded abnormalities detected are presented in Figure 4.13.

4.3 Statistical analysis of the results on unbanded data

Statistical analysis was performed on the data obtained on unbanded preparations from forty-four women on hormonal contraceptives and forty-four controls. (The reason for the exclusion of six study group cases and six cases from the control group is discussed in Section 4.7). The analysis on the two persons in the same pair (study and control individuals) was done simultaneously - a paired t-test was used after doing Hotelling's test (see Section 3.8).

The statistical results showed a highly significant increase ($p < 0,001$) in the total number of chromosome abnormalities per cell in the hormonal contraceptive group when compared with the control group.

Gaps are considered to be technical artefacts by many workers (Buckton and Evans, 1973) and chromatid breaks are the commonest abnormalities found in 'normal' individuals. The latter are also very likely to be, at least in some instances, technical in origin. For these reasons it was decided to subdivide the chromosome abnormalities into two groups for the purpose of statistical analysis.

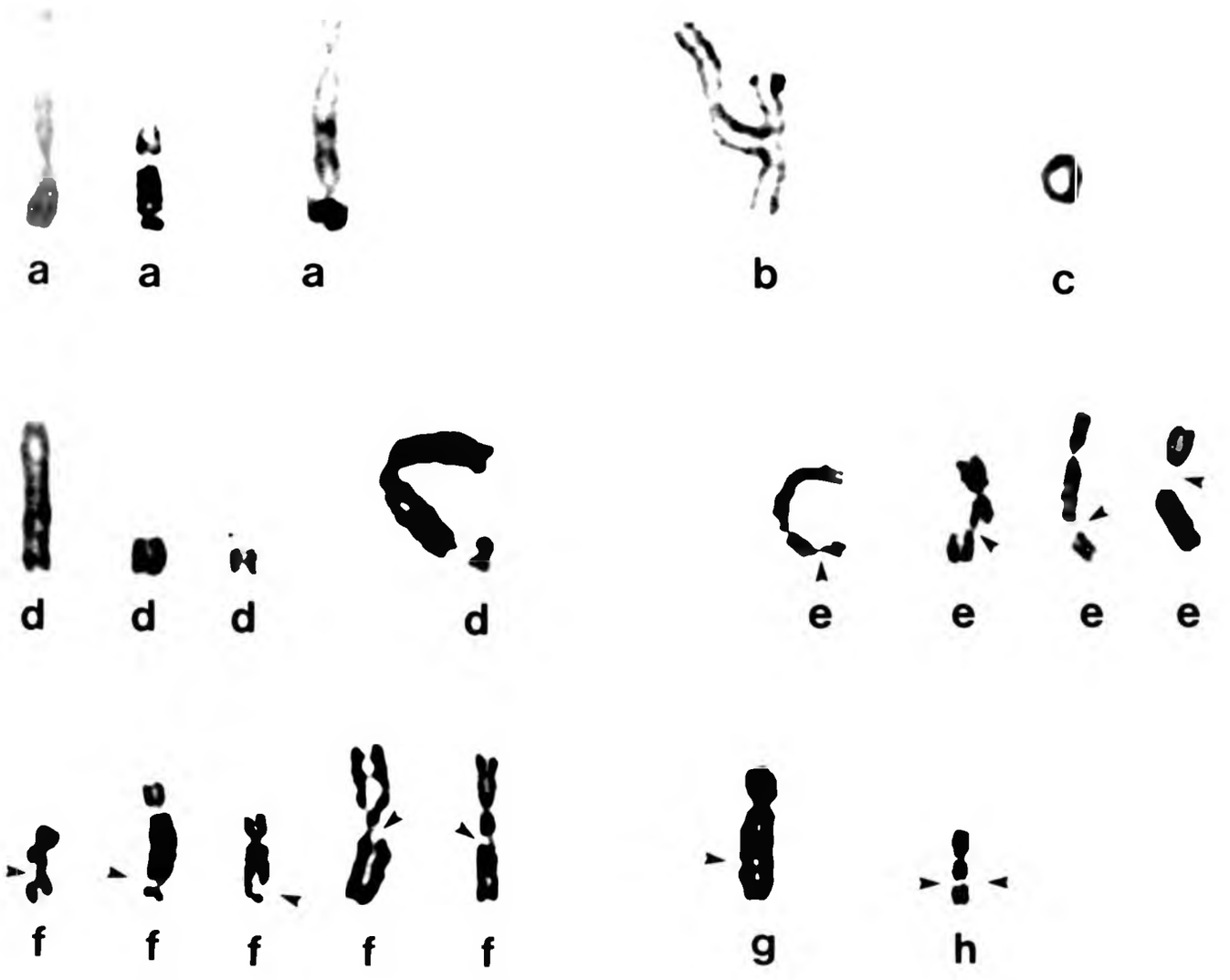

Figure 4.13. Examples of some of the unbanded abnormalities detected. a) Dicentrics. b) Rearrangement (triradial figure). c) Ring. d) Acentric fragments. e) Chromosome breaks. f) Chromatid breaks. g) Chromatid gap. h) Chromosome gap.

Group 1

This group included only chromatid and chromosome gaps and chromatid breaks, the abnormalities that are more likely to be caused by the technical handling of the chromosomes.

Group 2

The second group was comprised of chromosome breaks, deletions, rearrangements, rings, acentrics and dicentrics. These aberrations are likely to be nontechnical in origin, especially in view of the fact that only forty-eight hour cultures were used in this study.

After separating the data on chromosome abnormalities into these two subgroups, i.e. abnormalities likely to be due to technical factors and abnormalities not likely to be due to technical factors, the hormonal contraceptive group again showed a highly significant increase of abnormalities in both subgroups when compared to their controls ($p < 0.0001$).

Abnormalities were also separated according to the modal number of the cell in which they were found, i.e. abnormalities in cells with forty-six chromosomes and abnormalities in cells with less than forty-six chromosomes. This was not done to assess aneuploidy, because a cell with less than forty-six chromosomes was only included when an abnormality was found. It should be noted that for cells with less than forty-six chromosomes, the group on hormonal contraceptives had a lower average of technical abnormalities than the controls.

However, the nontechnical (relevant) abnormalities were significantly increased in the aneuploid cells as well as those with a normal modal number.

These results are presented in Table 4.1.

In conclusion, a highly significant increase was observed in the number of nontechnical abnormalities per cell in the hormonal contraceptive group when compared with the control group irrespective of the modal number of the cell.

Although there was no obvious indication of nonrandom accumulation of abnormalities in specific cells, a statistical analysis based on the number of abnormal cells was also performed. The ratio between the number of abnormal cells and the total number of cells was calculated for: a) cells with forty-six chromosomes, b) cells with less than forty-six chromosomes and c) total of (a) and (b).

The results of the t-test performed on the difference between control and hormonal contraceptive groups are shown in Table 4.2.

In conclusion the hormonal contraceptive group showed a highly significant increase in the total number of abnormal cells when compared with the control group. The same results were found when the abnormal cells were subdivided according to their modal number.

4.3.1 Chromosome abnormalities and type of hormonal contraceptive

Because of the small number of individuals taking a specific type of

(continued on page 85)

TABLE 4.1. t-tests performed on the differences in the number of abnormalities per cell between control and hormonal contraceptive groups

Type of abnormalities	Number of chromosomes per cell	Mean difference	t-value	p-value
Non-technical	46 and < 46	- 0.0868	- 7.52	< 0.0001
Non-technical	46	- 0.0631	- 6.30	< 0.0001
Non-technical	< 46	- 0.3771	- 3.41	< 0.005
Technical	46 and < 46	- 0.1011	- 4.63	< 0.0001
Technical	46	- 0.0912	- 4.69	< 0.0001
Technical	< 46	0.2687	2.00	0.056

TABLE 4.2 t-test performed on the difference in the number of abnormal cells between the control (C) and hormonal contraceptive (HC) groups

Ratio	HC	C	Mean difference	t-value	p-value
T	$^{410}/_{1286}$	$^{233}/_{1255}$	0.1306	6.47	< 0.0001
A	$^{305}/_{1286}$	$^{177}/_{1255}$	0.0942	6.98	< 0.0001
B	$^{105}/_{1286}$	$^{56}/_{1255}$	0.0364	3.14	< 0.005

Ratio T = Total number of abnormal cells/Total number of cells

Ratio A = Number of abnormal cells with 46 chromosomes/Total number of cells

Ratio B = Number of abnormal cells with less than 46 chromosomes/Total number of cells

hormonal contraceptive it was decided to categorize three different groups for the more common contraceptives, viz. Minovlar ED, Depo-Provera and Ovral or Nordiol, and a fourth mixed group for the remaining types either singly or in combination. The various groups were compared to each other by means of Bonferroni's procedure and only in the Depo-Provera group were there significantly more nontechnical abnormalities when compared to the Minovlar ED group ($p < 0.001$). No significant differences were found between the other groups.

Using the same four groups, the individuals in each group were compared with their own respective controls to determine if all groups of hormonal contraceptives produce a significant increase in chromosome abnormalities, or whether this is confined to a particular class of hormonal contraceptives.

For this purpose the difference in the number of nontechnical abnormalities per cell between each study individual and her respective control was calculated. The means of the differences between study and control subjects in each group is shown in Table 4.3.

The groups on Minovlar ED (19 cases), Depo-Provera (4 cases) and the mixed group (18 cases) all showed a highly significant increase in the number of nontechnical abnormalities per cell when compared with their own controls ($p < 0,001$). The small group on Ovral or Nordiol (3 cases) did not show a significant difference over their controls at the 2% level.

4.3.2 Chromosome abnormalities and duration of treatment

To determine if there was any dose-related relationship between the duration of use of hormonal contraceptives and the number of

abnormalities found, two groups were established according to the duration of use of the hormonal contraceptives. The two groups therefore consisted of those who had used hormonal contraceptives for more than 34,5 months and those who had used them for less than 34,5 months. The cut-off value of 34,5 months was chosen because it was approximately at the median duration of the joint sample. The χ^2 test showed no significant difference between the two groups.

4.3.3. Chromosome abnormalities and age

In both control and hormonal contraceptive groups no significant correlation (χ^2) was found between the number of chromosome abnormalities and age. The cut-off value for their ages was calculated to be 20,5 years for the control group and 24,5 years for the hormonal contraceptive group. (Again, the cut-off values were chosen approximately at the median age of the joint sample.)

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TABLE 4.3 t-tests performed on the mean difference in the number of non-technical chromosome abnormalities/cell between cases on a specific type of contraceptive and their own controls

Hormonal contraceptive	Mean difference	t-value	p-value (DF=40)
Depo-Provera (4 cases)	0.2068	5.68	< 0.001
Minovlar ED (19 cases)	0.0779	4.66	< 0.001
Ovral or Nordiol (3 cases)	0.1000	2.379	< 0.05
Mixed group (18 cases)	0.1203	7.01	< 0.001

4.3.4 Chromosome abnormalities and race

There was no significant difference (χ^2) in the number of chromosome abnormalities found in Black versus White populations in both the study or control groups (see Section 2.3.1).

4.3.5 Chromosome abnormalities and smoking

Smokers and nonsmokers were compared in both study and control groups. No significant differences (χ^2) in the number of abnormalities were found between smokers and nonsmokers of the same group.

4.4. Giemsa-banded studies

(Study group n = 188 cells; control group = 172 cells; see Section 4.5 for statistical analysis.)

Collective data on chromosome abnormalities found in both study and control groups is presented in Table 4.4. Two normal banded karyotypes (see Figures 4.14 and 4.15) and several examples of banded abnormalities (see Figure 4.16) are presented.

4.4.1 Dicentric chromosomes

Study group

One dicentric was found in the banded metaphases of the study group.

The dicentric resulted from a malunion of chromosomes 3 and 7. The dicentric described had an accompanying acentric fragment; this was the only dicentric in the study which had an accompanying fragment

TABLE 4.4. Banded studies in hormonal contraceptive group versus control group (combined data)

	Number of cells	Dicentrics	Acentric fragments	Rings	Rearrangements	Chromosome deletions	Chromatid deletions	Chromosome breaks	Chromatid breaks	Chromosome gaps	Chromatid gaps
Hormonal contraceptive group	188	1	4	4	2	8	1	5	14	0	0
Control group	172	0	0	0	1	1	0	1	2	0	1

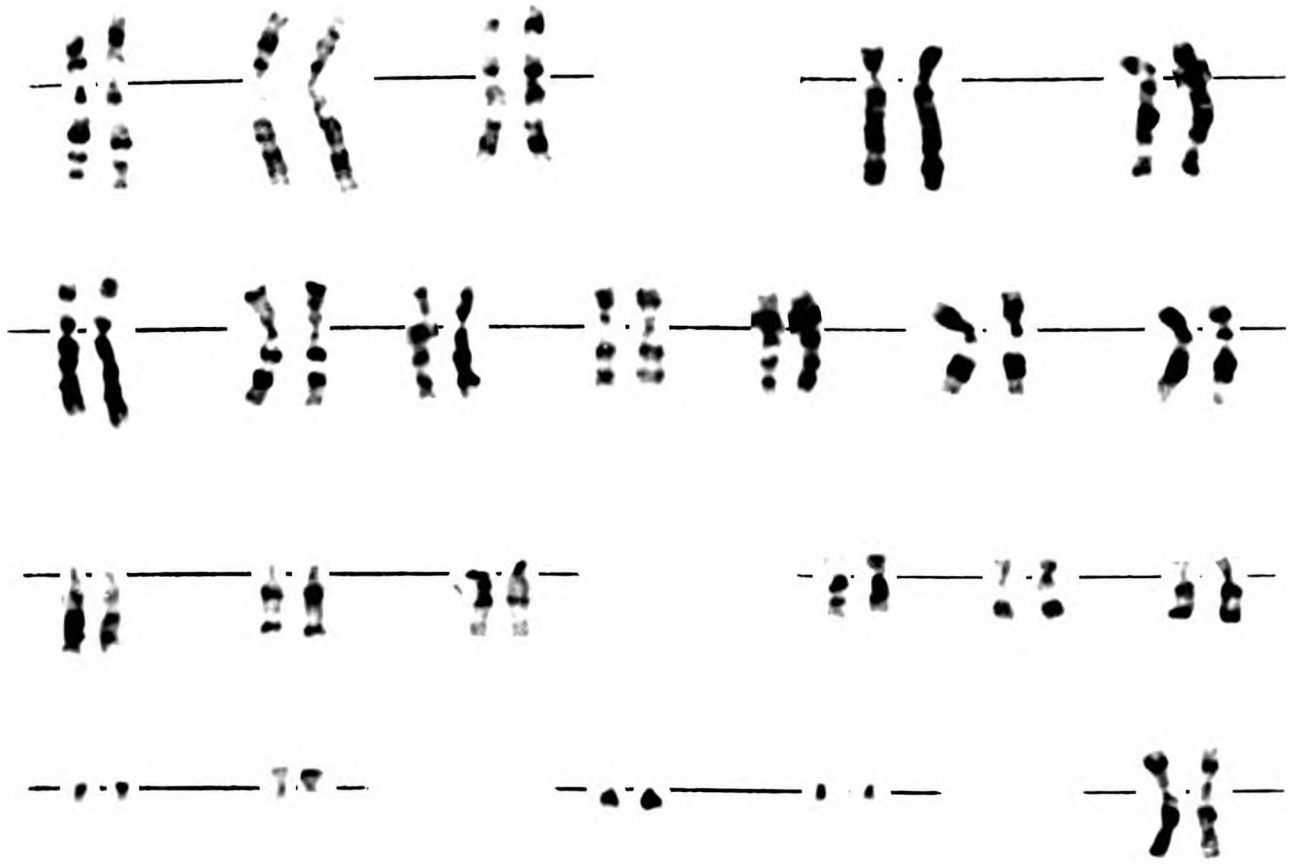

Figure 4.14. Giemsa banded karyotype obtained from one of the 'study' subjects.

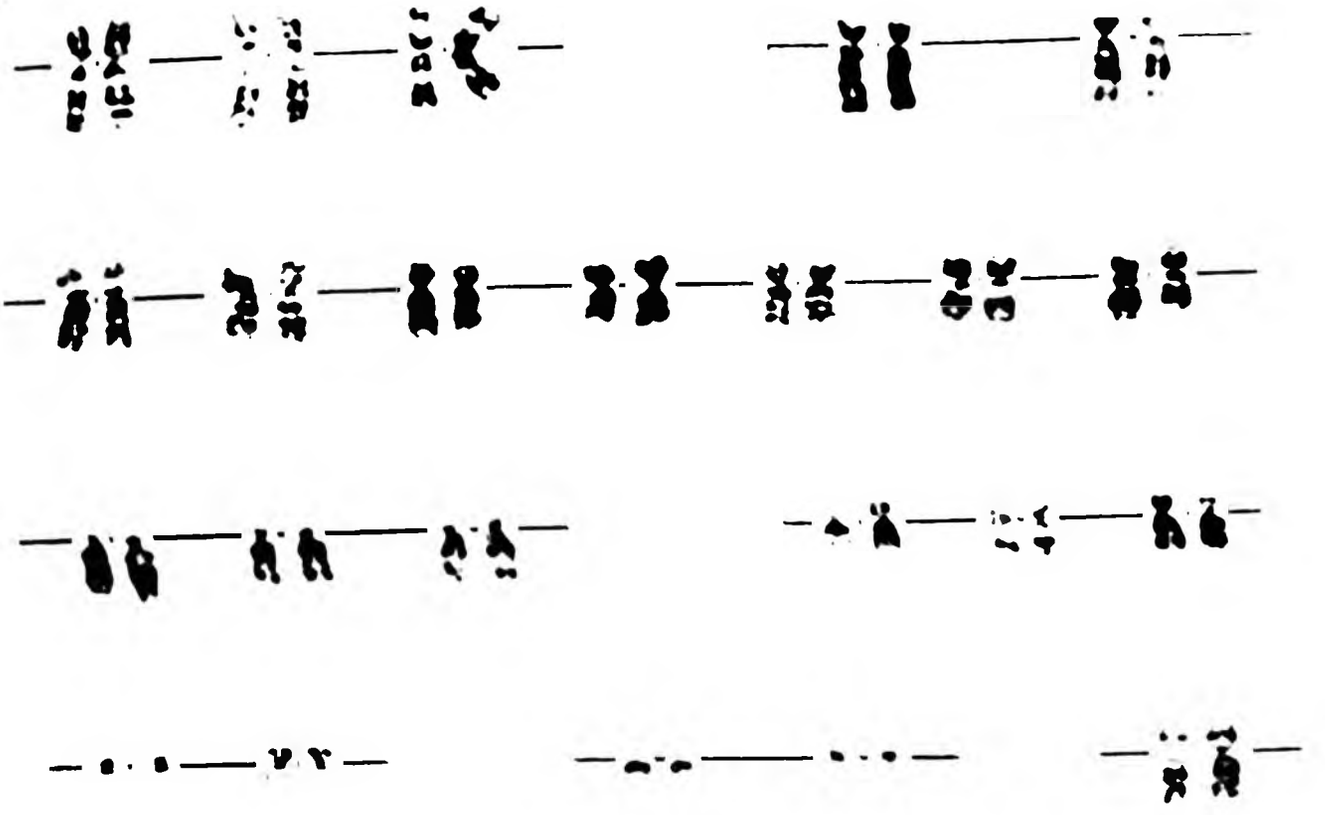

Figure 4.15. Giemsa banded karyotype obtained from another 'study' subject.

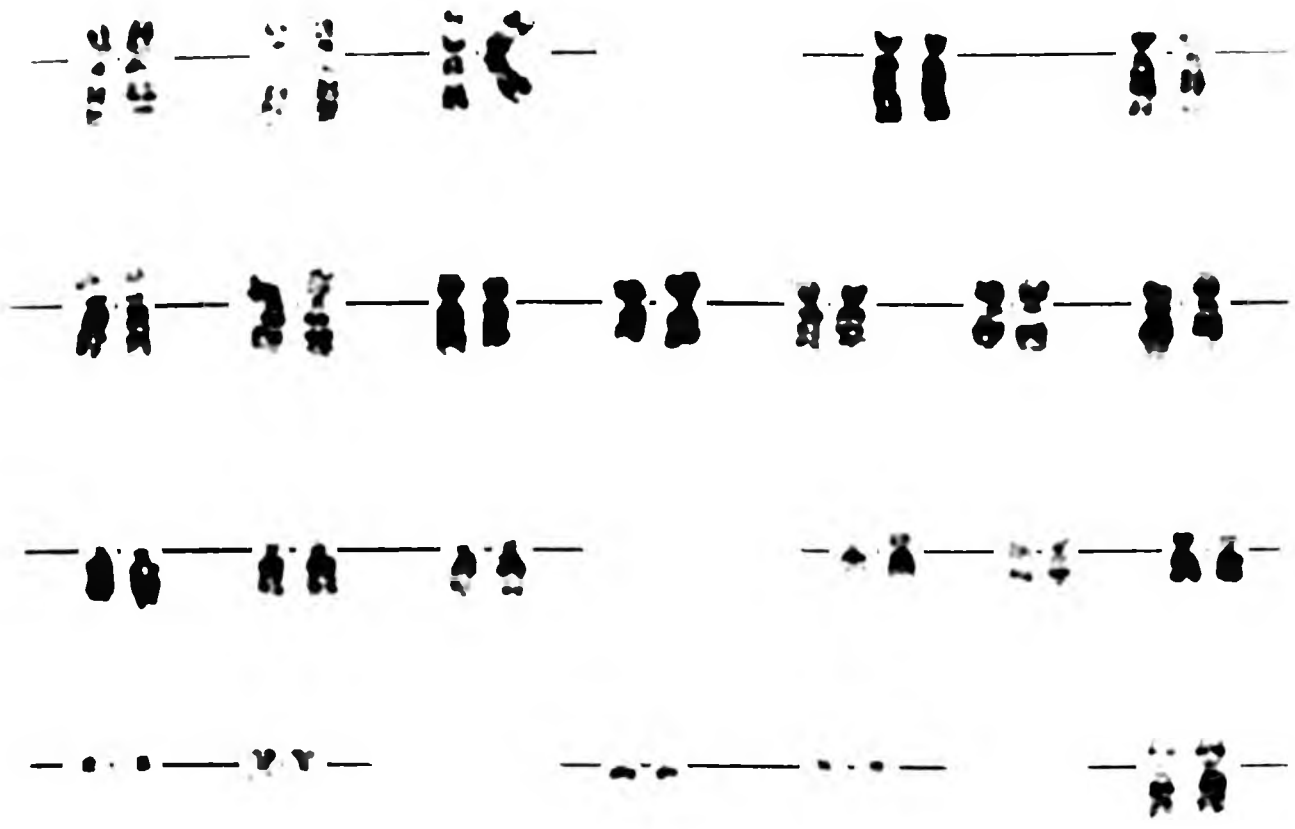

Figure 4.15. Giemsa banded karyotype obtained from another 'study' subject.

Figure 4.16. Examples of some banded abnormalities detected: b) Rearrangements quadriradial figures involving (10;18), (11;17), 16;16). c) Rings. d) Acentric fragment from a deletion on 2(p24). e) Chromosome deletions on 1(p36) and 20(q12). f) Chromatid deletion of 9p. g) Centromeric chromosomal break of chromosome 2. h) Chromatid breaks of 3, 15, 18 and 16 (the latter two showing rotation of the broken chromatids). i) Chromatid gap of 8.

in the same metaphase.

Control group

No dicentric chromosomes were found in banded preparations of this group.

4.4.2 Acentric fragments

Study group

A total of four acentric fragments were found in this group. One of these acentric fragments was the accompanying fragment from a dicentric formation mentioned previously in Section 4.4.1.

Control group

No acentric fragments were detected in this group.

4.4.3 Ring chromosomes

Study group

There were four ring chromosomes in this group. Two of them were ring X chromosomes, the third was a ring 10 and the fourth was also a C group chromosome but could not be accurately identified.

Control group

No ring chromosomes were detected in this group.

4.4.4 Rearrangements

Study group

Two rearrangements were detected in this group. One quadriradial figure involved both homologues of chromosomes 16 (case 14/2), and the

other quadriradial involved chromosomes 11 and 17 (case 31/2). Both of these two cases were already mentioned when rearrangements in unbanded metaphases were discussed (see Section 4.2.4).

Control group

One quadriradial figure involving chromosomes 10 and 18 was found; this control subject was a student.

4.4.5 Chromosome deletions

Study group

Eight chromosome deletions were detected in the study group. There was only one case (case 14/2) with two chromosome deletions; one involved the short arm of chromosome 8 and the other the long arm of chromosome 3. This case also had two rearrangements (one in a banded cell and the other in an unbanded metaphase).

Control group

One chromosome deletion involving chromosome 3 was detected in this group.

4.4.6 Chromatid deletions

Study group

Only one chromatid deletion was found on analysis of the hormonal contraceptive group.

Control group

No chromatid deletions were detected in the banded metaphases of this group.

4.4.7 Chromosome breaks

Study group

Five chromosome breaks were detected in the banded metaphases of this group. The five chromosome breaks belonged to four cases, i.e. one case showed two banded chromosome breaks (case 1/2) and also had several other abnormalities.

Control group

One chromosome break on chromosome 10 was found in this group.

4.4.8 Chromatid breaks

Study group

Fourteen chromatid breaks were found. The maximum number of banded chromatid breaks found in the same individual were four (case 16/2).

Control group

Two chromatid breaks were detected in two different control individuals.

4.4.9 Chromosome gaps

Study group

No chromosome gaps were detected in the 188 banded metaphases.

Control group

No chromosome gaps were detected in the 172 banded metaphases of this group.

4.4.10 Chromatid gaps

Study group

No chromatid gaps were detected in the study group.

Control group

One chromatid gap was detected in the 172 banded metaphases analyzed from this group.

4.5 Statistical analysis of the results on banded data

In view of the small number of banded cells (360) and small numbers of the different abnormalities observed, collective data of all chromosome abnormalities from all cases of the hormonal contraceptive group and from all cases of the control group had to be assessed and compared.

The collective data (analyzed by means of the χ^2 test) showed a highly significant increase in the incidence of chromosome abnormalities in the hormonal contraceptive group when compared to the control group ($p < 0,001$). These results are in agreement with the results of the unbanded studies.

4.6 SCE results

4.6.1. Sister chromatid exchanges (SCE)

SCE was done in ten additional cases - five pairs of study and control individuals. Of these five pairs, which were selected according to the protocol established (see Section 2.3), all except one pair were matched by smoking habits.

Of the four pairs matched by smoking habits, three pairs did not smoke and in one pair both 'pill' user and control smoked about twenty-five cigarettes per day.

In the fifth pair who were not matched by smoking habits, the control did not smoke and the 'pill' user smoked about twenty cigarettes per day.

The mean SCE/cell was calculated in all ten cases and these data, as well as relevant clinical data, are presented in Tables 4.5 and 4.6.

It is interesting to note that in all five different pairs the mean SCE/cell was always higher in the hormonal contraceptive group than in the control group. The highest difference of the means within a pair was 4,2; this was found in pair number 1, the pair that was not matched by smoking habits. In all the other pairs matched by smoking habits, the highest difference in the means was 3,5 in pair number 2 and the lowest difference in the means was 1,4 in pair number 4.

In the control group the highest value of SCE/cell was found in the control individual of pair 3, who was a 'heavy' smoker; in the study group the highest values of SCE/cell were found in 'pill' users who also smoked ('pill' users of pair number 1 and pair number 3).

The mean SCE/cell from all the control cases was found to be 10,42 and the mean SCE/cell from all of the hormonal contraceptive users was 13,02. Two photographs demonstrating SCE are presented: Figure 4.17 is stained with acridine orange and Figure 4.18 is stained with Giemsa after acridine orange.

TABLE 4.5. Distribution of SCE/cell in the hormonal contraception and control groups

	Hormonal contraception Mean SCE/cell	Control Mean SCE/cell
Pair I	13,2	9
Pair II	12,6	9,1
Pair III	16,1	14,1
Pair IV	11,2	9,8
Pair V	12	10,1
Total	13,02	10,42

TABLE 4.6. Use of hormonal contraceptives and smoking habits in ten cases subjected to SCE studies

		Type of hormonal contraceptive	Duration of treatment	Smoking habits
Pair I	Hormonal contraception	Lyndiol 2:5 + Minovlar ED	84 months	± 20 cigarettes/day
	Control	0	0	0
Pair II	Hormonal contraception	Minovlar ED	11 months	0
	Control	0	0	0
Pair III	Hormonal contraception	Nordette	60 months	20 - 30 cigarettes/day
	Control	0	0	20 - 30 cigarettes/day
Pair IV	Hormonal contraception	Nordette	12 months	0
	Control	0	0	0
Pair V	Hormonal contraception	Norinyl	6 months	0
	Control	0	0	0

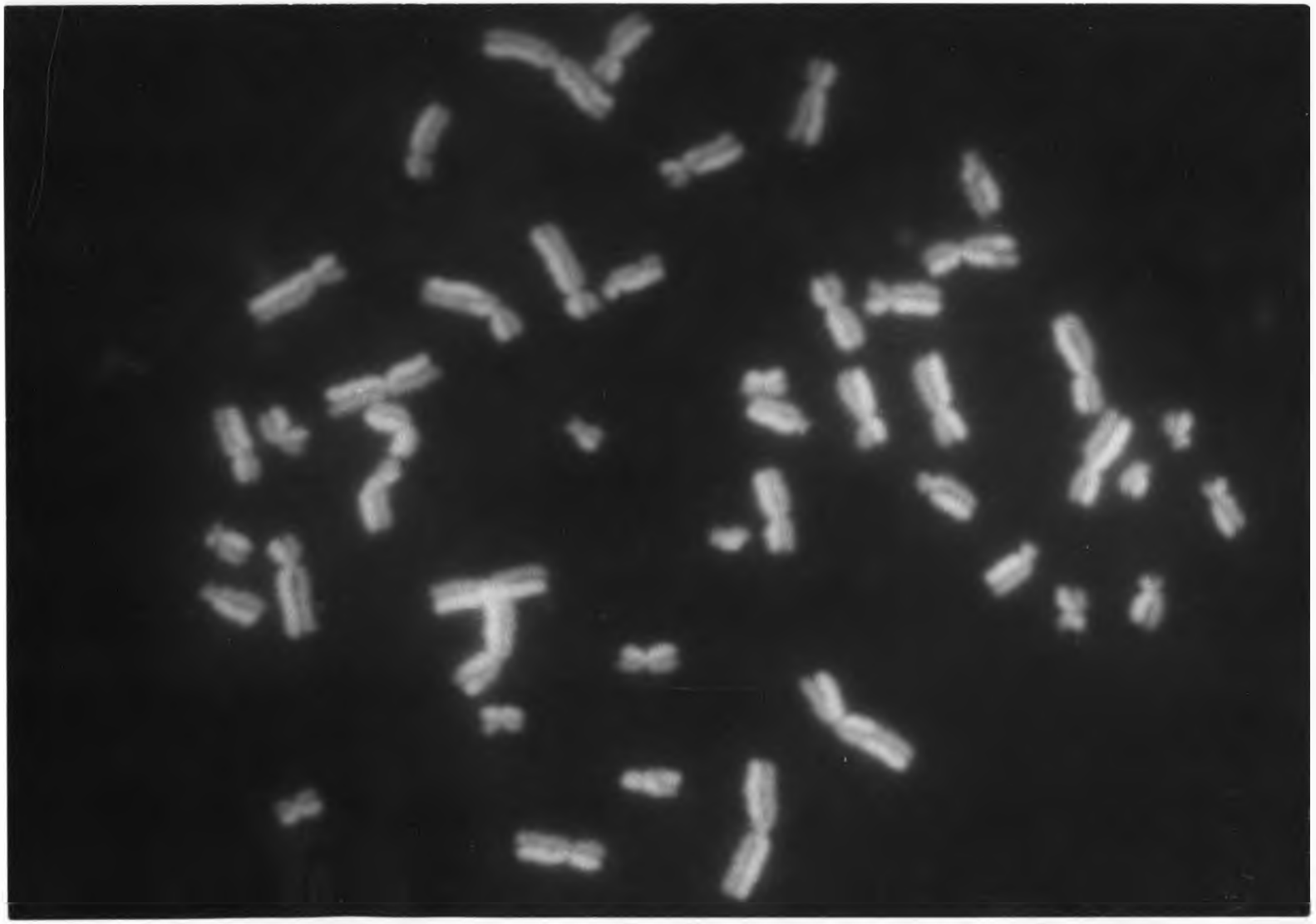

Figure 4.17. Black and white print of sister chromatid exchanges (SCEs) stained with acridine orange.

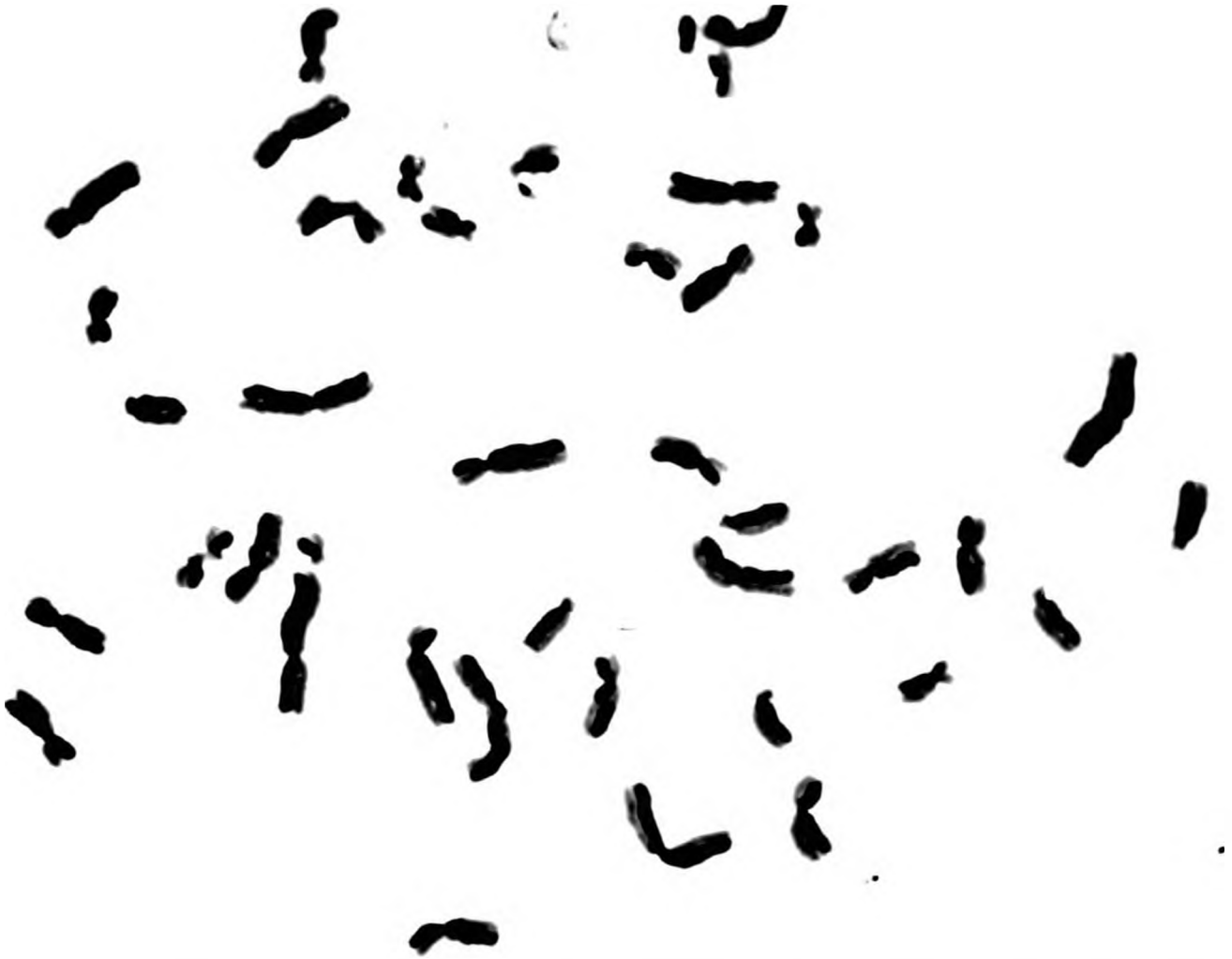

Figure 4.18. Sister chromatid exchanges (SCEs) stained with Giemsa stain after acridine orange staining.

4.7 Discussion of results

The exclusion of six pairs (twelve individuals) from the statistical analysis on unbanded data was decided on because of the choice of the statistical method. In consultation with the statisticians, it was decided to use a paired t-test in which each member of a pair (hormonal contraceptive user and control) was statistically analyzed one against the other, rather than other statistical tests where the totality of the study subjects is compared with the totality of the controls. It was therefore necessary to exclude the cases which were part of a set of three individuals done at the same time, i.e. two study individuals with a single control or two controls with a single study individual. These sets of three individuals were processed at the same time on several occasions when the author had three individuals suitable for study at the same time but could not find the fourth one.

However, what was lost by excluding these cases from the statistical analysis, was probably gained in the accuracy of the statistical method used, as the paired t-test is one of the more accurate statistical tests, when applicable (see Section 3.9.12).

For each individual, the total number of abnormalities per cell, as well as the number of abnormalities likely to be technical (group 1), and nontechnical (group 2), were calculated and compared with their own matched member of the same pair. The same procedure was adopted for complete cells with forty-six chromosomes and for cells with less than forty-six chromosomes. The total number of abnormal cells independent of the chromosome number, as well as those with forty-six chromosomes and those with less than forty-six chromosomes, were also calculated

(see Table 3.1 for procedure and Tables 4.1 and 4.2 for results).

The author is aware that the selection criteria of only incorporating aneuploid cells if they were abnormal, is subject to criticism although the reason for this selection criterion was explained before (see Section 3.9.9).

However, even if all the cells with less than forty-six chromosomes are excluded, the results obtained are still highly significant and do not invalidate the overall data and conclusions.

Because certain types of chromosome abnormalities in this study, e.g. dicentrics, rings and rearrangements were found very seldom, it was not valid on statistical grounds to compare the number of each type of abnormality per cell within the two members of each matched pair, nor

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to compare the number of each type of abnormality per cell from all cases of the study group, with the homologous value from all cases of the control group.

Before discussing the overall results, a few comments will be made on each type of chromosome abnormality found.

4.7.1 Dicentric chromosomes

The mean number of dicentrics per cell (unbanded and banded) was 2.9 times greater in the hormonal contraceptive group (0.00407) than in the control group (0,0014) (see Figure 4.19).

The fact that the great majority of dicentrics were found in cells with less than forty-six chromosomes is compatible with the mechanism of formation of this type of aberrant chromosome (see page 66).

Of a total of eight dicentrics found in this study all except one had no accompanying fragment. As stated by WHO (1973) in 'Methods for analysis of human chromosome aberrations', 'The presence of a dicentric or centric ring structure with no accompanying fragment is an almost certain indication that the aberrant cell has proceeded through at least one mitotic division since irradiation'. The lack of acentric fragments are therefore, surely indicative that cells with dicentrics found in this study had gone through at least one mitotic division, thus losing the fragment. Since these cells were all derived from forty-eight hour cultures it is extremely unlikely that the dicentrics had originated *in vitro*; the most likely explanation is that they already existed *in vivo* before blood specimen collection.

However, two other explanations are possible: a) the cells with dicentrics had non-randomly lost their accompanying acentric fragment or b) the dicentrics were not genuine dicentrics.

In relation to the second alternative (b) the author is well aware of the subjective nature of the scoring procedure in chromosome breakage studies; dicentrics may be problematic, because chromatid overlap can resemble a second centromere, or two separate chromosomes could by chance be exactly aligned and adjoining one another, thus resembling a dicentric.

4.7.2. Acentric fragments

The total (banded and unbanded) mean number of acentric fragments per cell was 16,4 times higher in the hormonal contraceptive group (0,023) than in the control group (0,0014) (see Figure 4.20).

It is worth noting that such acentrics were commonly found in women taking Depo-Provera.

4.7.3. Ring chromosomes

The mean number of ring chromosomes per cell in unbanded preparations was very similar in both the hormonal contraceptive group and the control group. However, in banded preparations there were four rings in the hormonal contraceptive group and none in the control group; this gave a total of six ring chromosomes for the hormonal contraceptive group and two for the control group, with a mean in the total banded and unbanded number of rings per cell that was 2,8 times greater in the hormonal contraceptive group (0,004) than in the control group (0,0014) (see Figure 4.21).

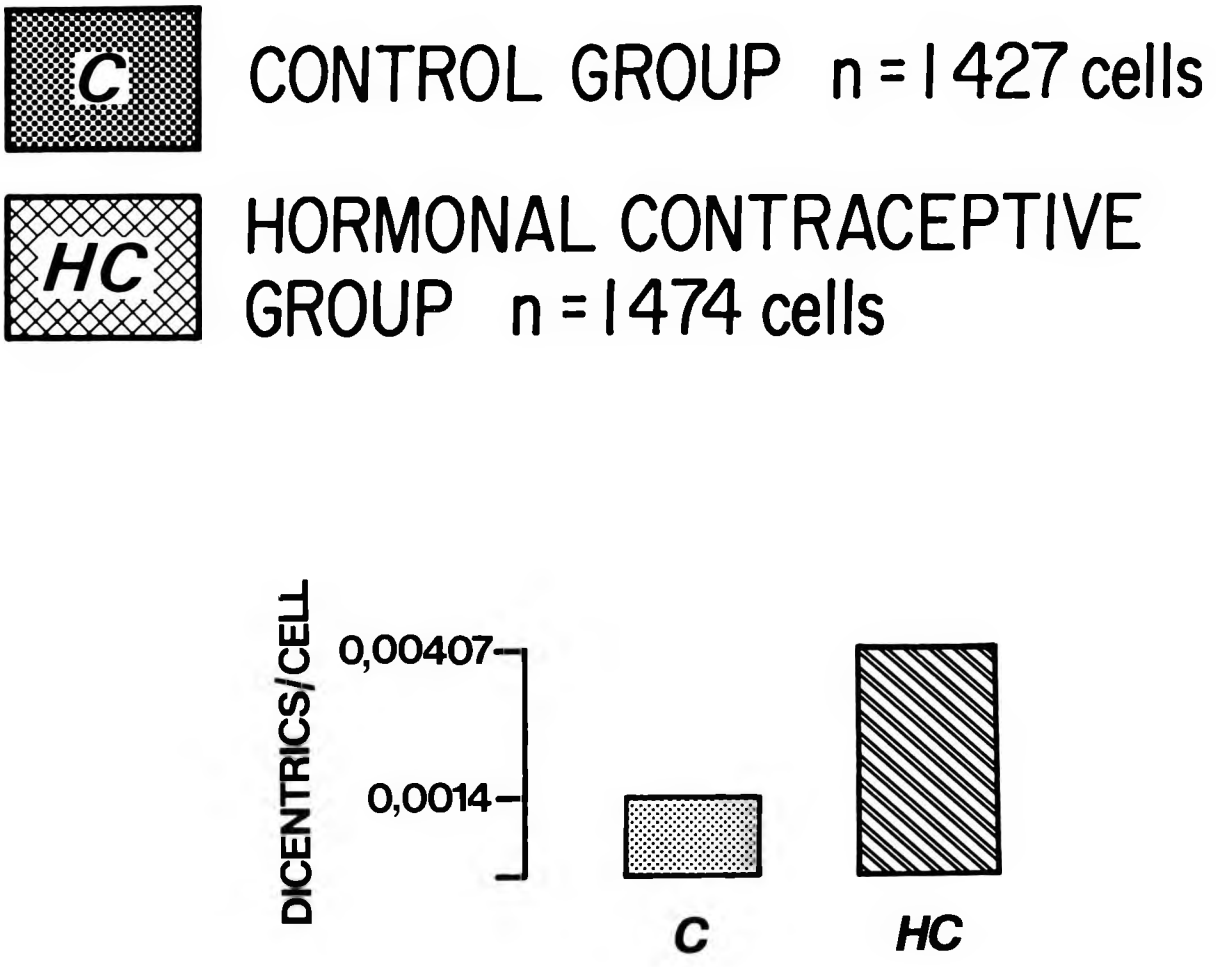

Figure 4.19. Comparison of the mean number of dicentrics per cell (unbanded and banded) between the hormonal contraceptive (HC) and the control (C) groups.

This apparent discrepancy in the number of rings found in the unbanded versus banded preparations with a much smaller number of analyzed cells in the latter, is probably a reflection of the difficulties and hesitations in distinguishing and identifying a

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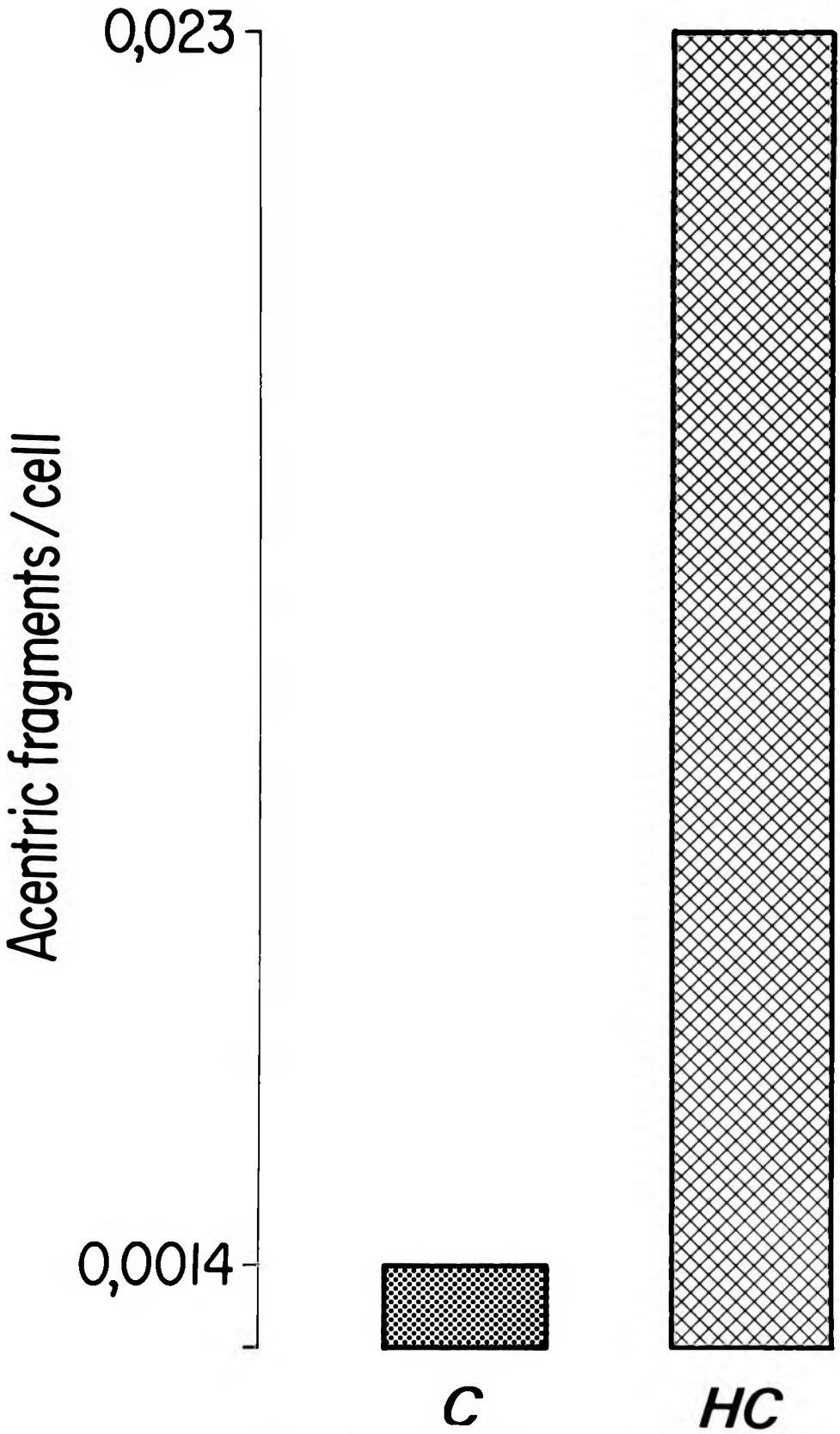

Figure 4.20. Comparison of the mean number of acentric fragments per cell (unbanded and banded) between the hormonal contraceptive (HC) and control (C) groups.

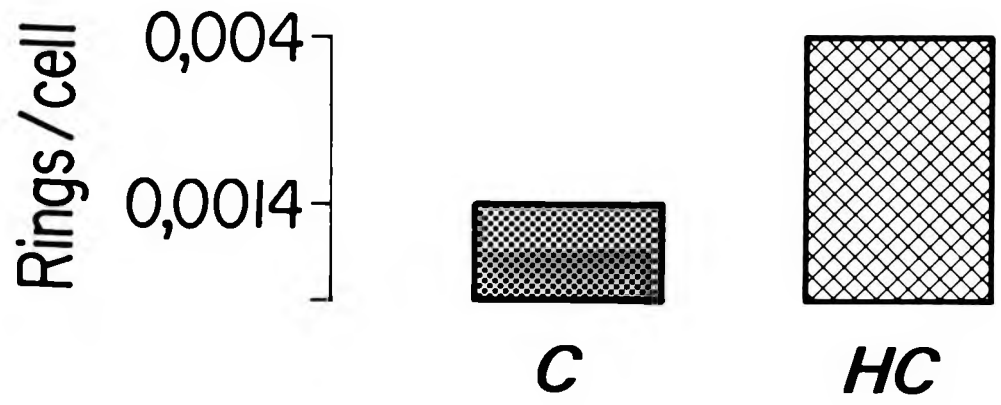

Figure 4.21. Comparison of the mean number of rings per cell (unbanded and banded) between the hormonal contraceptive (HC) and control (C) groups.

twisted chromosome in a rounded shape, especially long chromosomes, as a ring, unless the morphology was very clear. On the other hand, on banded preparations the pattern of the deletions in both short and long arms in a ring shaped chromosome will allow a more confident classification of such aberrations.

Ring chromosomes provided an example of a type of chromosome abnormality where banding was of value for a confident scoring of such an abnormality.

4.7.4 Rearrangements

The total (banded and unbanded) mean number of rearrangements per cell was 4,3 times greater in the hormonal contraceptive group (0,0061) than in the control group (0,0014) (see Figure 4.22).

The fact that all the rearrangements were found in hypodiploid cells was expected in view of the definition and mechanism of formation of these aberrant chromosomes.

4.7.5 Chromosome deletions

Again, in this group of abnormalities there was a discrepancy in the number of chromosome deletions found in unbanded preparations of the hormonal contraceptive group - two versus eight found in the banded preparations of the same group. The same discrepancy was found in the control group where no chromosome deletions were found in unbanded preparations and one was found in banded preparations.

These discrepancies emphasise the value of banding in the detection of this particular type of chromosome aberration. Unless the deletion is so large that it will be noticed in an unbanded

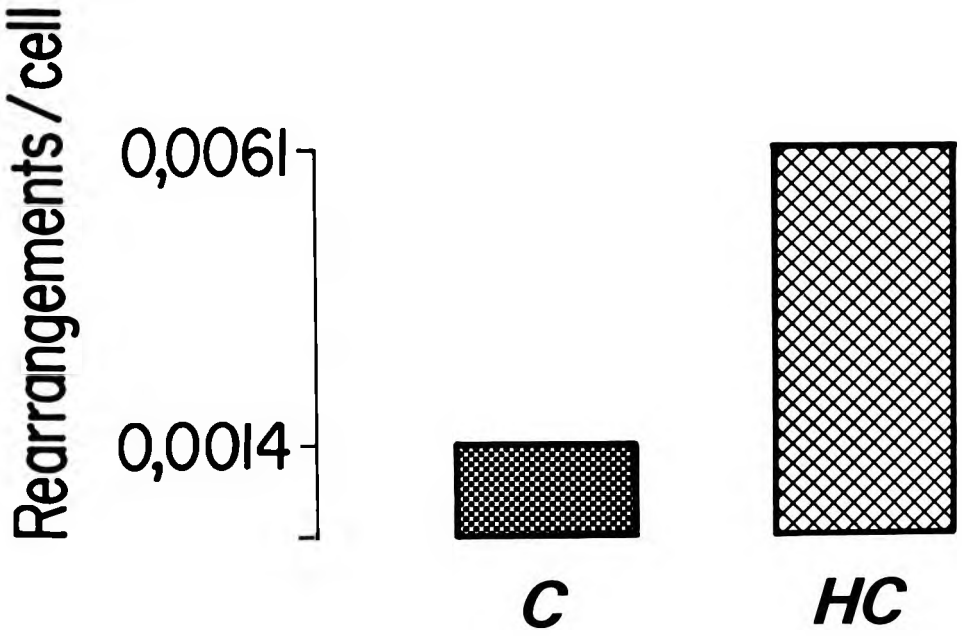

Figure 4.22. Comparison of the mean number of rearrangements per cell (unbanded and banded) between the hormonal contraceptive (HC) and the control (C) groups.

karyotype classified by groups, the abnormality is likely to be missed on unbanded preparations.

This seemed to be the case in this study where, although the great majority of cells analyzed were unbanded, the majority of chromosome deletions were found in banded preparations.

The total (banded and unbanded) mean value of chromosome deletions per cell was 9,5 times greater in the hormonal contraceptive group (0,0067) compared to the control group (0,0007) (see Figure 4.23).

4.7.6 Chromatid deletions

The total (banded and unbanded) mean value of chromatid deletions per cell in the hormonal contraceptive group (0,014) was twenty times greater than the mean value found for the same abnormality in the control group (0,0007) (see Figure 4.24).

4.7.7 Chromosome breaks

The hormonal contraceptive group had a five times greater total (banded and unbanded) mean value of chromosome breaks per cell than the control group (0,05) versus (0,01) (see Figure 4.25).

The relatively greater proportion of chromosome breaks found in the unbanded preparations versus banded preparations (see Sections 4.2.7 and 4.4.7) in both groups is an indication of the difficulties in screening aligned breaks in banded preparations especially if they occur in G-banded negatively stained regions.

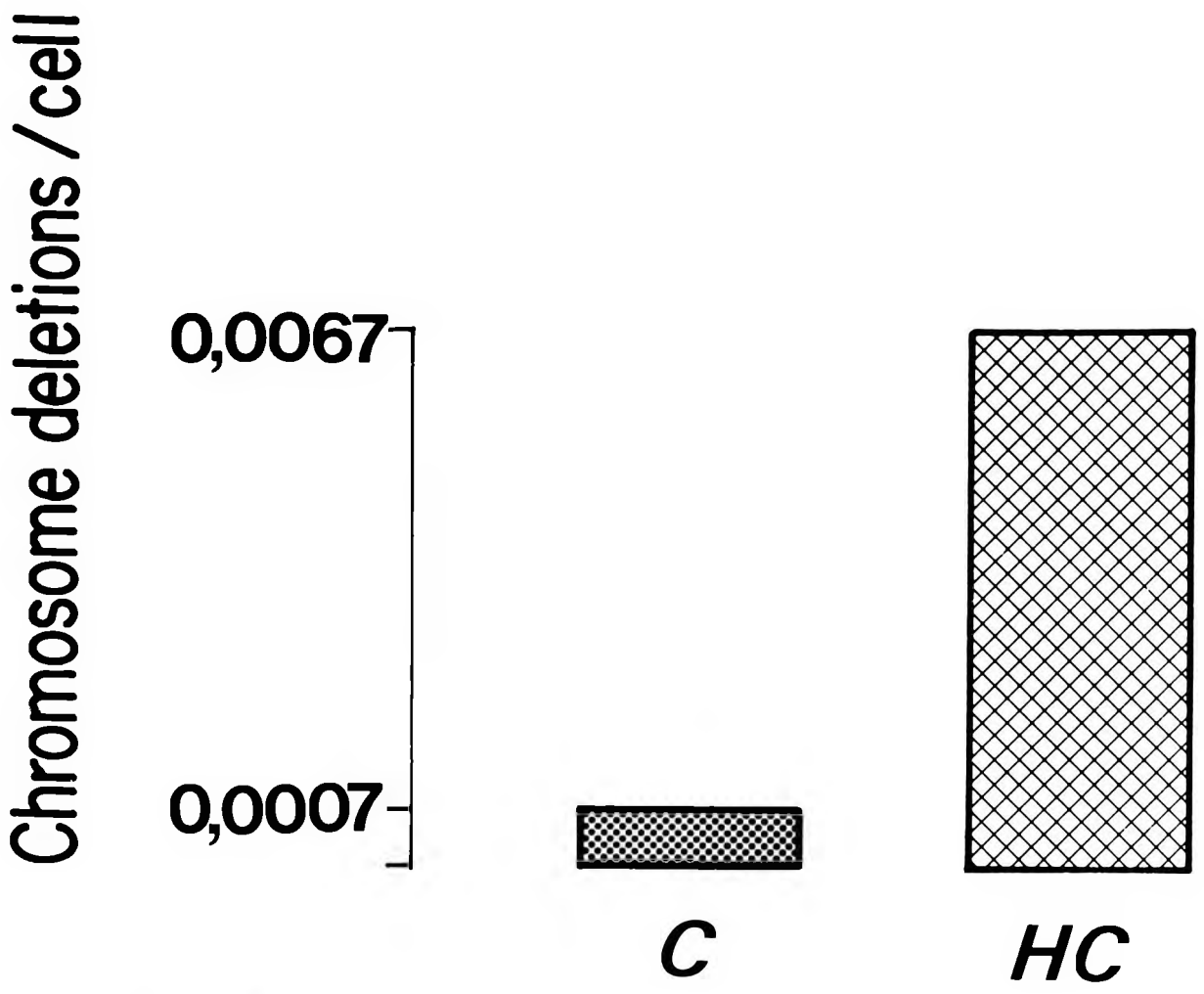

Figure 4.23. Comparison of the mean number of chromosome deletions per cell (unbanded and banded) between the hormonal contraceptive (HC) and control (C) groups.

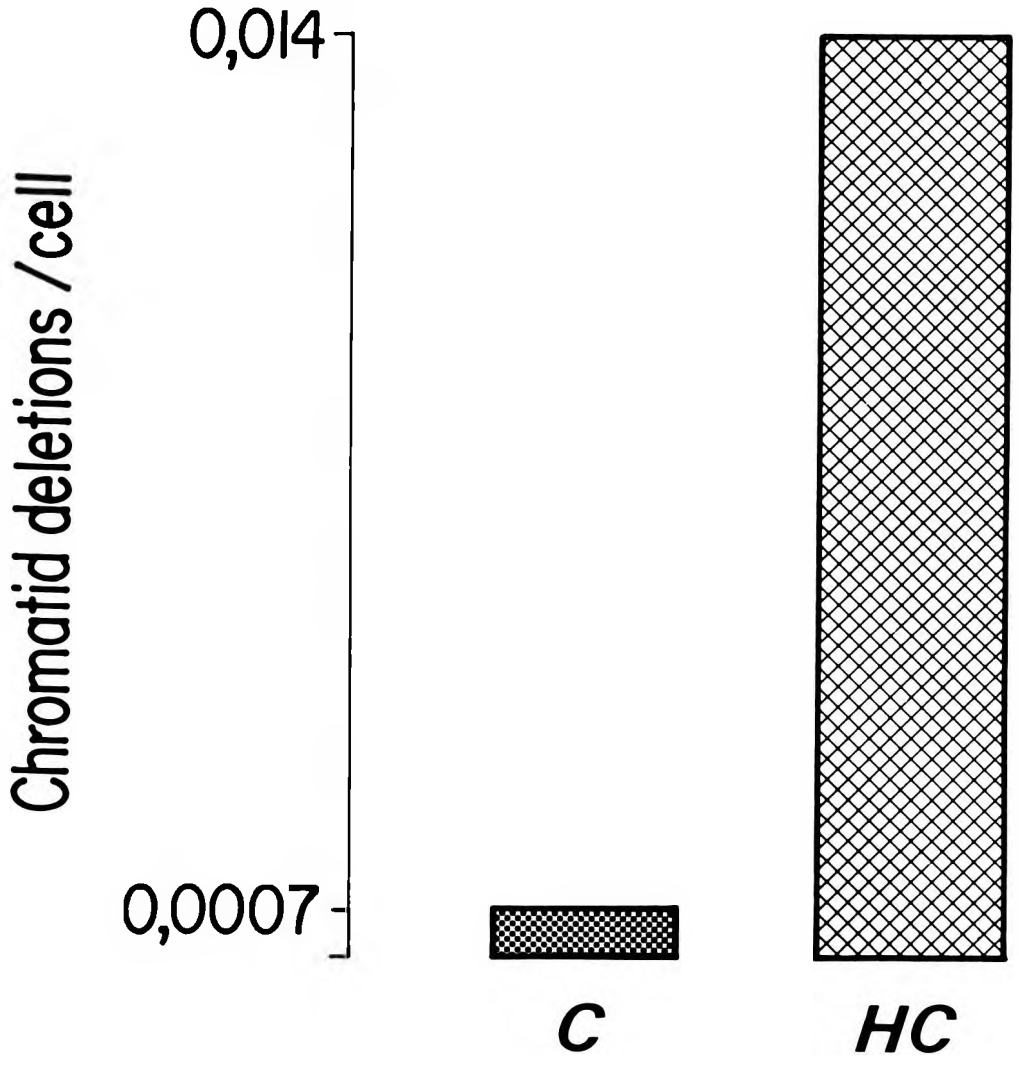

Figure 4.24. Comparison of the mean number of chromatid deletions (unbanded and banded) between the hormonal contraceptive (HC) and control (C) groups.

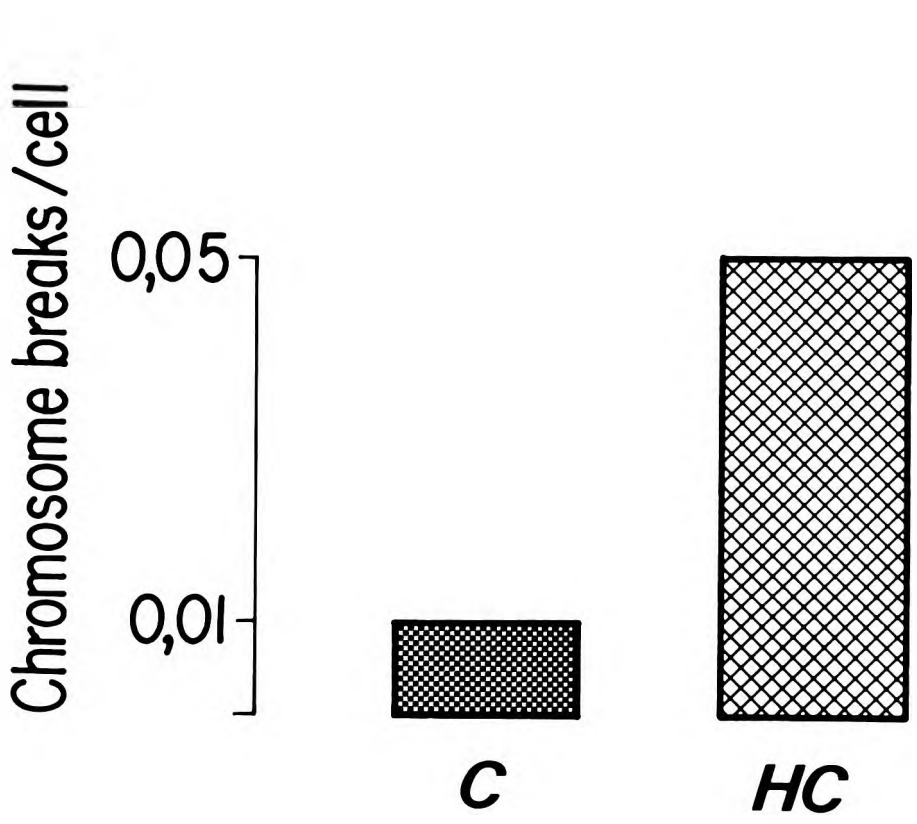

Figure 4.25. Comparison of the mean number of chromosome breaks per cell (unbanded and banded) between the hormonal contraceptive (HC) and control (C) groups.

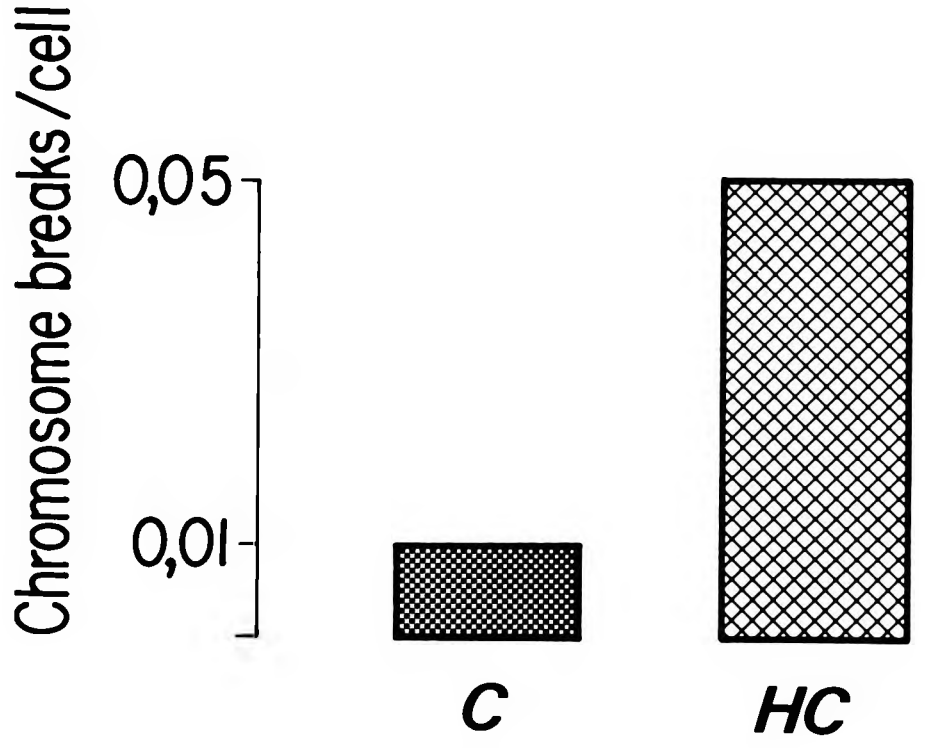

Figure 4.25. Comparison of the mean number of chromosome breaks per cell (unbanded and banded) between the hormonal contraceptive (HC) and control (C) groups.

4.7.8 Chromatid breaks

The total (banded and unbanded) mean value of chromatid breaks per cell was 1,8 times greater in the hormonal contraceptive group (0,24) compared with the control group (0,13) (see Figure 4.26). Again, there was a proportionally higher number of chromatid breaks detected in unbanded metaphases compared with the number detected on banded metaphases in both groups. The reason for this observation is presumed to be the same as that put forward for chromosome breaks.

4.7.9 Chromosome gaps

The total (banded and unbanded) mean value of chromosome gaps per cell was 2,8 times greater in the hormonal contraceptive group (0,002) than in the control group (0,0007) (see Figure 4.27).

4.7.10 Chromatid gaps

This was the only group where more abnormalities were found in the control group than in the hormonal contraceptive group.

In this group of abnormalities the total (banded and unbanded) value of chromatid gaps per cell was 1,48 times greater in the control group (0,037) than in the hormonal contraceptive group (0,025) (see Figure 4.28).

On screening for gaps (as for breaks), the expected figures for these abnormalities in banded preparations, in relation to the number of cells screened, was also lower. The reason put forward for the discrepancies in breaks again pertains. The number of gaps and nonaligned breaks were definitely underestimated in banded preparations.

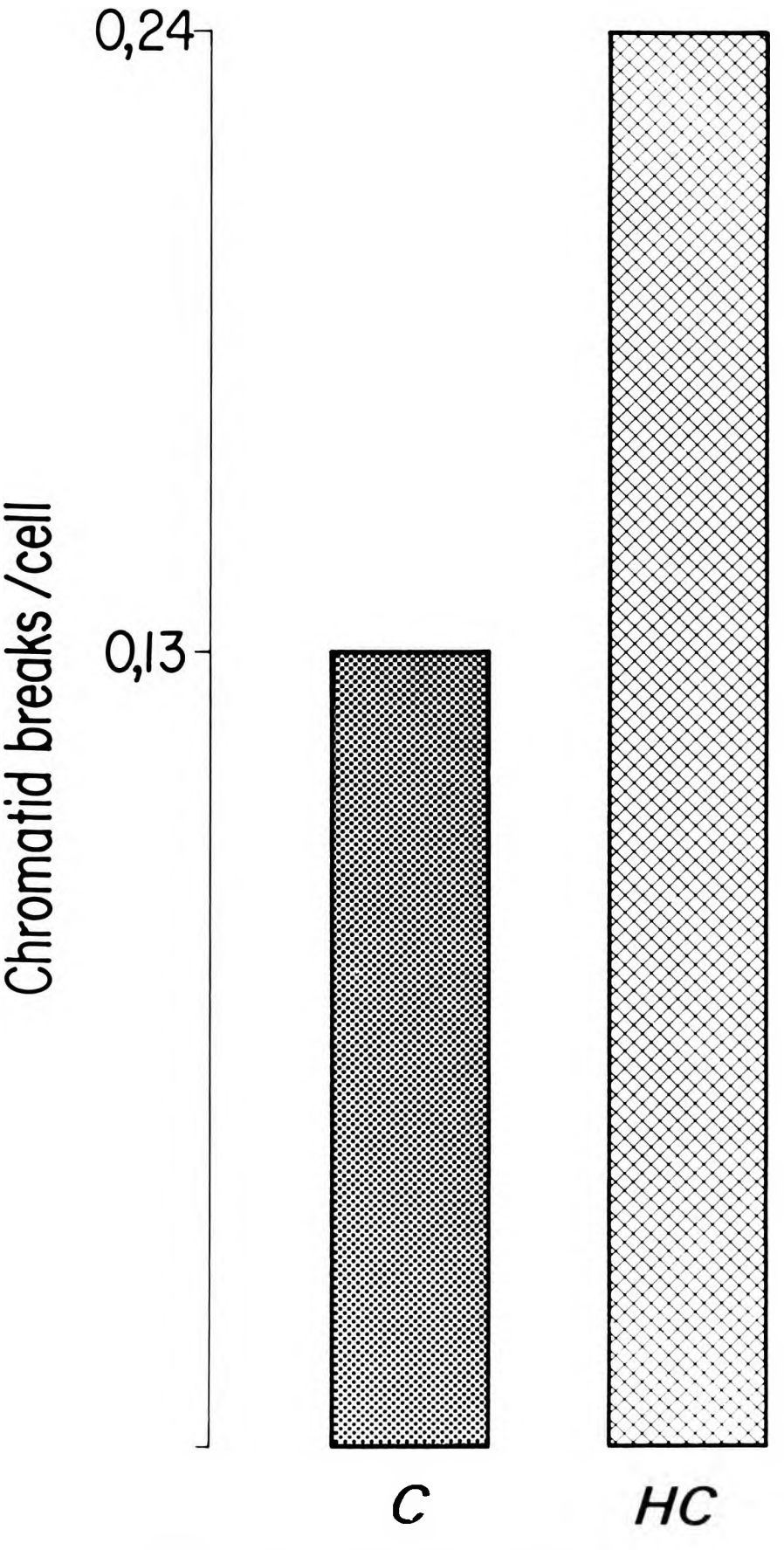

Figure 4.26. Comparison of the mean number of chromatid breaks per cell (unbanded and banded) between the hormonal contraceptive (HC) and control (C) groups.

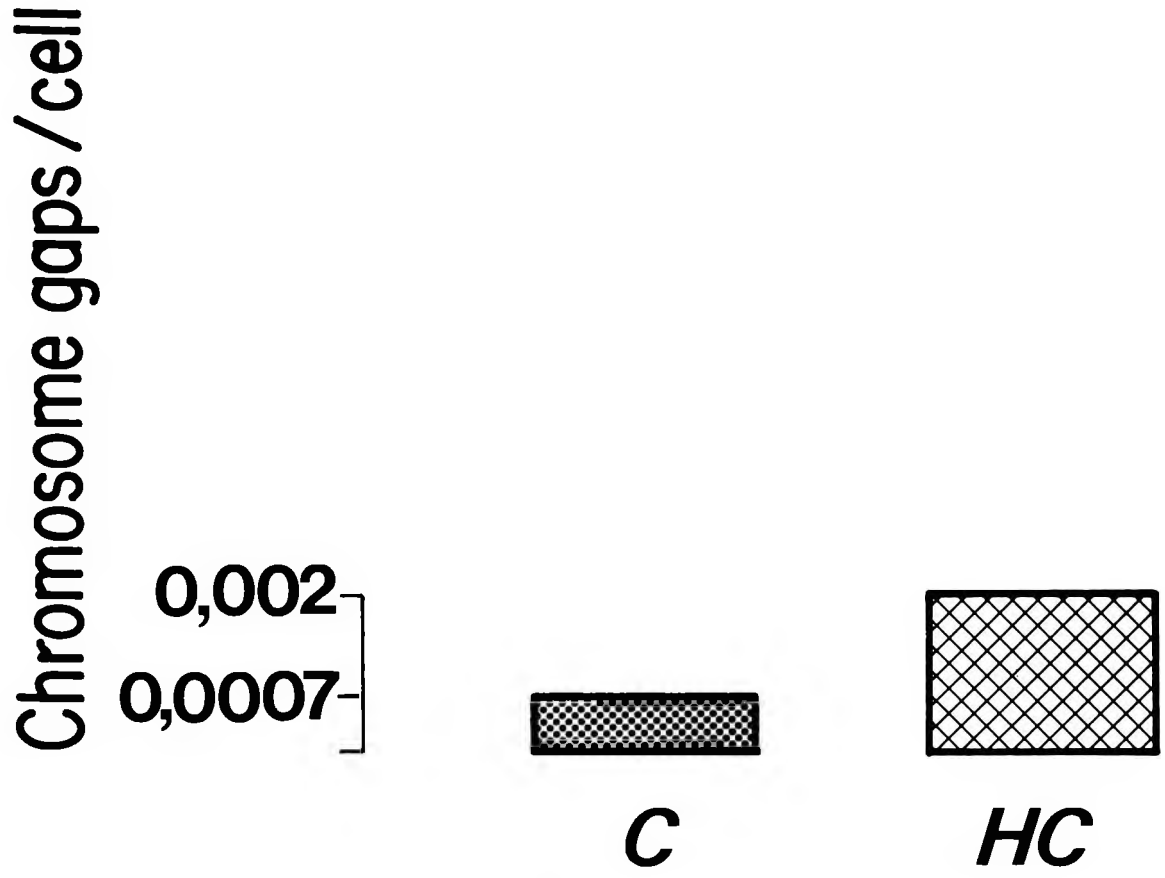

Figure 4.27. Comparison of the mean number of chromosome gaps per cell (unbanded and banded) between the hormonal contraceptive (HC) and control (C) groups.

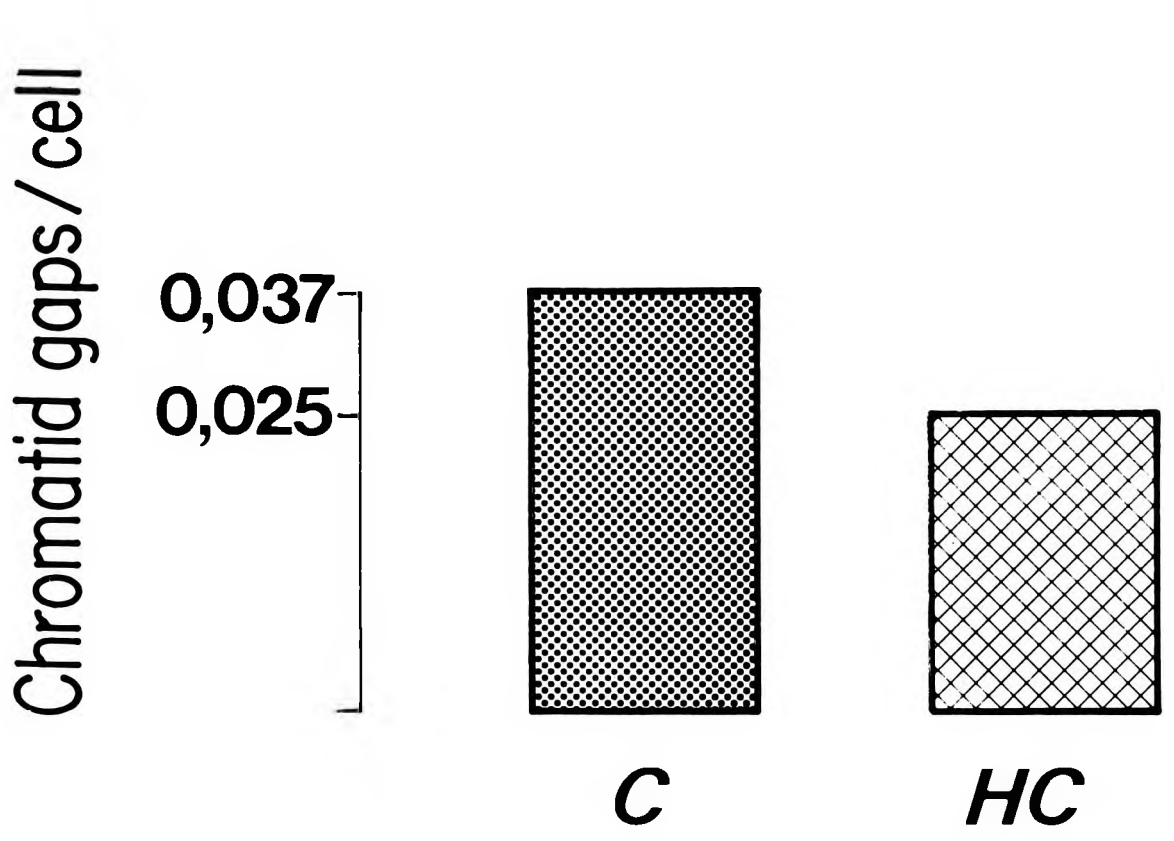

Figure 4.28. Comparison of the mean number of chromatid gaps per cell (unbanded and banded) between the hormonal contraceptive (HC) and control (C) groups.

The exact mechanisms producing gaps is not yet known and their biological significance is still questionable (Beckman *et al.*, 1979). However, it is worth noting that gaps and chromatid breaks, the types of abnormalities that were considered as possibly technical in origin, were the ones where the smallest differences between the two groups, hormonal contraceptive group and control group, were found.

With the exception of the deletions, the absence of stable abnormalities, such as small translocations and inversions, could be due to the difficulty, and in some instances, impossibility of detecting such abnormalities in unbanded metaphases, from which the great majority of data used in this study were obtained. The advantages and disadvantages of banded versus unbanded metaphases for chromosome breakage studies have already been discussed in Section 3.9.10.

4.7.11 SCE results

In view of the small number of cases studied and the small differences in SCE/cell found between the two groups, there would be no value in submitting these data to a statistical analysis; a nonsignificant difference would be expected. However, there is the possibility that this difference might become significant if the size of the sample was increased.

The biological significance of sister chromatid exchanges is at the moment still obscure (Latt *et al.*, 1980). However, analysis of SCE has been used as a sensitive and rapid test of chromosome damage by chemical and physical agents (Perry and Evans, 1975). In this

context SCE analysis has been used as an assay for testing mutagens and carcinogens.

4.8 Conclusions from results (see Table 4.7)

The overall conclusions that emerge from these results are that the use of hormonal contraceptives is associated with a highly significant increase in the incidence of *chromosome abnormalities per cell* and *the number of abnormal cells* when compared with their normal controls. Furthermore, when the total chromosome abnormalities per cell, were sub-divided into abnormalities that were likely to be technical and those abnormalities not likely to technical in origin, again a highly significant association between the use of hormonal contraceptives and an increase of these two subtypes of abnormalities was found. The above results applied irrespective of the modal number (forty-six and less than forty-six chromosomes), apart from the subgroup of technical abnormalities per cell in aneuploid cells. This latter observation, in the statisticians opinion, could be random fluctuation due to the fact that there were very few cells with less than forty-six chromosomes and thus the estimation showed a high variation.

In any kind of study, to find an association, either positive or negative, is not a difficult task; what is extremely difficult is to determine whether the association found is a *causal* one or not.

However, because of the care taken in the selection of the subjects, as well as in the technical procedure (and within the limits of practicality and human nature), the author would like to go further and suggest that the association found in this study is

a causal one, i.e. that hormonal contraceptives have a clastogenic effect.

These results are in agreement with some similar published work on the subject and, as expected in this kind of study, in

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TABLE 4.7. Overall summary of results from both unbanded and banded studies

	Hormonal contraceptive group	Control group	p-value
I.	Unbanded cells (n=1 286)	Unbanded cells (n=1 255)	
	1. Abnormalities per cell		
	a) non-technical	0.104	0.0175	<0.0001*
	b) technical	0.297	0.1968	<0.0001*
	2. Abnormal cells	410 (31%)	233 (18%)	<0.0001*
II.	Banded cells (n=188)	Banded cells (n=172)	
	1. Abnormalities per cell	0.207	0.0348	<0.001Ψ

* p-value obtained by paired t-test

Ψ p-value obtained by chi-square test

disagreement with others (see below).

In fact, in all published studies on the possible clastogenic effect of a certain chemical agent, apparently no single agent exists where total agreement between the different workers has been reached. Many factors have contributed in the past and will still contribute in the future, to the controversies concerning clastogenic effects, especially in *in vivo* studies. The adequate choice of subjects and their own controls is of primary importance; furthermore, the elimination of all known confounding factors, e.g. drug and radiation history, is very important. From the technical aspect, standardization of techniques, time of culture and criteria for classification and scoring of abnormalities are essential prerequisites for comparable studies. Coded samples, and a single observer for the entire chromosome analysis are also mandatory.

4.9 Conclusions from other similar surveys

4.9.1 Studies in agreement with the present results

Goh (1968) was the first to report on a higher incidence of chromosome breakage in women taking birth control 'pills'. He reported on five women taking three different types of oral contraceptives for a period of three months. He found a frequency of breaks of six to twelve per cent in the oral contraceptive groups compared with 0,8% in the controls. He further found that the percentage of abnormalities returned to normal in six to twelve weeks after discontinuation of the oral contraceptives.

Badr *et al.* (1971) reported on the effects of oral and injectable contraceptives on human chromosomes. They selected four groups of patients:

Group 1

Patients who took oral hormonal contraceptives regularly for periods ranging from four months to four years.

Group 2

Patients receiving injectable hormonal contraceptives every three months for a period of one year.

Group 3

Patients receiving injectable hormonal contraceptives every six months.

Group 4

Control group.

Their results showed a significantly higher incidence of chromosome aberrations especially in the first two groups when compared with the control group. Group 3, however, showed a decrease incidence of chromosome aberrations when compared with groups 1 and 2.

McQuarrie *et al.* (1970) also reported an increase in chromosome aberrations and a very high frequency of group D/G chromosome satellite association in a group of twenty women using sequential mestranol and mestranol with chlormadinone acetate cyclically, for extended periods, and in a group of three women who had used norethindrone (a contraceptive with only progestagen) in a daily dose of 0,35 mg for six months.

The authors mentioned that the choice of subjects was based on medical and genetic records. Their results were obtained from seventy-two and forty-eight hour cultures.

It is interesting to note that other studies also confirmed a higher frequency of acrocentric chromosome associations in women taking oral contraceptives (Bishun *et al.*, 1972; Müller and Ritter, 1978). The exact implications of these findings, especially in the incidence of Down syndrome is still controversial (Zellweger *et al.*, 1966; Muller and Ritter, 1978).

At the 4th International Congress of Human Genetics (1971), Littlefield *et al.* reported a significantly higher incidence of breakage in a group of twenty-nine women who had been taking oral contraceptives. In these women, serial chromosome studies had been performed every six weeks during a period of two years. The results were compared to similar concurrent studies done on ten control men and twenty control women.

Littlefield *et al.* (1975) confirmed their previously reported preliminary studies (Littlefield *et al.*, 1971). In an extended study over a period of four years, chromosome examinations were performed every four to seven weeks in a group of ten men and fifty-four women. The group of fifty-four women included controls, pregnant women and women taking oral contraceptives. All the subjects were between twenty and forty years of age, and the choice of participants for this study, as well as information on possible exposure to clastogenic agents, seem to have been carefully evaluated. The authors reported the lowest frequency of chromosome breakage in the cultures from men, and the highest in 'pill' users. A significantly higher average breakage in lymphocyte cultures was found in nulligravidas who were taking oral contraceptives when compared with nulligravidas who had never taken the 'pill'. It was also found that current 'pill' users had a significantly

higher breakage rate than the ones that had discontinued the use of the 'pill'.

It should be noted that these authors did not find a significant difference in the frequency of chromosome aberrations between 'pill' users and nonusers if the subjects had already had a previous pregnancy.

The present results are not, however, in agreement with others such as those detailed below.

4.9.2 Studies not in agreement with the present results

Shapiro *et al.* (1972) reported their cytogenetic findings on seven young women before and after one and two months of oral contraceptive use (norethindrone 1 mg with mestranol 0,05 mg). In addition, four women who had been using hormonal contraceptives for more than three years (three to five years) were also studied cytogenetically and compared with four controls. The metaphases were obtained from seventy-two hour cultures and sixteen cells were analyzed from each individual. Only chromatid and chromosome breaks were found in this study, and no significant increase in the number of abnormalities was noted with the use of oral contraceptives.

The main criticism of this study is its small sample size, both in the small number of individuals and the few cells analyzed. Another criticism relates to the short duration of contraceptive use in the group of seven individuals on whom chromosome studies were done prior to and after treatment (only one and two months of treatment).

This was the only study where an attempt was made to relate

the duration of 'pill' usage to the frequency of breakage; the study failed to show such a relationship, but this was not surprising. In fact, it does not seem logical to expect a detectable increase in the number of chromosome aberrations after only one to two months of using a drug that, although synthetic, is similar to a normal female's own sex hormones.

The observation that no increase in abnormalities was found in the long-term users, could perhaps be due at least partially to lack of careful selection of subjects. The authors did not mention their criteria for the selection of individuals, apart from the fact that they were 'pill' users and controls.

Fitzgerald *et al.* (1973) published their results on seventeen women who had used different types of hormonal contraceptives for a period ranging from four and a half years to ten years, and on eighteen matched controls.

The metaphases obtained were derived from forty-eight hour cultures and whenever possible 100 metaphases were analyzed. It is of interest that in spite of the large number of cells analyzed, ten of the seventeen cultures in the hormonal contraceptive users and twelve of the eighteen cases from the control group did not have a single chromosomal break or gap. The authors found a mean incidence of cells with structural abnormalities in the oral contraceptive group nearly three times greater than in the controls; however, the incidence of such cells in individuals was, according to them, not significantly different between the two groups.

In the same year Gutierrez and Lisker (1973) reported their

cytogenetic findings on thirty-five women taking an oral contraceptive (100 µg mestranol and 1 mg ethynodiol diacetate) for at least six months. The authors used mixed cultures as controls, i.e. they mixed lymphocyte rich plasma from normal male controls with lymphocytes from women being tested. The cultures were harvested after sixty-five hours and a variable number of cells were analyzed from each subject. All the subjects were recalled for reinvestigation after one year and only seventeen subjects cooperated. Of these seventeen, none had continued to take the same contraceptive.

No significant differences in the number of chromosome abnormalities between 'patients' and 'male controls' were found.

Apart from the fact that the study group used hormonal contraceptives and that controls were medical students who had not been exposed to X-rays for at least six months, no mention was made as to how the subjects were selected. The authors acknowledge that the finding of a decreased number of structural chromosome abnormalities some months after discontinuation of hormonal contraceptives, is a possible indication that oral contraceptives do have some deleterious effect on chromosomes, in at least some women.

Matton-van Leuden *et al.* (1974) published their results of chromosome studies in thirty-two women on different types of oral contraceptives and one woman on an injectable hormonal contraceptive for different periods of time. Forty-two patients (twenty-six males and sixteen females) who were referred for cytogenetic investigations for a variety of reasons were used as controls.

Cultures were harvested at seventy-four hours and approximately

fifty metaphases per individual were analyzed by two different observers, each observer screening different cases. The data had to be analyzed separately because the results obtained by the two different observers were different. These authors found an incidence of chromosome abnormalities below their usual technical breakage rate in routine cytogenetic analysis in both groups.

Apart from the main criticism that in this study two observers were used, other criticism is the choice of subjects and controls; no attempt was made to match them by sex or age, and no mention is made of previous exposure to known clastogenic agents. The authors, however, acknowleged all the limitations in their study, and stressed the caution with which this kind of study had to be interpreted.

Studies by Bishun *et al.* (1975), Mills *et al.* (1975), Bishun (1976) as well as Briggs and Briggs (1971), on mothers who had taken hormonal contraceptives and their babies, cannot be compared with the present study, as there was at least a nine months's interval between the cessation of the therapy and obtaining the blood samples for chromosome study.

The fact that some lymphocytes have a very long life-span, is well illustrated by the presence of unstable abnormalities in survivors of atomic bombings in Japan twenty years after the event (Bloom *et al.*, 1966; Ishihara and Kumatori, 1967); however, the exact life-span of a lymphocyte is still uncertain (Lloyd and Dolphin, 1977). These authors mention the work of Sharpe *et al.* (1968) in which they found that after extracorporeal irradiation, using an arteriovenous shunt, the lymphocytes with aberrations disappeared

to the extravascular pool in about five minutes.

Apart from the studies based on *in vivo* hormonal contraceptives reviewed so far, a few *in vitro* studies have also been done. These results, however, cannot be compared with or extrapolated to *in vivo* studies as the metabolic processes that occur *in vivo* have been excluded.

4.10 Chromosome abnormalities and type of hormonal contraceptives

Apart from the group using Minovlar ED in the present study (nineteen cases), there were great difficulties in trying to establish any relationship between the different types of hormonal contraceptives and the frequency of abnormalities found, because of the small number of patients on each type of hormonal contraceptive.

Only after close contact with the Family Planning Clinic, did the author realize how few women found a 'pill' which was satisfactory to them at the first attempt, and also how easily women change from one type of hormonal contraceptive to another. Minovlar ED was the commonest type of hormonal contraceptive used because it is one of the lowest dosage 'pills' available at the local Family Planning Clinics.

The impression gained in this study that more abnormalities were noted in the group using Depo-Provera (refer to Section 4.3.1) cannot be compared with any other study, as the majority of previous studies were done on users of oral contraceptive preparations and very few on injectable preparations. One patient reported in Matton-van Leuven's study (1974), had used Depo-Provera for fifteen months. On the other hand, the small number of individuals in the Depo-Provera

group (only four), precludes any definite conclusions from the finding that the highest incidence of chromosome aberrations occurred in this group.

There were also a few individuals who were using or had used Depo-Provera, but they were not included in this group because they also received other oral contraceptives. It is obvious that in these cases, if a significant increase in abnormalities was found over and above the other groups of hormonal contraceptive users, those abnormalities could not have been specifically attributed to Depo-Provera or to any other oral contraceptive used. Interestingly, certain alterations in meiotic chromosomes have been reported in relation to Depo-Provera usage.

Camacho *et al.* (1972) reported on a series of four male patients suffering from constitutional sexual precocity who were given medroxy-progesterone for periods ranging from eighteen to seventy-five months, and in whom alterations in testicular histology and on meiotic chromosomes were noted. Testicular biopsies were obtained on three of the patients while receiving the drug and on the fourth patient, who had been treated for seventy-five months, the biopsy was obtained one year after discontinuation of the therapy. Spermatogenesis was arrested near the end of the first meiotic prophase and the chromosomes showed the characteristic 'stickiness' and condensation commonly associated with degeneration. Aneuploidy and polyploidy were also present. Although the author is aware that extrapolation from meiosis in spermatocytes to oocytes, as well as extrapolation from meiosis to mitosis is unreliable, the fact that these alterations have been noticed in human male meiosis, seems worthy of attention.

Not only was the increase in chromosome abnormalities significant for all hormonal contraceptive users combined, but when users of specific classes of hormonal contraceptives were individually compared with their controls, women on Minovlar ED, Depo-Provera and the mixed group also showed a highly significant increase in nontechnical abnormalities ($p < 0,001$). Only the three women using Ovral or Nordiol did not show a significant increase in these abnormalities above their respective controls. This is probably due to the small sample size.

(continued on page 127)

4.11 Chromosome abnormalities and duration of treatment

The dose-response relationship could not be established *in vivo* because of ethical considerations; therefore, the aberration frequency was related to the duration of treatment. No such relationship between duration of treatment and number of abnormalities was found. This was not surprising, since dose-response relationships for *in vivo* lymphocyte chromosomal damage are rarely found (Kirkland *et al.*, 1978) because severely chromosomally damaged cells tend to die, and mildly damaged cells are likely to repair the damage before sampling or during subsequent cultures (Buckton *et al.*, 1978).

Funes-Cravioto *et al.* (1977) found that in their studies, individuals "who had been exposed most heavily to organic solvents did not necessarily have the highest frequency of cytogenetic changes", and also that "there was a low correlation between the frequency of chromosome breakage and the number of years that the subjects had been exposed to organic solvents".

It has been suggested (Funes-Cravioto *et al.*, 1977) that "the degree of exposure, cell turnover and the degree of damage" as well as "genetically determined qualitative and quantitative differences in the metabolism of mutagens and DNA repair" are all confounding factors in the research for dose-response relationships in *in vivo* studies.

In fact, genetically determined differences, giving rise to individual variations are probably one of the most important factors in the search for dose-response relationships *in vivo*. This individual variation was observed in the present study, as well as in many other *in vivo* studies (Funes-Cravioto *et al.*, 1977; Purchase,

1978; Fischer *et al.*, 1978; Kemmar *et al.*, 1978; Funes-Cravioto *et al.*, 1977; Beckman *et al.*, 1979) using other chemical or physical agents.

4.12 Chromosome abnormalities and age

As expected, in view of the age limits introduced in the choice of subjects, there was no positive age-dose interaction such as had been noted in other studies (Evans *et al.*, 1979).

4.13 Chromosome abnormalities and race

In the discussion of materials (see Section 2.7.1) the question of possible differences in chromosome breakage and DNA repair in a Negroid and Caucasoid population was put forward. The idea that the chromosomal biology of Caucasoids may be different from Negroids was suggested by Stark and White (1977). Certainly no racial differences in chromosome breakage were found in this study. This can be tentatively interpreted as indicating either that there are no genetically determined racial differences, or if these differences do exist they were not apparent, because this was a selected and small sample of urbanized Blacks.

4.14 Chromosome abnormalities and smoking

The fact that no increase in chromosome abnormalities was associated with smoking in either control or study groups is probably due to the fact that there was a considerable number of cases in whom smoking habits were unknown, or it may be that none of the subjects (either from the control or study groups) were so-called 'heavy' smokers.

CHAPTER FIVE

5. MATERIALS

A preliminary pilot study was performed on 2 baboons with the object of studying a cell-line exposed to hormonal contraceptives *other* than peripheral blood lymphocytes. Such studies were not possible in humans for obvious ethical reasons.

A nonhuman primate, the Chacma baboon *(Papio ursinus)*, was selected as the experimental animal of choice on the recommendation of a World Health Organisation Symposium (1973) on pharmacological models in contraceptive development. They advised the use of an animal model closely related to man. Furthermore, female baboons have a known menstrual cycle of thirty to thirty-five days, with ovulation at mid-cycle and a similar hormonal profile to that of the human female (Mitruka, Rawnsley and Vadehra, 1976).

The Chacma baboon *(Papio ursinus)*, is the most readily available nonhuman primate in this region of southern Africa (Figure 5.1).

These animals reach puberty at about four years of age, but are not fully grown until seven years (Mitruka, Rawnsley and Vadehra, 1976). The weight of a female Chacma baboon is approximately 13 Kg (half of the male weight) (Mitruka, Rawnsley and Vadehra, 1976).

The baboons used in this study were wild-caught and housed in the Animal Unit (Frankenwald) of the University of the Witwatersrand. They were visually inspected daily by the staff of both the Central Animal Unit and of the Department of Psychology and Primate Behaviour Research Group to determine signs of ovulation, menses, abnormal bleeding or any diseases.

Figure 5.1. Photo of an adult Chacma baboon (*Papio ursinus*).

5.1 Female meiotic chromosomes *(Papio ursinus)*

The first experimental project was the study of the possible effects of hormonal contraceptives on meiotic chromosomes of baboon females. To establish the techniques, ovaries of female baboons sacrificed after the completion of experiments in other departments, were used. The ovaries were collected in a sterile McCartney bottle containing M150 medium and were immediately transported to the author's laboratory where several meiotic techniques were attempted (see Section 6.1).

5.2 Female mitotic chromosomes *(Papio ursinus)*

A pilot study of the possible effects of hormonal contraceptives on mitotic bone marrow chromosomes as compared to peripheral blood chromosomes, was carried out on two adult female baboons:

Baboon 1. Healthy adult female, age unknown, weight 15 Kg.

Baboon 2. Healthy adult female, age unknown, weight 16,5 Kg.

As far as is known neither animal was submitted or exposed to factors known or suspected to be harmful to chromosomes within a month prior to the beginning of this experimental work; this interval was established on an empirical basis.

Bone marrow aspirate and peripheral blood specimens were collected for chromosome analysis from the two anaesthetized animals (see Section 6.2).

After these first samples of bone marrow and blood were obtained, one year's treatment with Depo-Provera 150 mg [medroxyprogesterone (17 hydroxy-6-methyl-progesterone acetate)] was instituted. The

treatment consisted of five courses of intramuscular injections at twelve week intervals, i.e. thirteen months in all. The dosages were calculated to be equivalent on a weight for weight basis to those given to humans for contraceptive purposes. In the first two courses, 0,25 mℓ was used but in subsequent ones, 0,3 mℓ was given as the baboons were gaining weight (Baboon 1 - 2 Kg; Baboon 2 - 3,5 Kg) and both showed signs of possible ovulation, i.e. gross enlargement and engorgement of the skin of the anogenital area (Vagtborg, 1965). The dosage was increased as it was thought that an increase in weight would lead to a decreased supply of the drug to the target cells, and therefore inadequate suppression of ovulation.

After twelve months, in which period the baboons remained healthy and isolated from known agents which could possibly damage the chromosomes, repeat bone marrow and peripheral blood specimens were removed from each baboon.

Mitotic chromosomes obtained from these posttreatment samples were then studied and compared with those found in pretreatment samples.

5.3 Discussion of materials

5.3.1 Female meiotic chromosomes (*Papio ursinus*)

Although early chromosome studies in man were mainly carried out on testicular meiotic chromosomes, the subject of meiosis in males is still not all that well documented. Very little information is available on meiotic chromosomes in human females. This is due to the difficulty in obtaining material, partly because of the internal

location of the ovaries, and partly because most stages of female meiosis take place in foetal life (Sharma and Talukder, 1974). Thus, oogonia complete their multiplication phase in the fifth month of intrauterine life, and the first meiotic prophase of oocytes is completed several weeks before the end of maternal gestation. The oocytes then remain at dictyotene stage for a period of approximately twelve to fifty years during the female's reproductive life span (Sharma and Talukder, 1974; Klášperská *et al.*, 1976).

At puberty and in every menstrual cycle thereafter, the oocytes usually complete meiosis singly; completion of meiosis I occurs in a mature follicle on the day of ovulation and meiosis II commences in an ovum after ovulation in the fallopian tube (Sharma and Talukder, 1974; Klášperská *et al.*, 1976).

Methods are available for the study of female meiotic chromosomes both *in vivo* and *in vitro*. The latter is more practical because of the difficulties encountered in acquiring the *in vivo* oocyte at the correct stage. The availability of the above techniques initiated the first experimental project.

To the author's knowledge an *in vivo* study on the effects of hormonal contraceptives on female baboon meiotic chromosomes has not been done. Jagiello and Lin (1974) reported on studies of mammalian oocytes, meiosis and oral contraceptives in several species (mouse, cow, rhesus monkey and baboon), but their work on baboons was an *in vitro* study, therefore the results cannot be extrapolated to the *in vivo* situation. Normal *in vitro* results do not guarantee that a test substance is harmless (Mailhes, 1976; Balson and Roberts, 1977). It must be remembered that many chemical agents are metabolized

into active products by the living organism.

Two reports in particular were relevant to this project. Carr's work (1970) had shown an increased incidence of chromosomally abnormal embryos among spontaneously aborted foetuses if conception took place within a few months of discontinuing oral contraceptives.

Poland (1970) reported a significant increase in spontaneously aborted abnormal embryos showing gross disorders of development among fifty-eight patients who used oral contraceptives for at least six months prior to conception when compared with controls.

5.3.2 Female mitotic chromosomes *(Papio ursinus)*

The effects of a certain chemical agent are not necessarily the same in different cell-lines (Balson and Roberts, 1977). Therefore, comparison of the possible effect of medroxyprogesterone on peripheral blood and bone marrow chromosomes of the baboon was attempted.

The choice of a long-acting injectable progestin instead of an oral hormonal contraceptive was based largely on the convenience of using a long-acting injection rather than the oral form. The use of a daily tablet for the baboons would have meant a tedious daily journey (± 20 Km) to the Animal Unit where they were housed. Even if the drug had been administered by the staff of the Animal House, close observation of the animals to ensure that the drug was indeed taken would have been necessary.

Incidental episodes of diarrhoea or vomiting could have interfered with absorption of the drugs and thus introduced unwanted variables.

To counteract these practical problems, medroxyprogesterone acetate (Depo-Provera), which has a slow absorption rate because of its insolubility in water, was used. It was assumed that Depo-Provera would be an adequate substitute for the oral preparations.

The long lasting effect of Depo-Provera is due to the quick absorption of the diluent after injection of the aqueous suspension; this leaves the active material as a deposit which slowly dissolves over the course of ± weeks.

CHAPTER 6

6. METHODS

6.1 Meiotic chromosomes

6.1.1. Techniques using ovarian tissue

The author tried several methods in an attempt to culture baboon (*Papio ursinus*) oocytes to study meiotic chromosomes. Because all attempts at culture were unsuccessful, the methods used are merely mentioned and not fully described. These included:

a) Brinster's method (1963).

b) Ohno's method (1965).

c) Method used by Jagiello (1977).

All these methods used hormone-free medium.

The principle of these methods was briefly: a) to remove the follicles from the ovarian stroma; b) to puncture the follicles, aspirate the follicular fluid and hopefully the oocyte; c) to incubate the oocyte in culture medium for a few hours; d) to transfer the oocyte to a slide; e) to observe a stained squash preparation of the oocyte for meiotic chromosomes.

6.2 Mitotic chromosomes

6.2.1 Direct bone marrow culture

Bone marrow metaphases were obtained from two experimental baboons, before and after they were subjected to injections of Depo-Provera (see Section 5.2), by modification of the direct bone marrow technique described by Rowley (1973). This method involves a short incubation of an aspirated bone marrow suspension in the presence

of colchicine.

The baboon was sedated with Sernylan (phencyclidine hydrochloride), 0,2 mℓ IM per animal (dosage 0,8 - 1 mg/kg) after net-trapping. Sternal bone marrow was aspirated under local anaesthesia through suitably cleaned and shaved skin, using a human sternal marrow needle. The bone marrow was transferred without delay to a sterile bottle containing 5 mℓ of culture medium M150 + 0,1 mℓ of heparin (Boots - 5 000 units per mℓ). Colchicine in a final concentration of 0,25 µgm/mℓ was added immediately on arrival at the laboratory (one to two hours after aspiration) and this was followed by twenty-five minutes incubation at 37°C.

The harvesting procedure was similar to that used for the peripheral blood culture preparations (see Section 3.2.5), and in order to avoid undue repetition only the differences will be described:

a) The hypotonic pretreatment period was twenty minutes, rather than ten to twelve minutes, used in peripheral blood culture.

b) The cell button was left undisturbed in the first fixative (one part glacial acetic acid to three parts absolute methanol) for at least half an hour, instead of fifteen minutes as in the case of peripheral blood.

c) A freshly prepared mixture of forty-five per cent glacial acetic acid and fifty-five per cent absolute methanol was then used as a fixative which was changed at least three times. The cell button was left undisturbed in the last fixative and kept in the deep-freeze (-4°C) overnight.

d) Chromosome spreads were prepared on the following morning, after

another change of fixative (45% AA/55% AM), using the flame-drying technique (see Section 3.2.6) and stained in ten per cent Giemsa stain (see Section 3.3.7). Banding techniques were not attempted as it was felt that they were not fundamental in this particular experimental work.

6.2.2 Peripheral blood culture

Forty-eight hour cultures were established using 6 mℓ of M150 and 3 mℓ of AB serum + 0,4 mℓ PHA and 1 mℓ whole blood. The rest of the procedure was the same as that used for human peripheral blood cultures (see Section 3.2).

6.3 Discussion of methods

6.3.1 Ovarian tissue

Edwards (1965) found that oocytes dissected from follicles could resume meiosis *in vitro*, and could pass through first and second meiotic divisions.

Since then, other workers have reproduced this phenomenon, and although reinduction of meiosis by unprimed mammalian oocytes *in vitro* has not been well explained, several methods for different species are now available.

From the different methods described in the literature for culturing *Papio* oocytes, only those where a hormone-free medium was used were attempted, even though the author realized that the probability of successful results would be greater by using media supplemented with hormonal additives (Jagiello *et al.*, 1975). The reason for this choice was to avoid any additional hormonal agent

over and above those introduced by the treatment protocol which could have interfered with the results.

After several unsuccessful attempts this aspect of the project was abandoned. Some reasons for the failure could have been:

a) Lack of a CO_2 incubator.

b) Shortage of suitable ovarian material from baboons.

c) Previous reports from the literature indicate that the percentage of atretic oocytes in monkeys is very high; preliminary observations on the ultrastucture of *Macaca mullata* oocytes suggest that monkey oocytes are less suited for *in vitro* maturation experiments than those of other species (Zamboni, 1972).

ANIMAL STUDIES

CHAPTER 7

7. RESULTS

7.1 Bone marrow results

Baboon 1

Pretreatment

Chromosome analysis was performed on thirty unbanded metaphases obtained from a direct bone marrow culture prior to initiation of the Depo-Provera treatment. In the thirty cells analyzed three chromatid breaks were found.

Posttreatment

Chromosome analysis of metaphases obtained from a direct bone marrow culture after one year of treatment with Depo-Provera revealed the following abnormalities in twenty-five unbanded cells analyzed:-

One acentric fragment,

one ring chromosome,

one chromosome break,

five chromatid breaks,

one marker chromosome.

Baboon 2

Pretreatment

Direct bone marrow culture performed before initiation of treatment revealed the following abnormalities in thirty unbanded cells analyzed:-

One chromatid deletion,

one chromosome break,

two chromatid breaks.

Posttreatment

After one year's treatment the following abnormalities were detected in thirty unbanded metaphases obtained from direct bone marrow culture:-

Three acentric fragments,

one ring chromosome,

one chromosome break,

nine chromatid breaks.

7.2 Peripheral blood results

Unfortunately, peripheral blood cultures established before initiation of the treatment were infected and therefore not successful. When it was realized that the cultures had failed, the baboons had already been given their first dose of Depo-Provera, and therefore another control had to be used as detailed below.

After one year of treatment when bone marrow and blood were collected from the two experimental animals, blood from a third, healthy, untreated adult female baboon was collected to be used as a control for the blood samples of Baboons 1 and 2.

Control baboon

In this baboon thirty peripheral blood unbanded metaphases were analyzed and the following abnormalities were found:-

One chromosome break,

five chromatid breaks.

Baboon 1

Posttreatment

In Baboon 1, twenty peripheral blood metaphases were screened and

the following abnormalities were detected:-

Two chromosome breaks,

six chromatid breaks,

two marker chromosomes.

The two marker chromosomes were found in two different slides but originated from the same culture bottle. These two markers are similar to each other and also resembled the marker found in the post-treatment bone marrow preparations (see Figure 7.1).

Unfortunately the preparations obtained from Baboon 1 were of poor quality and this marker chromosome could not be more definitively identified by quinacrine banding. There were thus three cells with a similar abnormal marker chromosome, which, if they were the same, would denote an abnormal clone according to the criteria of the First International Workshop on Chromosomes and Leukemia (1977). However, due to the poor quality of the metaphases, it would be more accurate to say that these findings in Baboon 1 are only suggestive of an abnormal clone in this animal, which was not detected prior to the onset of treatment (in bone marrow preparations).

Baboon 2

Post-treatment

The following chromosome abnormalities were found in thirty unbanded metaphases obtained from peripheral blood cultures established after one year's treatment:-

Two acentric fragments,

one chromatid deletion,

nine chromatid breaks,

one chromatid gap.

No markers were found in Baboon 2.

See Table 7.1 for results on both baboons.

Figure 7.1. Similar marker chromosomes (M) found in 3 different cells obtained from Baboon 1 after one continuous year's treatment with Depo-Provera. Two cells (i and ii) were derived from a peripheral blood culture and the third (iii) was from a direct bone marrow preparation.
The easily identifiable variant chromosome 10 (Bernstein *et al.*, 1980) of the Chacma baboon is presented side by side with the marker for size comparison, but the origin of the marker is unknown.

One unbanded metaphase (Figure 7.2) and one banded karyotype (Figure 7.3) of baboons are presented (the banded karyotype does not belong to any of the animals in this study, but is from another *Papio ursinus* presented in an article in which the author participated (Bernstein *et al.*, 1980). Some examples of the abnormalities found are presented in Figures 7.4, 7.5, 7.6 and 7.7.

7.3 Discussion of results

The results of chromosome analysis in the two experimental baboons, both on bone marrow and peripheral blood samples, tended to indicate an increased incidence of chromosome abnormalities in both cell-lines after one year of treatment with Depo-Provera. It was realized that these results would not be statistically valid because only two animals were used. However this study was merely performed as a preliminary pilot experiment on a mammal close to man, to compare breakage in marrow and peripheral blood metaphases. It appears that the above animal studies are the first of this nature to be attempted; no reference to similarly conducted experimental studies was found in the literature, and therefore these results cannot be compared with others.

Future aims

The results do suggest that it may be worthwhile to institute a well designed formal study to confirm the validity of these preliminary observations; the use of a larger number of animals studied over a shorter time period is envisaged.

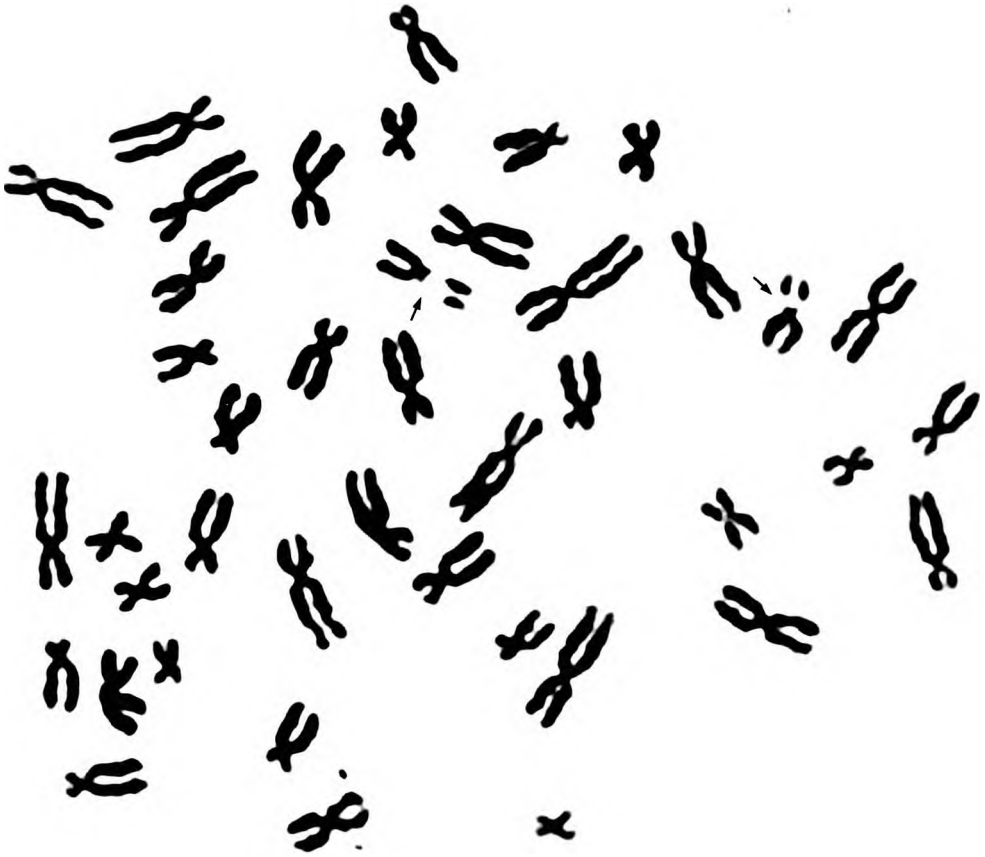

Figure 7.2. Unbanded metaphase of a female *Papio ursinus* baboon obtained from a 48 hour peripheral blood culture, karyotype 42,XX. The readily identifiable chromosome 10 homologues are arrowed (Bernstein *et al.*, 1980).

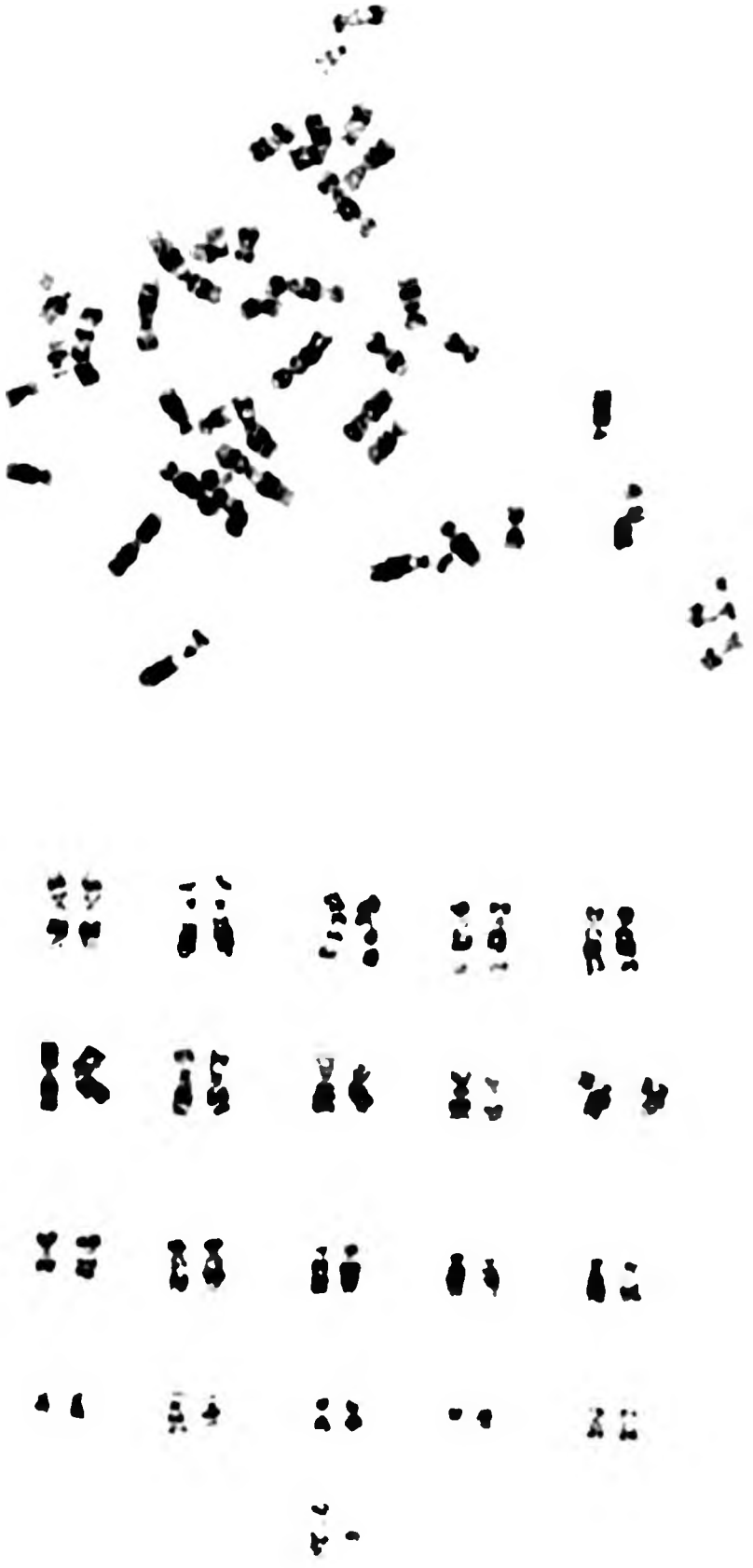

Figure 7.3. Giemsa banded metaphase and respective karyotype of a male baboon *Papio ursinus*. The nomenclature used in this karyotype was modified from that of Dutrillaux *et al.* (1978) as specified by Bernstein *et al.* (1980).

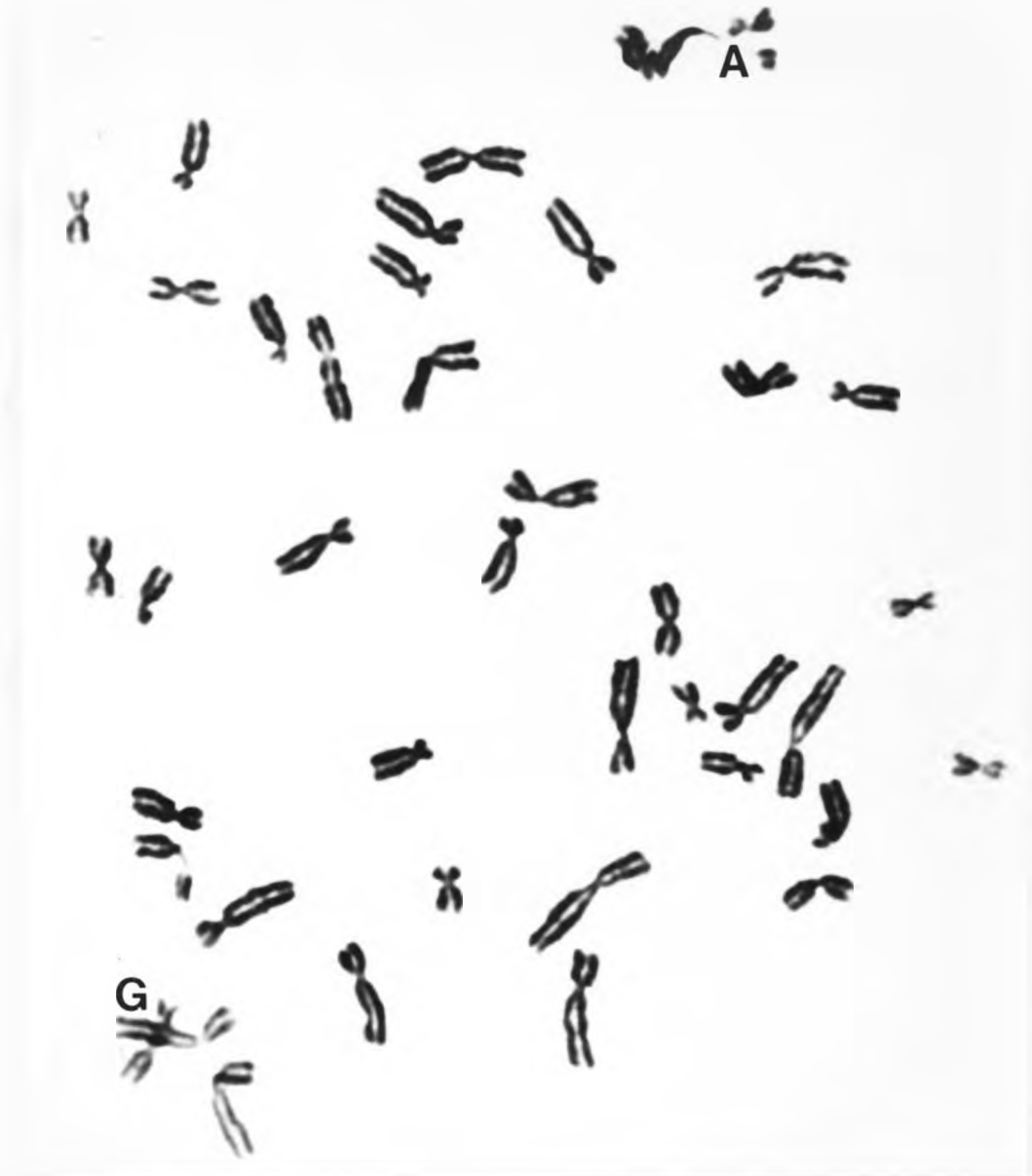

Figure 7.4. Unbanded metaphase obtained from Baboon 2 posttreatment, showing an acentric fragment (A) and a chromatid gap (G).

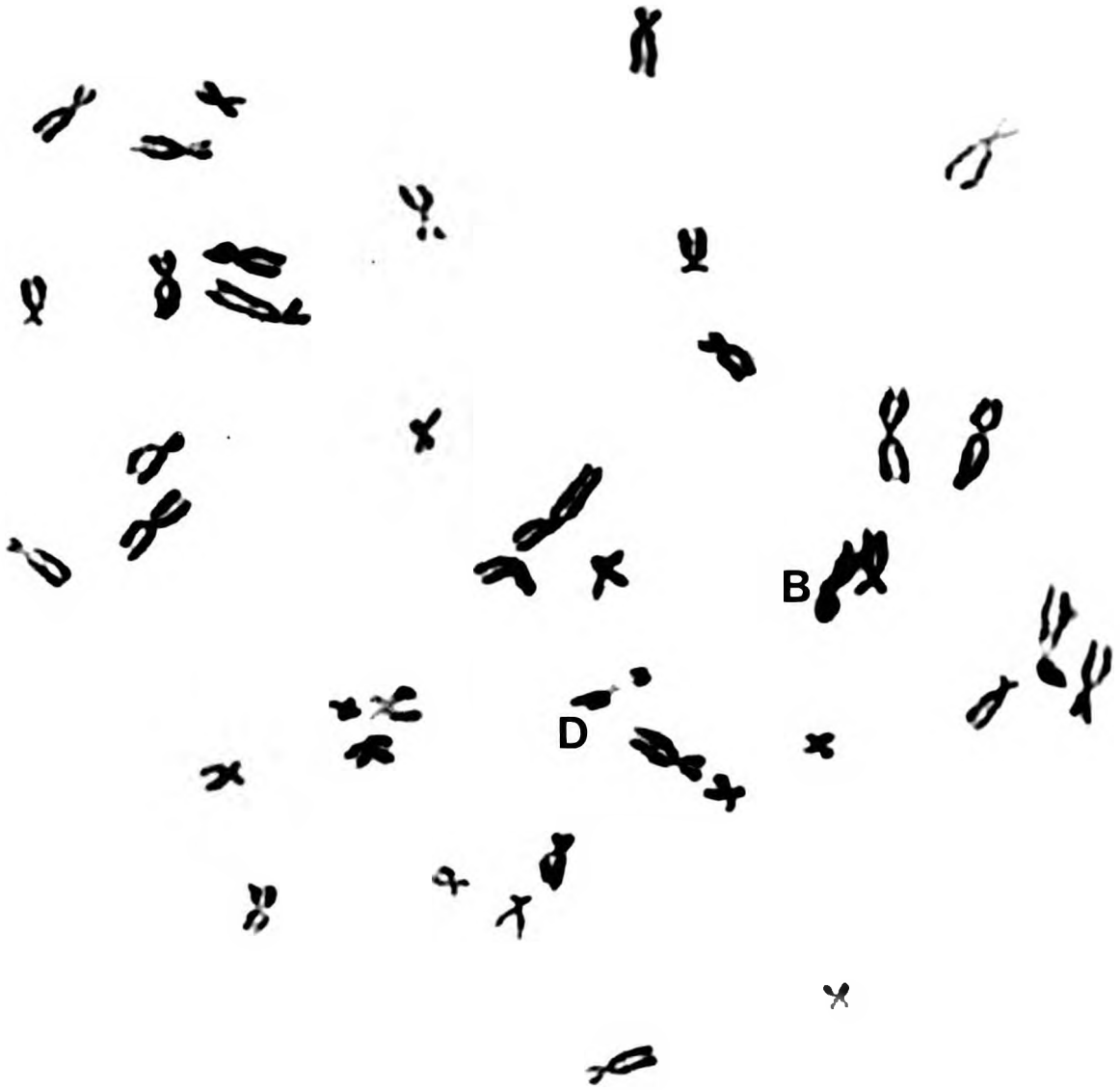

Figure 7.5. Unbanded metaphase obtained from Baboon 2 posttreatment, showing a chromatid deletion (D) and a chromatid break (B).

Figure 7.6. Unbanded metaphase obtained from the control baboon, showing a chromatid break with displacement (B).

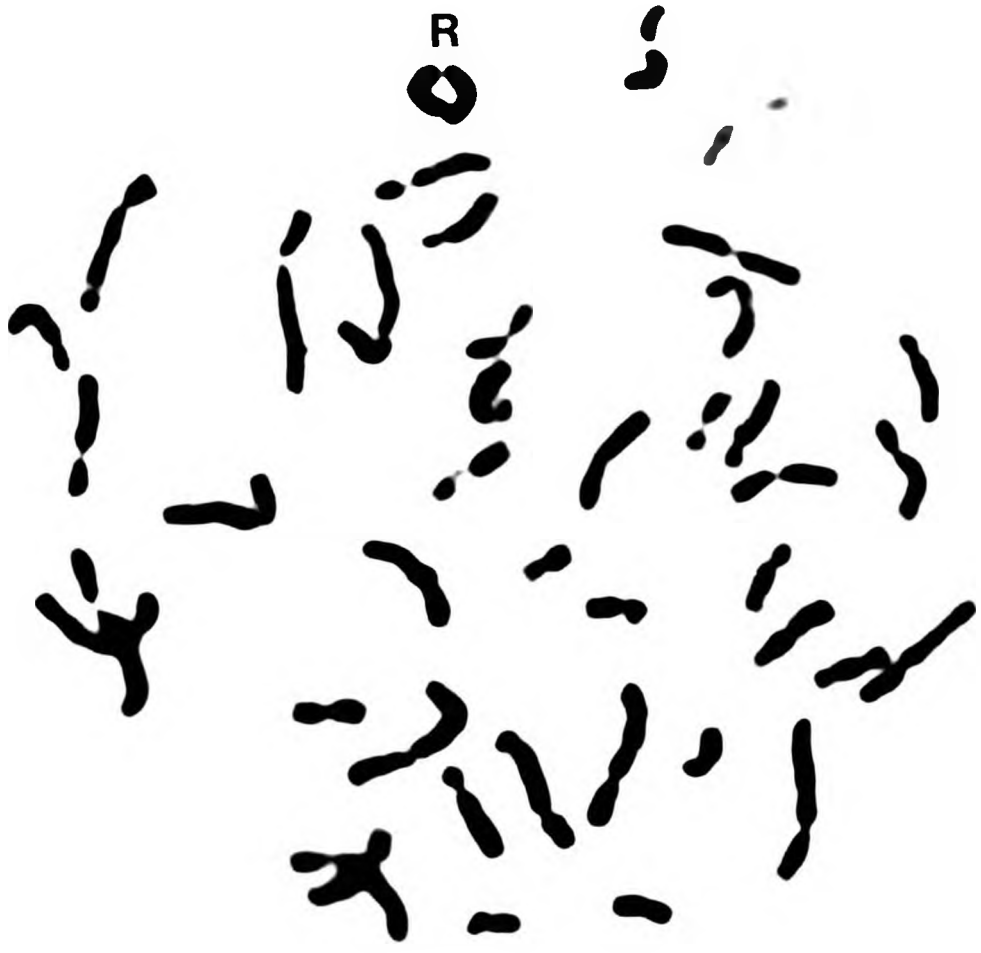

Figure 7.7. Unbanded metaphase obtained from Baboon 2 after treatment for one year with Depo-Provera, showing a ring chromosome (R) (bone marrow preparation).

CHAPTER 8

8. CLINICAL IMPLICATIONS OF CHROMOSOME BREAKAGE

It is well established that chromosome aberrations in somatic cells may well be regarded as indirect visible evidence of damage to the genetic material (Mitelman and Pero, 1979). Chromosome aberrations are often accepted as an indicator of mutagenicity and are thus a potentially malignant event (Brøgger, 1979; Mitelman and Pero, 1979). This acceptance (Mitelman and Pero, 1979) is based on the following:-

1. Some agents capable of producing chromosome damage are carcinogenic (e.g. radiation, viruses and certain chemicals).
2. Individuals with chromosome breakage syndromes, e.g. Fanconi's anaemia, Bloom's syndrome, ataxia telangiectasia and xeroderma pigmentosum have an increased risk of developing a malignancy.
3. Many malignant tumours show the presence of chromosome abnormalities.
4. Chromosome abnormalities in many experimental and human neoplasms are nonrandom and in some instances specific (see Section 8.3.2.1).

Because of the apparent relationship between chromosome aberrations, mutagenesis and carcinogenesis, chromosome analysis has become a useful tool in the evaluation of potential hazards caused by environmental agents. In fact it is the most practical test for detecting genetic damage directly in man. It goes without saying that the great limitation of this approach is that only gross genetic

damage is visible and a negative finding does not exclude other types of genetic damage, e.g. single gene point mutations. The problem is that up-to-date there is no available method which is capable of visibly and directly showing changes at the gene level. Although such changes are probably an essential part of carcinogenesis, our inability to examine chromosomes at the level of the gene contributes to our lack of knowledge in understanding the causation and role of chromosome changes in malignancy. The practical questions at this point in time are: What are the clinical implications of chromosome breakage? How is man's health and wellbeing affected when there is genetic damage?

The phenotypic expression of chromosome damage, either as a visible chromosome aberration or single mutation is dependent on: a) the moment in time when the damage occurs, i.e. pre- or post-natal life; b) the type of cell in which the damage occurs, i.e. somatic or germ cells.

8.1 Genetic damage in prenatal life

Genetic damage occurring in embryonic and foetal life can affect either somatic and/or germinal tissues.

If such damage occurs in somatic cells of the developing embryo it can lead to malformations. These malformations could be the result of: a) chromosome breakage with consequent extensive cell death; b) unbalanced chromosome abnormalities due to nondisjunction or a stable structural aberration; c) single gene mutations. In extreme cases if genetic damage is so extensive that it is incompatible with life, death will supervene and the foetus will be spontaneously aborted.

Theoretically it is also conceivable and accepted that a mutation occurring in somatic cells, especially later in embryonic life, can give rise to clones of mutant cells with a low grade proliferative advantage which may later transform into a neoplasm, i.e. a pre-malignant clone. Malignancy presenting at birth, or during childhood, and the apparent correlation between intrauterine X-irradiation and childhood leukaemias are pertinent in this context (German, 1979).

If genetic damage occurs in germ cells of the developing embryo this will give rise to mutant germinal clones which may or may not produce viable gametes. If such gametes are indeed viable, abnormal offspring could be produced. If they are nonviable, sterility will result.

8.2 Genetic damage in postnatal life

As in the instance of prenatal life, genetic damage occurring in post-natal life can affect either the soma or the germ line. The effects of genetic damage on somatic cells could possibly be associated with cancer, and is a purely speculative idea proposed by German (1979) with the ageing process.

If damage occurs in germ cells this could lead to sterility, abnormal offspring, or childhood neoplasias in offspring (Purtilo *et al.*, 1978; German, 1979).

8.3 Chromosomes and cancer

The history of chromosome studies in association with cancer dates back to the nineteenth century. The first recorded observation of cell-division in tumours was published by Arnold in 1879. It

gradually became clear that the great majority of spontaneous or induced tumours studied had chromosomal and mitotic irregularities (Hungerford, 1969; Makino, 1975). Boveri (1914) first postulated that chromosome changes played a causal role in the origin of cancer.

For general purposes, cancer can be defined as a neoplastic tissue, originating from preexisting normal cells, which develops through uncontrolled proliferation, i.e. has a capacity for autonomous growth. Since growth of a tissue is dependent on the process of cell division, which in turn is controlled by the genetic material in the chromosomes (DNA), any 'inbalance' of DNA can, theoretically, lead to among other things, malignant transformation. Genetic imbalance, either inherited or acquired, and its association with neoplasia is well documented (German, 1973; Harnden, 1977; Purtilo *et al.*, 1978).

8.3.1 Inherited genetic imbalance and neoplasia

Inherited genetic imbalances either as single gene mutations or chromosome aberrations are in certain instances associated with an increased risk of developing malignancy (Purtilo *et al.*, 1978).

8.3.1.1 Single gene diseases

Well over 240 syndromes showing a simple mendelian inheritance pattern, e.g. dominant, recessive or X-linked, have been recognized as malignant or premalignant conditions (Purtilo *et al.*, 1978).

8.3.1.1.1 Single gene dominant

Von Recklinghausen's neurofibromatosis is an example of an autosomal dominant condition, with a predisposition to neuroblastomas and

neurogenic sarcomas. Neurofibromatosis alone accounts for almost half of the childhood tumours with a single gene aetiology (Bernstein, 1981).

8.3.1.1.2 Single gene recessive

Autosomal recessive disorders associated with a high risk of malignancy are very well documented in a group of diseases characterized by a basic defect in DNA repair mechanism - the so-called chromosome breakage syndromes.

Individually these diseases, in the homozygous form, are rare but the chromosome damage in the form of breakage in these syndromes and their oncogenic associations provide a classic model of inter-relationship of chromosome breakage and neoplasia. Of important clinical significance is the observation that even the heterozygote carriers of these disorders may also have an increased risk of developing cancer (Swift *et al.*, 1976). If this is confirmed, at least three to five per cent of the population (combined frequency of heterozygotes for these syndromes) may be predisposed to develop malignancy (Bernstein, 1981). Bloom's syndrome, Fanconi's anaemia, ataxia telangiectasia and xeroderma pigmentosum are classic examples of the chromosome breakage syndromes. These are also known as hypersensitivity diseases (German, 1973; Purtilo *et al.*, 1978). The first two are usually associated with the development of leukaemia, the third with leukaemia and lymphomas and the fourth with skin cancer (Purtilo *et al.*, 1978).

8.3.1.1.3 Single gene X-linked

An example of an X-linked recessive mutation associated with a

predisposition to develop malignancy is the so-called X-linked lymphoproliferative syndrome. Affected males have a remarkably increased predisposition for malignant lymphomas (Purtilo *et al.*, 1978).

8.3.1.2 Chromosome disorders

Various malignancies have long been known to occur at higher frequencies than expected in individuals with constitutional chromosome abnormalities, especially those with an aneuploid chromosome complement.

8.3.1.2.1 Numerical chromosome disorders

Even before the chromosomal aetiology of Down syndrome was identified, an increased incidence of leukaemia was noted in children with Down syndrome (Krivit and Good, 1957; Gericke *et al.*, 1977). An increased incidence of breast cancer in males is found in patients with Klinefelter syndrome (Harnden *et al.*, 1971), and there is an increased incidence of ovarian cancer in female patients with Turner syndrome (Purtilo *et al.*, 1978) when compared with the incidence in the normal population.

8.3.1.2.2 Structural chromosome disorders

The possible association of structural chromosome abnormalities and neoplasia is more difficult to demonstrate for two main reasons:

1. In live-borns the incidence of structural chromosome abnormalities, especially the unbalanced forms, is relatively low when compared with the incidence of numerical abnormalities (Hamerton *et al.*, 1975).
2. Numerical chromosome abnormalities in live-borns are limited to the

sex chromosomes and several autosomes (21, 18, 13 and very rarely 8), whereas structural abnormalities can involve any of the forty-six human chromosomes in the human complement in a multitude of different loci.

This means that for a specific gross structural abnormality, the actual number of cases described in the literature is minimal and therefore possible associations with cancer are extremely difficult to establish. Furthermore, it is possible that two gross structural chromosome abnormalities that appear identical with presently available methods of chromosome analysis, may well not be identical at the gene level. This must always be remembered and considered as a limitation to our capacity for establishing associations of this kind at the present time. However, at least two constitutional unbalanced chromosome abnormalities have already been related to malignancy. Deletion of chromosome 13 long arm (region q14) has been associated with multiple anomalies and with the presence of retinoblastoma (Walbaum *et al.*, 1978). Deletion of chromosome 11 short arm (region p13) is associated with aniridia, genitourinary tract anomalies, mental retardation and with Wilm's tumour - the so-called Aniridia-Wilm's tumour association - AWTA (Riccardi *et al.*, 1978).

Of potential clinical importance is the new concept of constitutional genetic 'imbalance' introduced by Cohen *et al.* (1979). The authors described a familial chromosome translocation where the carriers of an apparently balanced form of the translocation t(3;8) have a greatly increased risk of developing renal cancer. Sub-microscopic deletion or 'position' effect were the mechanisms postulated to explain this tendency to develop malignancy in these members affected by an apparently balanced constitutional

chromosome translocation.

It is conceivable that many other numerical and unbalanced structural chromosome abnormalities could also predispose to malignancy. Perhaps the majority of these are not viable. If indeed, some of these are compatible with life, it may be that affected individuals are so severely handicapped that they do not generally survive long enough for the malignancy to become manifest (Bernstein, 1981).

8.3.2 Acquired genetic imbalance and neoplasia

The association between acquired genetic 'imbalance' in the form of visible chromosome changes and cancer can be demonstrated in two different ways:

1. The presence of chromosome abnormalities in human solid tumours and leukaemias.
2. The effects of environmental agents both clastogenic and carcinogenic.

8.3.2.1 Chromosome abnormalities in human cancer and leukaemias

It is a well known fact that the majority of human cancers and leukaemias exhibit gross chromosome changes and in the majority of cases these are confined to the malignant tissue (Harnden, 1977; First International Workshop on Chromosomes and Leukemia, 1977; Rowley, 1978; Sandberg, 1980). In many of these malignancies the changes are nonrandom and in some instances specific. A few examples will be set out.

The following review embraces the situation mainly in the

leukaemias as they are the most studied of the malignancies. This is mainly due to the fact that haemopoietic tissues in the blood or bone marrow are more easily available for cytogenetic studies.

The Philadelphia chromosome is the first example cited here because it constitutes a milestone in the history of cytogenetics and is by far the most important (and puzzling) chromosome finding in human and experimental oncology. Described by Nowell and Hungerford (1960) as a deleted G-group chromosome, and later precisely identified by Rowley (1973) as a translocation between chromosomes 9 and 22 (in the majority of cases), the Philadelphia chromosome was until recently the only acquired chromosome anomaly closely related to a specific neoplastic disease - chronic myelocytic leukaemia (CML).

Other chromosome abnormalities can be identified as the disease progresses and often they precede the clinical and haematological features of blastic transformation. Again, in the majority of cases, these changes in the Ph^1 positive cell-line are not random (second Ph^1; extra 8; i(17 q) (Rowley, 1978; Sandberg, 1980).

In the acute nonlymphocytic leukaemias (ANLL) a specific translocation between chromosomes 15 and 17 has been correlated with FAB M_3-ANLL (acute promyelocytic leukaemia) (Rowley *et al.*, 1977; Yunis *et al.*, 1981) and a translocation between chromosomes 8 and 21 is specifically associated with FAB M_2-ANLL (acute myeloblastic leukaemia with differentiation) (Second International Workshop on Chromosomes and Leukemia, 1980; Yunis *et al.*, 1981).

Apart from these specific chromosome changes, nonrandom loss of chromosomes 7 and 5 as well as trisomies for 8 and 21 have also been described in ANLL (Sandberg, 1980) - especially in 'secondary

leukaemias' (Second International Workshop on Chromosomes and Leukemia, 1980; Paquin and Purtilo, in press).

In other myeloproliferative disorders, nonrandom chromosome abnormalities have also been reported. For example, polycythaemia vera has been associated with deletion of the long arm of 20 (20 q^-) (Testa *et al.*, 1978) and with 1 q^+ in the form of partial trisomy of the long arm of 1 (Swolin *et al.*, 1981).

In acute lymphoblastic leukaemia (ALL) a considerable number of non-random chromosome changes are found. These include gain of chromosomes 21, 4, 6, 10, 13, 14 and 18 and loss of chromosomes 7, 9 and 20. Re-arrangements involving chromosomes 6, 9, 14 and 22 are also common (Third International Workshop on Chromosomes and Leukemia, 1981).

In chronic lymphoblastic leukaemia (CLL) the most common abnormality is trisomy 12 followed by abnormalities of chromosome 14, deletion of the chromosome 6 long arm (6 q^-) and translocation between chromosomes 11 and 14 t(11;14) (Emberger *et al.*, 1981). In Burkitt's lymphoma and also in B-cell ALL the following translocations were nonrandomly found: t(8q;14q), t(2p;8q) and t(8q;22q) (Sandberg and Wake, 1981). It is interesting to note that in these three translocations described in B-cell malignancies, chromosome 8 is the common denominator and also that chromosomes 2, 14 and 22 are known sites of immunoglobin chain loci. This is the first definitive demonstration of a link between the chromosome site involved in an acquired chromosome abnormality and the gene loci involved in the normal functional activity of the cell-type which has become malignant (Klein, 1981). The 14 q^+ abnormality present in Burkitt's lymphoma and other lymphoproliferative disorders is slowly emerging as a candidate for a specific karyotypic anomaly comparable to that of the Ph^1 in CML.

Chromosome aberrations are likewise found in almost all human solid tumours. These tumours usually show multiple chromosome abnormalities and aneuploidy (Paquin and Purtilo, in press). Due to difficulties in processing the material and obtaining a suitable number of good quality metaphases, the present status of chromosome studies in solid tumours is difficult to assess. However, with improvement of techniques a pattern of nonrandom abnormalities is emerging (Sandberg and Wake, 1981; Mitelman, 1981).

Monosomy 22 was the first nonrandom abnormality described in a solid tumour; it is associated with meningioma (Paquin and Purtilo, in press).

Abnormalities of chromosome 1 long arm are common in breast cancer and melanoma (Sandberg and Wake, 1981). Chromosome 6 long arm is nonrandomly involved in ovarian carcinoma (Sandberg and Wake, 1981), and deletion of chromosome 3 short arm is nonrandomly found in lung cancer (Trent, 1981). In mixed parotid tumours a translocation involving chromosomes 3 and 8 is nonrandomly found (Sandberg and Wake, 1981).

With the availability of better techniques and the possibility of future automation for chromosome screening, the day may come when specific (or at least nonrandom) chromosome abnormalities will be demonstrated in all solid neoplasias and leukaemias.

8.3.2.2 Environmental agents both clastogenic and carcinogenic

With the natural desire to maintain and improve the standard of living, the scientific and industrial fraternities, especially in developed

countries, constantly develop and introduce hundreds of new and varied compounds to the world market annually (Legator and Rinkus, 1979). The fact that some of these chemicals are effective and useful, either in combating disease, increasing the food supplies, or in industry, is unquestionable. However, the fact that some of these chemicals can have adverse effects on the health of the population is also without doubt.

This massive production of chemicals creates serious toxicological problems as it is far easier to synthesize new chemicals than to fully evaluate their potential risks to man. If the problem is serious for the assessment of acute toxic effects, it is very much worse in the case of long term effects such as inheritable changes and cancer induction. It may well be impossible to observe any such long term effects until several decades have passed. There is, however, an increasing awareness of the role of environmental agents in the aetiology of malignancy (Meretoja and Vainio, 1979). This awareness was well demonstrated at one of the latest meetings of the International Society of Haematology (1981) where several papers stressed several exogenous factors in the causation of leukaemia.

The exogenous factors incriminated in the aetiology of leukaemias included occupational exposure to certain chemical agents (e.g. chemical solvents, insecticides and petrol products, as well as chemotherapeutic and radiotherapy agents for diverse malignancies).

Acute nonlymphocytic leukaemia (ANLL) is the usual type found in these secondary leukaemias and in the context of the present study it seems important to mention some of the cytogenetic findings in these patients with ANLL.

In those patients with ANLL who gave a positive history of environmental exposure, seventy-five per cent had a detectable chromosomally abnormal clone. On the other hand, in those patients with ANLL but with a negative history of environmental exposure to known leukaemogenic agents, only thirty-two per cent showed a chromosomally abnormal clone (Nilsson *et al.*, 1981).

In 1775 Percival Pott had already observed the association between scrotal cancer and exposure to soot in chimney sweeps.

Many such observations have been made since then. It is now believed that at least eighty per cent of cases of human cancer are environmentally induced and that chemical carcinogens predominate as oncogenic agents (Higginson, 1969; Meretoja and Vainio, 1979; Doll and Peto, 1981). This does not imply that all chemicals can cause cancer or induce mutations. It is a fact, however, that not enough information on the health consequences of exposure to chemicals is known. Some are definitely detrimental, and several carcinogenic agents have been identified (e.g. benzene, vinyl chloride).

Radiation was the first environmental agent recognized as being both clastogenic (Muller, 1927) and carcinogenic in man. March, (1944) reported that leukaemia was ten times more commonly recorded as a cause of death in radiologists as compared with other physicians. Subsequent studies confirmed these findings (Ulrich, 1946; March, 1950), but undoubtedly the best *'in vivo'* example of the effects of radiation was provided by studies on the survivors of the atomic bombing of Hiroshima and Nagasaki. This population showed a high frequency of unstable and stable chromosome aberrations such as dicentrics, rings, acentrics, translocations, and so on (Bloom *et al.*, 1966; Ishihara

and Kumatori, 1967), and a few years later demonstrated a significantly higher than statistically expected mortality from leukaemia (German, 1973), as well as a higher incidence of other

(continued on page 164)

neoplasms (Bloom *et al.*, 1970; Sandberg, 1980).

Additional information on the leukaemogenic effects of radiation is supplied by the high incidence of leukaemia in ankylosing spondylitis patients who received X-ray therapy (Sandberg, 1980). These patients also presented with a high percentage of unstable chromosome aberrations in their lymphocytes such as dicentrics, rings and acentric fragments.

All the references mentioned above refer to high doses (more than 300 rads) of radiation exposure, being either accidental, in war, or for therapeutic purposes. Little is known about exposure to low doses of radiation for diagnostic purposes. A recent article (Linos *et al.*, 1980) on low dose radiation (less than 300 rads) and leukaemia, seems reassuring in showing that low levels of exposure to medical radiation most probably does not increase the risk of developing leukaemia.

A host of chemical substances to which man is exposed either accidentally or through daily use have been incriminated in the production of chromosome damage either *in vivo* or *in vitro* (Shaw, 1970). A number of these substances have been shown to be carcinogenic to man and others have been classified as potentially carcinogenic hazards.

Benzene, a known clastogen (Tough and Court Brown, 1965; Forni, 1966; Forni and Moreo, 1967; Forni *et al.*, 1971) is also a proven carcinogen; studies in populations occupationally exposed to benzene showed that they have an increased risk of developing acute leukaemia (Vigliani and Forni, 1976; Infante *et al.*, 1977). It is interesting to mention the sequence of events in some patients occupationally exposed to benzene in whom chromosome studies were performed prior

to the development of leukaemia (particularly in the preceding period of aplastic anaemia) and immediately after the development of the leukaemia. In the chromosome study performed before the leukaemia supervened, changes consisting primarily of breaks, deletions and gaps were found in the subjects exposed to benzene. Later the emergence of a chromosomally abnormal stable clone and clinical leukaemia were observed (Sandberg, 1980).

Other chemicals including lead (Sandberg, 1980) and arsenic (Beckman *et al.*, 1979) also produce changes similar to those described in benzene poisoning; that is breaks, deletions, gaps, rearrangements, acentrics and others. Some of these chemicals have also been implicated in the genesis of cancer and leukaemia.

Vinyl chloride provides an example of clastogenicity, mutagenicity and carcinogenicity in a single agent. Vinyl chloride, a known clastogenic agent (Ducatman *et al.*, 1975; Funes-Cravioto *et al.*, 1975; Purchase *et al.*, 1975) has been shown to cause angiosarcoma of the liver in rats, and recently angiosarcoma has also been found in human vinyl chloride factory workers in the USA (Bishun *et al.*, 1978).

Apart from the field of industry, chemical agents used for therapeutic purposes have also been associated with leukaemia, the so-called therapy-linked leukaemia (Editorial, Lancet, 1977). An increased incidence of acute leukaemias has been noticed after chemotherapy or radiotherapy for diverse malignant diseases, e.g. Hodgkin's disease, and cancers of breast, lung and ovary (Editorial, Lancet, 1977). Even in nonmalignant conditions such as chronic hepatitis (Silvergleid and Schrier, 1974), chronic renal disease (Roberts and Bell, 1976) and others (Editorial, Lancet,

1977) where cytotoxic immunosuppressent drugs were used, an increased incidence of acute myeloid leukaemia (AML) has been observed.

Finally, something must be said about a biological environmental agent and its association with malignancy - the virus.

Chromosome aberrations such as breaks, gaps, deletions and others are increased in human lymphocytes after various viral infections and virus vaccinations, e.g. hepatitis, German measles, meningitis, German measles vaccination (Makino, 1975).

Animal studies have demonstrated both chromosome breaking and tumourogenic effects of viruses (Makino, 1975). In cell cultures infected by oncogenic viruses it has been shown that the chromosome abnormalities induced by the virus are associated with multiple changes. These changes include alterations in cell behaviour involving cell morphology, and abnormal accelerated cell growth with increased cloning efficiency together with the chromosome aberrations (Makino, 1975). However, in humans, apart from the Epstein-Barr (EB) virus, which has been closely associated with Burkitt's lymphoma and with nasopharyngeal carcinoma (Paquin and Purtilo, in press), there is not, to-date, definitive evidence that a virus can cause any human cancer or leukaemia. It has to be stressed that not even with EB virus is there absolute proof that it is an oncogenic virus (Paquin and Purtilo, in press). There is, doubtless, a possibility that viruses may play a role in the genesis of human cancer, the extent of which is still, however, unknown but suspected by many (Klein, 1979).

Many years have passed since Boveri (1914) postulated that chromosome changes were causally related to malignancy; but the precise relationships and mechanisms still elude us!

As time goes by accumulative evidence seems to support that causal relationship (Ohno, 1979).

This evidence is mainly based on three observations:

1. Carcinogenic agents, either ionizing radiation, chemical or viral, generally cause chromosome damage in the form of chromosome breakage.
2. Certain inherited diseases characterized by an increased liability to chromosome breakage carry an increased risk of malignancy.
3. Malignancies are generally associated with visible chromosome changes.

This important triad of observations which have been discussed in previous pages, seems to provide us with the links of the chain between chromosome breakage and malignancy.

However, the questions which can still be asked are: a) What is the possible sequence of events between chromosome damage and the acquisition of malignant properties by the cell? b) What is the role of chromosome change in environmentally induced tumours? In summary, what is the role played by chromosomal change in the initiation and progression of any human tumour?

The previous pages have reviewed briefly certain of the possible teratogenic and oncogenic clinical implications of genetic damage in the light of our present incomplete state of knowledge. A review of the literature will be reported on concerning the ill effects of hormonal contraceptives which may be interpreted as direct or indirect manifestations of genetic damage. Speculation on the future possible implications of genetic damage due to hormonal contraceptives will

also be set out.

8.4 Hormonal contraceptives and genetic material

Following the same trend of thought mentioned earlier in this chapter, reference will be made to the available literature concerning the possible ill effects of hormonal contraceptives when taken around the time of conception and in early pregnancy (in many cases it is difficult to know when conception has occurred).

The different mechanisms implicated in teratogenesis will not be discussed and it will be assumed that the abnormalities could be the result of genetic damage in prenatal life by any of the mechanisms previously discussed (see Section 8.1).

8.4.1 Possible genetic damage in prenatal life caused by hormonal contraceptives - somatic cell

Although the larger surveys concerning oral contraceptives and congenital abnormalities have been reassuring in their negative findings (Royal College of General Practitioners, 1976; Klinger and Glasser, 1977), there have been a number of reports of foetal abnormalities in pregnancies when oral contraceptives were inadvertently continued during early pregnancy or around the time of conception.

One of the earliest reports on the possible teratogenic effects of oral contraceptives came from Nora and Nora (1973). They described a group of ten patients with multiple congenital abnormalities described by the acronym VATEL or VACTERL (Vertebral, Anal, Cardiac, Tracheal, Esophageal, Renal, Limb) and two patients with DiGeorge syndrome (congenital absence of thymus and parathyroid glands), where there was a history of exposure to progestagen-oestrogen compounds

either as a pregnancy test or as contraceptive pills during a critical early period of embryogenesis. These same authors also report an association between isolated congenital heart disease and hormonal contraceptives. Nora and Nora (1975) reported on an expanded survey of patients with Vater syndrome (anomalad) where the same association was found.

Levy *et al.* (1973) reported a high incidence of hormone exposure in pregnancies leading to infants affected by transposition of the great vessels. The hormonal treatment implicated included therapy for threatened abortion, hormonal pregnancy tests, insulin and thyroid therapy. Although the chemical compounds used for threatened miscarriages and pregnancy tests are the same as the ones used in the 'pill' the results cannot be directly extrapolated to the contraceptive preparations, as will be explained later (see page 175).

Janerich *et al.* (1973) suggested an association between limb-reduction deformities and the use of small doses of oestrogens or progestogens, either as contraceptive pills or hormone pregnancy tests. The following year, the same authors, confirmed their previous data in an investigation of 108 infants with congenital reduction deformaties of the limbs; fifteen of these mothers gave a positive history of exposure to exogenous sex steroids during pregnancy. Six of these fifteen mothers presented a history of 'breakthrough' pregnancies while on the pill (Janerich *et al.*, 1974).

Robertson-Rintoul (1974) reported that four of five women, known to have continued taking oral contraceptives during pregnancy produced infants with serious malformations: two with congenital heart defects,

one with exomphalos and the other with skeletal malformations. Bracken *et al.* (1978) reported on exposure to oral contraceptives during pregnancy in a study of 1 370 cases of congenital malformations and 2 968 controls. They found that exposure to oral contraceptives during pregnancy was associated with a greater risk for anencephaly, cardiac septal defects, heart valve anomalies, alimentary and digestive tract problems, talipes and Down syndrome. An attempt was made in this study to examine exposure to other risk factors, and possible synergistic effects with the 'pill' on foetal development. A higher risk was found in oral contraceptive users who smoked more than twenty cigarettes per day.

Janerich *et al.* (1978, 1980) reported on two retrospective studies on children born with congenital defects (neural tube defects, hypospadias, Down syndrome, cardiovascular defects and others). These children were compared with a corresponding number of normal children, and they found a small increase of oral contraceptive exposure, at approximately the time of conception, in the pregnancy histories of birth defects cases. In this study the authors mention their concern about the tendency for malformed children to be conceived immediately following discontinuation of the 'pill'. Although stressing that the small size of their sample makes it difficult to draw any conclusions, the authors imply that there is a higher incidence of malformed infants during the first two menstrual cycles after stopping the 'pill'. They also attempt to correlate the 'fertility lag' among normal births during the same time interval in order to postulate that conception in the first cycles after discontinuation of 'pill' use, may pose some risks to the foetus.

It is interesting to recall at this particular point a survey

done by Kasan and Andrews (1980) in which they reached similar conclusions in relation to a 'critical' interval after discontinuation of oral contraceptives and incidence of neural tube defects (NTD). In their study, 10 479 single births were surveyed over a period of two and an half years; they found an increased incidence of neural tube defects in babies born to mothers who had taken the 'pill' within three months of falling pregnant. Neural tube defects are polygenically inherited conditions, i.e. there is a genetic component plus an environmental contribution. Recently, the environmental factor (Smithells *et al.*, 1980, 1981) has been identified as a possible vitamin deficiency. It is therefore also feasible that the increase of NTD noticed in babies conceived within three months after stopping the 'pill' are due to the disturbance in vitamin metabolism found in women on oral contraceptives (Briggs, 1978).

Rothaman and Louik (1978) published the results of a survey on 5 535 infants whose mothers had used oral contraceptives within three years preceding the birth of the child included in the survey, and on 2 373 infants from a control group. They found a slight increase of congenital malformations in the group of recent oral contraceptive users (less than one month between cessation of medication and conception) when compared to the control group. The abnormalities included Down syndrome, heart defects, hypospadias, undescended testes and hydrocele. Among the abnormalities noted in this group who recently took oral contraceptives, undescended testes was the commonest problem. The main criticism of Rothaman's study lies in the choice of their control group which includes babies born to women that were not taking the 'pill' three years prior to the birth of the present child, but had taken the 'pill' at some stage before

that time. In choosing these women, Rothaman assumes that an interval of three years is probably long enough for the potential ill effects of the 'pill' on the offspring to have disappeared. In fact, the image of the oocytes 'resting' in the ovary like a flock of sitting ducks, sometimes waiting for many years until their respective month of ovulation occurs, seems to provide an ideal target for germ cell mutations (German, 1979). If any germ cell mutation due to ingestion of oral contraceptives does occur, the particular affected oocyte or oocytes can be released at any time until the end of the reproductive life of the women; therefore it seems logical that a 'safe' period of three years does not exclude potential effects on offspring. Controls should be chosen from women who have never taken exogenous female sex hormones in their lives.

The majority of surveys conducted in order to determine the possible teratogenic effects of the hormonal contraceptives have been retrospective studies, with all the deficiencies and criticisms attached to this kind of study. In a retrospective study, we are dealing with referral cases, i.e. cases with a certain disorder or group of disorders which are compared with controls in respect to the exposure to the possible teratogenic agent in question (in this instance, the hormonal contraceptives). Because they are referred cases the parents of a child with a defect are more likely to remember previous exposure to environmental agents than the controls and therefore an inaccurate impression can be apparent.

The other limitation of the retrospective studies is that they provide no idea of the prevalence of the defect at birth, and neither attributable nor relative risk can be derived from them

(Leck, 1979). The few prospective studies reported are most reassuring with regard to negative findings (see below). In a prospective study the group exposed to a possible teratogenic is compared to an unexposed group in respect of the frequency of the defect.

In a prospective study of 50 282 mother-child pairs, undertaken to evaluate the association between cardiovascular birth defects and exposure to female sex hormones in the first four months of pregnancy, an association between the use of oral contraceptives and congenital cardiovascular anomalies was suggested although a causal relationship could not be drawn (Heinonen *et al.*, 1977). Other investigators have found less pronounced or only marginal indications of a positive relationship between the use of oral contraceptives and abnormalities in the offspring.

Robinson (1971) failed to show a statistically significant difference in foetal outcome between mothers who took estrogen-progestin contraceptives and controls; there were, however, more congenital heart defects and other life-threatening anomalies in the group of infants born to mothers who took oral contraceptives.

In a study by Rice Wray *et al.* (1971), four abnormal infants were born among thirty-two women who continued to take oral contraceptives while pregnant. The abnormalities included: one meningoencephalocele and three cases with minor unspecified abnormalities. Four abnormalities in thirty-two cases appears to be quite high, especially in view of the fact that of the original thirty-two women, four were lost to follow-up, two had induced abortions and five had spontaneous abortions. An interesting paper by Yalom *et al.* (1973) concerns hormonal influences during pregnancy and postnatal psychological

development in males. These authors concluded that male children who had been subjected to high levels of oestrogen and progesterone prenatally were less aggressive and athletic, and also generally less 'masculine'; two out of the forty males studied also had hypospadias. The possible relevance of prenatal oral contraceptives on the psychological development of males can, however, only be speculated on as the years go by.

The references mentioned in the previous pages indicate the possible teratogenic effects of the 'pill'. The mechanism of these effects is at present unknown. It is speculated that this could be due to: a) extensive cell death; b) mutation of gene or genes; c) chromosome abnormality (this subject will be dealt with in Section 8.4.11).

Indirect evidence for mutation is the interesting observation of possible sex-linkage, in that the excess of males among malformed infants who had been exposed to oral contraceptives *in utero* is remarkable (Royal College of General Practitioners, 1976; Janerich *et al.*, 1980). Combined figures from three different studies (two prospective and one retrospective) give a distorted sex ratio in malformed infants, eighty-two per cent of whom were males (Janerich *et al.*, 1980). This unexpected sex-ratio is difficult to reconcile with a noncausal association between oral contraceptive exposure and birth defects, especially in view of the fact that in the few published data relating to oral contraceptive use and sex of offspring, an increase in normal males has not been observed (Royal College of General Practitioners, 1976; Klinger and Glasser, 1977). On the contrary, two surveys (Crawford, 1973: Kaserü *et al.*, 1974) reported an excess of females among the normal infants born after the use of oral contraceptives.

These findings could indicate an increase in X-linked mutations which may be manifested as: a) increased incidence of males in the malformed babies; b) if an X-linked mutation is lethal there will be an increased incidence of females among normal infants at term. To-date there is no certainty as to the causal relationship between the use of oral contraceptives near and after conception and the risks of having abnormal offspring. The present imperfect understanding of the aetiology of birth defects associated with oral contraceptives is partly due to the fact that in the majority of published studies the data on hormone pregnancy tests, supportive hormonal therapy and oral contraceptives have all been grouped together, i.e. they have not been selective enough!

The reason for collectively assessing all these compounds was based on their similar chemical composition but their dosages are very different (oral contraceptives containing the smallest doses). The length of exposure as well as particular time when the embryo is exposed to these drugs is also very different; oral contraceptives are the most likely to be ingested at conception, and continued for a few weeks thereafter. On the other hand, exposure to other types of exogenous sex hormones usually starts at least some weeks after the first menstrual period has been missed and last a few days when used as pregnancy tests, or many weeks or months in cases of threatened abortion, and in some instances intermittently. Because of all these differences the risk to offspring of mothers with accidental pregnancies while on the 'pill' should be considered separately from other cases of female sex hormone exposure.

Another problem in detecting the possible teratogenic effect of the 'pill' has been the comparatively small number of affected children.

It is relatively easy to detect the cause of a 'disaster' as in the case of thalidomide; but it is very difficult to uncover a teratogen unless the 'attack' rate is high or the anomaly rare. These difficulties are discussed by Miller and Yasuda (1978) in a paper wherein they also comment on the difficulties of investigating the possible teratogenic effects of hormonal contraceptives. Another factor which has contributed to our present state of confusion on the subject is that the epidemiologic studies done on oral contraceptives have been assessed in different ways by different workers; either by grouping many types of malformations together due to the small number of affected children in their sample (cohort studies) or by looking at large numbers of identical malformations, e.g. cardiac malformations as in case-control studies. If oral contraceptives cause a specific malformation this could become submerged in a larger category and therefore its relationship to oral contraceptive use would be diluted (Janerich *et al.*, 1980).

At this point emphasis must be placed on the fact that up to now the studies on offspring of hormonal contraceptive users have only recorded abnormalities noticed at birth. Serious consideration should be given to abnormalities not easily detectable in infancy but sometimes only detected later in life, such as the possibility of cancer among others! The unfortunate 'episode' of diethylstilboestrol (DES) should be a warning at this point, notably because this compound has been used as a postcoital contraceptive (Bishun *et al.*, 1975) and is chemically related to hormonal contraceptives. DES, a synthetic oestrogen, was largely used for the treatment of threatened abortion during the period from 1948 to 1971. Herbst *et al.* (1970, 1071) published the first reports of

vaginal adenocarcinoma in girls and young women prenatally exposed to DES (taken by their mothers). Subsequently, numerous case reports have confirmed these findings (Wilson, 1977). To-date such exposure has been largely associated with abnormalities confined to the vagina and cervix; these include clear cell adenocarcinoma of vagina and cervix, and several nonmalignant uterine and vaginal lesions. mainly vaginal adenosis (Herbst *et al.*, 1975; Driscoll and Taylor, 1980). The transplacental effects of DES do not seem to be limited to females because abnormalities of the urogenital system (e.g. undescended testes, hypotrophic testes, hypospadias, urethral stenosis) and semen changes in density and motility have also been reported in human males (Driscoll and Taylor, 1980) prenatally exposed to DES. However, no increase in malignancy related to the male urogenital tract has apparently been reported to-date (Driscoll and Taylor, 1980).

The exact mechanism of action of DES in these cases is not yet clear; but stilboestrol is a known carcinogen and it has been postulated that this compound may initiate changes in the embryo which can later be triggered by the hormonal changes during puberty (Davies, 1977). It is possible that a clone of mutant premalignant cells may later in an adequate environmental milieu become malignant with a selective proliferative advantage. The postulates made in this regard are the same as those made for childhood leukaemia in children who received prenatal X-irradiation (German, 1979) mentioned in Section 8.1.

DES provides the first convincing evidence of transplacental carcinogenesis induction in man.

8.4.1.1 Chromosome abnormalities in live-borns and hormonal contraceptive use

None of the surveys so far have individually been able to demonstrate an association between the use of hormonal contraceptives and chromosome abnormalities in live-born children. This is possibly due to the small number of affected children and the small number of chromosome studies performed. It is suspected, however, that chromosome abnormalities may be increased in babies born after oral contraceptive failures (Harlap *et al.*, 1979). Rothaman and Louik (1978) observed an excess of Down syndrome among the offspring of women who conceived within a month of discontinuing the 'pill' and also in those who fell pregnant while taking the 'pill'.

A two-fold excess of Down syndrome was also noted by Bracken *et al.* (1978) in the offspring of women who had been taking oral contraceptives at the time of conception and after conception.

In two other clinical trials (Harlap *et al.*, 1979) a further two cases of trisomy 21 were observed among 215 breakthrough pregnancies. Yet another case was reported by Harlap *et al.* (1979) in a retrospective cohort study of 103 breakthrough pregnancies.

When the figures of all cohort studies are totalled, the incidence of Down syndrome in breakthrough pregnancies is very unlikely to be due to chance when compared with the expected values for Down syndrome at birth, related purely to maternal age in the general population.

Similar findings were reported in an extensive cohort study of 33 551 mothers by the Kaiser-Permanent Birth Defects Study; one case of trisomy 21 and two cases of trisomy 18 were found among

814 babies whose mothers continued taking oral contraceptives after conception (Harlap *et al.*, 1979). Again, because of the small size of the study, no definite conclusion could be drawn. It was stressed by these authors that it was most important to publish the results of such studies relating oral contraceptives and chromosome abnormalities in live-borns, even if the studies were considered unsuitable for publication, either due to the negative findings or because the sample was too small. It is possible that the combined data so revealed would throw some light on the subject. In the same year, 1979, Lejuene and Prieur in a retrospective study of 730 cases of trisomy 21, found a significant increase of Down syndrome progeny among mothers aged thirty to thirty-eight years, who took oral contraceptives. This excess was significant when the 'pill' was taken for more than one year and the child conceived within six months of discontinuation of the therapy.

Although the present author is well aware of the practical difficulties and time-consuming nature of chromosome studies on consecutive new-borns, the limitation of the presently available data on hormonal contraceptives and chromosome abnormalities in live-borns have to be looked at critically. The incidence of chromosome abnormalities in consecutive live-born infants has been calculated to be 1:800 for autosomal trisomies, 1:2 000 for autosomal structural unbalanced abnormalities (the only two categories that are likely to be detected as MCA at birth), 1:500 for autosomal structural balanced abnormalities, 1:400 for a sex chromosome abnormality in males and 1:700 for a sex chromosome abnormality in females (Hamerton *et al.*, 1975). It is therefore quite obvious that the previous few studies done are not adequate because of the

small sample size. Furthermore, in the majority of reports of pregnancy outcomes after oral contraception, no mention was made of chromosome studies in any of the abnormal children reported. Where Down syndrome was diagnosed it is not clear whether this diagnosis was based on cytogenetic studies or on clinical grounds.

Cytogenetic studies on the progeny of women who had used oral contraceptives are unfortunately scanty. McQuarrie *et al.* (1970) published a cytogenetic study on fifty babies born to mothers who had used oral contraceptives. The contraceptives used were sequential mestranol and mestranol with chlormadinone; the exposure to the drugs prior to conception varied from six to forty-one months with an average of 19,7 months. No mention was made of the time interval between discontinuation of the drug and conception. Among the fifty babies studied, one had a 'de novo' D/G chromosome translocation. Chromosome breakage in these babies was significantly higher than those of published controls. The authors did not, however, have adequate controls of their own in this study. Although the finding of the D/G translocation could very well be due to chance, and no conclusions can be drawn from a single case, it could very well be that the breakage on a D and G chromosome which preceded the formation of the translocated chromosome, was caused by the same agent (the 'pill') that also produced the chromosome breakages reported in the other babies.

In the same year, 1970, Rice-Wray *et al.* reported the results of chromosome studies in sixty-one children born to mothers who had used several types of hormonal contraceptives for an average period of twenty months. Seven of these sixty-one women continued the medication while pregnant; no numerical, stable or unstable chromosome

abnormalities were found. No controls were done in this study.

The following year the same authors (1971) published a further report on the offspring of oral contraceptive users. Chromosome studies were performed on seventy-three of a total of 516 offspring surveyed. Again no chromosome abnormalities, either numerical or structural were detected. However, in their table of abnormalities a case of 'mongolism' was reported. How the diagnosis was made is not specified. There was no control group in this study and the results were compared with published data from other sources.

Briggs and Briggs (1971) published a cytogenetic study on fifty mothers who had used oral contraceptives before conception and their babies. Each mother and baby had a control pair which was carefully selected for clinical, gynaecological and obstetrical histories and also for the consumption of pharmaceutical products. In this well conducted study no significant differences in either stable or unstable chromosome abnormalities were found between the two groups. However, it should be stressed that at least a minimum interval of nine months had elapsed between the discontinuation of the oral contraceptives and the time when the cytogenetic studies were done.

Bishun *et al.* (1975) reported chromosome studies on 185 babies and their respective mothers who had used different types of oral contraceptives sometime before conception. The results were compared with the chromosome findings obtained from a group of 181 babies and their mothers who had never practised any form of oral contraception. The results showed a significant increase in the incidence of chromosomal gaps (an increase of nearly five times) as well as an increase in hypomodal cells in babies whose mothers had taken oral

contraceptives. The abnormalities found were related to only certain types of hormonal contraceptives. Apart from the fact that the two groups were chosen based on previous history of use or nonuse of hormonal contraceptives, no reference was made to any other criteria essential in the choice of suitable subjects. Mention was made of the fact that oral contraceptives were used for a long time but the exact duration of therapy was not stated.

Klinger *et al.* (1976, 1977) in their studies provide the largest sample so far. In these studies, chromosome analyses were performed in 1 670 consecutive new-borns, 720 of whom had a preconception history of maternal oral contraception. This was the first study reported where banding was done in some of the cases. Six of the 720 infants had chromosome abnormalities, three of which were inherited. The authors acknowledge the small number of chromosomally abnormal cases as a limitation for their statistical analysis of possible relationships.

8.4.1.2 Chromosome abnormalities in abortions and hormonal contraceptive use

As discussed previously, if damage to the embryo is so extensive that it is incompatible with life, the conceptus will almost always be spontaneously aborted. Spontaneous abortions therefore provide an excellent (probably the best) source of material to investigate human malformations, particularly chromosome abnormalities. It is clear from data obtained from the various cytogenetic studies performed on spontaneous abortions, that chromosome abnormalities are the commonest single recognized cause of foetal death in man (Hassold *et al.*, 1980). It is also known that most of the abnormalities found in

spontaneous abortions, are only rarely, if ever, seen in live-born children (Alberman *et al.*, 1976; Hassold *et al.*, 1980). It is therefore logical to approach the problem as to whether or not hormonal contraceptives induce chromosomal abnormalities by performing chromosome studies on spontaneous abortions from women with a previous history of hormonal contraceptive use.

Carr's initial study published in 1970 was the 'trigger' for all subsequent publications on the subject. In his original work he reported an increase in triploidy and probably other aneuploidies among abortuses from women who became pregnant within six months of ceasing oral contraception. These findings were confirmed in a further study by the same author published in 1971. In Carr's reports there is reference to the time of exposure to oral contraceptives as well as to the time 'off the pill' prior to conception; there was, however, no reference to the type of oral contraceptive involved.

A similar report to that of Carr appeared in Lancet (Dhadial *et al.*, 1970) on chromosome studies in 331 spontaneous abortuses of which sixty came from mothers who had used oral contraceptives. They found that an excess of chromosomally abnormal foetuses originated from women with a history of previous oral contraceptive ingestion, as compared to nonusers, but the difference was not significant. No reference was made to the type of oral contraceptive or the time on hormonal contraceptive therapy.

Lazar *et al.* (1973) found a small but nonsignificant excess of chromosome abnormalities in spontaneous abortions after the 'pill'; however, the rate of use of oral contraceptives was extremely low.

No banding techniques were used in any of the previously mentioned studies.

Lauritsen (1975) conducted a more detailed cytogenetic survey using banding techniques on 286 consecutive spontaneous abortions. Of the 286 abortions, 124 originated from women who had previously used oral contraceptives and 122 from nonusers. In the remainder, chromosome studies were unsuccessful. Several types of oral contraceptives had been used by the women in this survey and the duration of therapy ranged from two to sixty months. No statistically significant difference in the incidence of chromosome abnormalities between the two groups was found except for a specific abnormality - 45,X, which was significantly increased in abortuses from women who used oral contraceptives.

There was, however, a factor in the above survey that the authors recognized as a possible source of error, viz, that the mean age of women who had used oral contraceptives was significantly lower than the mean age of the controls. It is of course well known that chromosome abnormalities, particularly trisomies, are dependent on maternal age at conception (Lauritsen, 1975; Alberman *et al.*, 1976; Hassold *et al.*, 1980) and this could have influenced the results obtained in this and other similar surveys.

Klinger and Glasser's study (1977), mentioned previously in connection with live-born investigations also included chromosome studies on ninety-eight spontaneously aborted foetuses of which twenty-eight had a previous history of exposure to oral contraceptives before conception. Four of these twenty-eight abortuses had an abnormal karyotype, which included triploidy (three cases) and one

case of trisomy E. The authors pointed out that their sample on spontaneous abortions was too small to be analyzed in detail.

The largest survey relating chromosome abnormalities in spontaneous abortuses with history of hormonal contraception is that of Alberman *et al.* (1976). Chromosome studies were performed on 992 of 2 620 spontaneously aborted foetuses. Various maternal factors, e.g. age, oral contraceptive history, social class, etc., were also assessed. There was a significant increase of chromosomally abnormal foetuses related to the use of oral contraceptives. An excess of about twenty per cent in the proportion of abnormal karyotypes was noted in aborted foetuses of women who had used oral contraceptives for more than eighteen months. Furthermore, the number of chromosomally abnormal foetuses appeared to correlate with the length of exposure to the drug. In summarizing these various reports, it seems that spontaneous abortions in mothers who have used oral contraceptives may have a small excess of chromosomally abnormal foetuses when compared to the nonusers.

8.4.2 Possible genetic damage in prenatal life caused by hormonal contraceptives - germ cell

The theoretical possibility that hormonal contraceptives taken during pregnancy could induce mutations in the germ cells of the foetus cannot be ignored. This effect would only be manifested when individuals reached the reproductive age, either in the form of sterility or abnormal offspring, but we must remember that the first generation from 'pill-users' are still in infancy!

8.4.3 Possible genetic damage in postnatal life caused by hormonal contraceptives

In this section reference will be made to possible genetic damage

in the woman who ingests hormonal contraceptives.

Available literature concerning possible associations between hormonal contraceptives and tumours will be reviewed in the context of phenotypic expression of mutations on the soma.

8.4.3.1 Somatic cells

8.4.3.1.1 Hormonal contraceptives and hepatocellular adenoma

Since 1971 there has been an apparent increase in reported cases of nonmalignant and possibly malignant liver cell tumours in women of childbearing age in the USA (Editorial, Br Med J, 1977; Nissen *et al.*, 1978; Ham *et al.*, 1978). Significantly, most of the women in these case reports had used oral contraceptives for several years.

Prior to the 1960's, hepatic adenomas were considered to be extremely rare. The dramatic increase in this benign tumour has been observed only in 'pill' users. At present the literature on the subject is so extensive that the link between oral contraceptives and this type of neoplasia appears incontrovertible. It does not appear coincidental that liver tumours were the first neoplasias to be related to the use of hormonal contraceptives. After all the liver is the major organ where these compounds are metabolized, and it is well documented that contraceptive steroids can impair hepatic functions in some hormonal contraceptive users as measured by liver function tests (Bloustein, 1978).

So far all the data have been obtained from retrospective studies, with all the deficiencies attached to this method of ascertainment.

However, several trends appear from the collected data:

Age - More than seventy-five per cent of the women affected were between the ages of twenty and thirty-five years (Nissen *et al.*, 1978).

Duration of exposure - The data on duration of exposure to hormonal contraceptives appears to be highly significant - over eighty-five per cent of the affected patients were exposed for more than four years (Nissen *et al.*, 1978).

Type of hormonal contraceptive and histopathological classification - Data correlating the type of hormonal contraceptive with the histopathological tumour type has been more difficult to assess because of the multitude of the oral contraceptive preparations available on the market.

8.4.3.1.2 Hormonal contraceptives and cervical carcinoma

A review of the literature on the possible association between cervical carcinoma and the use of hormonal contraceptives allows for no conclusions to be drawn as to a direct association so far (Peritz *et al.*, 1977; Ferenczy, 1978; Bibbo *et al.*, 1979; Swan and Petitti, 1982). Various factors contribute to this lack of knowledge. The most important variable in the incidence of cervical cancer, viz, the age of first coitus (Coppleson, 1970; Ferenczy, 1978) has not been included in many studies. Other factors, for example, the number of sexual partners, exact classification of cervical lesions, and the wide variety of contraceptive formulations used contributed to the difficulties in drawing any positive conclusions from these studies.

Lastly, the most essential requirement in order to try to establish

a negative or positive causal relationship between the use of oral contraceptives and cervical carcinoma, is an adequate number of patients followed up for a long period. This we do not yet have.

Although cervical erosion is considered benign and sometimes even an asymptomatic pathological lesion, it is interesting to note that cervical erosions are significantly increased in women taking oral contraceptives (Goldacre *et al.*, 1978).

Lerner (1978) obtained data on 3 000 000 American women from a large computerized file for a case control study. He found a significant association between the use of oral contraceptives and cervical dysplasia. In this study the patient's age, age at first pregnancy and gravidity were compared and carefully matched. These findings were confirmed by Meisels *et al.* (1977) who also found that the prevalence rate of dysplasia was significantly higher in users of oral contraceptives. They analyzed a total of 370 502 cervical smears from 'pill' users and controls. Dysplasias are considered, at least in some cases (Coppleson, 1970), true precursor lesions of carcinoma '*in situ*' and of squamous invasive carcinoma of the cervix. In fact, Stern *et al.* (1977) in a prospective study of women with cervical dysplasia showed an increase in severity of dysplasia and of conversion to cancer '*in situ*' in users of oral contraceptives when comparison was made with users of other contraceptive methods. They calculated that the risk of conversion of a cervical dysplasia to cervical cancer is six times greater for oral contraceptive users.

8.4.3.1.3 Hormonal contraceptives and endometrial carcinoma

After approximately three decades of relative stability in the

incidence of endometrial cancer, it has been reported in the USA that subsequent to 1970 the incidence of this type of malignancy has been rising sharply. The increased frequency is most marked among middle-aged women, but the incidence has also increased in younger and elderly women (Weiss *et al.*, 1976). There is strong evidence that oestrogens are related to endometrial cancer (Weiss *et al.*, 1976; Berendes, 1977), and there is every reason to believe that the use of oestrogen preparations in menopausal women is at least partially responsible for the increased incidence of endometrial cancer recently observed in postmenopausal women. These claims are based on the following:-

1. Animal studies have demonstrated a causal relationship between the use of oestrogens and endometrial cancer (Barron, 1978).
2. There is a definite association between excessive endogenous oestrogen production and increased risk of endometrial cancer, e.g. in women with oestrogen-secreting ovarian tumours, polycystic ovaries and Stein-Leventhal syndrome (Weiss *et al.*, 1976; Berendes, 1977).
3. Oestrogens when given to women with gonadal dysgenesis are associated with an increased risk of carcinoma of the endometrium (Weiss *et al.*, 1976; Berendes, 1977).
4. There is a higher frequency of oestrogen use seen among women with endometrial cancer and precursor lesions of such cancer.

As mentioned above, the increased incidence of endometrial cancer has also been noticed in premenopausal women. The spectacular increase in the use of oral contraceptives in recent times, has been incriminated as being possibly responsible for the rising incidence of endometrial

cancer in younger women (Weiss *et al.*, 1976). By 1975 sequential contraceptive 'pills' had already been withdrawn from the market in the USA because it was found that a high proportion of women under forty years of age with endometrial cancer had used sequential 'pills' (Berendes, 1977; Hanson, 1977; Silverberg, 1978).

8.4.3.1.4 Hormonal contraceptives and breast cancer

Among the proven 'ill effects' of the 'pill', thromboembolic disease is probably one of the most serious. However, the increase in mortality due to thromboembolic accidents in females using hormonal contraceptives is only slightly raised because this particular pathological entity is rare.

The picture will, however, be completely different if the incidence of breast cancer is rising due to the use of hormonal contraceptives. Breast cancer is common enough for even a small rise associated with the use of hormonal contraceptives to have dire consequences (Royal College of General Practitioners, 1981).

There is indirect evidence of endocrinological influences in the causation of breast cancer (Fasal, 1978; Editorial, Br Med J, 1981), and many of the established risk factors for women developing the disease are probably also related to hormones. Parity, age of first pregnancy, age at menarche, age at menopause, history of benign breast disease and obesity are amongst the factors known to influence the risk of developing breast cancer (Fasal, 1978).

As in the case of endometrial cancer, the incidence of breast cancer has increased over the past forty years (Paffenberger *et al.*, 1977; Fasal, 1978).

Several studies have been conducted to determine the possible effects of oral contraceptive use on the incidence of breast cancer, but the results thus far appear to be contradictory, confusing and inconclusive (Paffenberger *et al.*, 1977; Stadel, 1978; Royal College of General Practitioners, 1981; Vessey *et al.*, 1981).

Paffenberger *et al.* (1977) reported 452 cases of breast cancer in the San Francisco Bay area, USA. They found a significantly increased risk of breast cancer in women who had used the 'pill' for two to four years and who had presented with previous benign breast disease. The same was noted in women who started to take oral contraceptives before their first childbirth. In a survey of women under thirty-three years of age suffering from breast cancer, a statistically significant increased risk of breast cancer was found in those women who had used oral contraceptives for more than four years before their first full-term pregnancy (Pike *et al.*, 1981).

A prospective study of 1 541 women (Lees *et al.*, 1978) also showed a tendency for an increased risk of developing breast cancer in women using oral contraceptives for one to five years.

The two largest prospective studies done so far were conducted by the Royal College of General Practitioners (1981) and by the Oxford Family Planning Association (Vessey *et al.*, 1981). The first of these studies mentioned, reported on the incidence of breast cancer in 23 000 women using oral contraceptives and on a similar number of controls. The two groups were matched according to age, parity, etc., but no information was available on the most important determinant on the subsequent risk of developing breast cancer - age at first full-term birth (MacMahon *et al.*, 1970). This study failed to show an increase in

the incidence of breast cancer related to the use of oral contraceptives, though in the group of women between thirty and thirty-four, the risk is reported to be on the borderline of statistical significance.

The other major study (Vessey *et al.*, 1981) also failed to show an association between the use of oral contraceptives and breast carcinoma in a survey performed on 17 000 women. In this study, the observation noted in the thirty to thirty-fours' old group by the study of the Royal College of General Practitioners (1981), was not confirmed, but the authors point out that only fourteen women who developed breast cancer were below thirty-four years of age.

Although the findings in these last two studies were reassuring, both groups of authors expressed their consciousness of the difficulties and limitations of this kind of survey. As pointed out in the first paper (Royal College of General Practitioners, 1981), women who use oral contraceptives are a selected population *per se*. They are self selected and medically selected since they are not given oral contraceptives if there is a poor medical history. Also the lack of information on the age of first birth has been recognized by the authors as an important deficiency in assessing the risk for breast malignancy.

As noted in the second study (Vessey *et al.*, 1981), most of the women started using the 'pill' after giving birth to at least one child.

Apart from all the possible confounding factors which could account for the above conflicting observations, the unknown (possibly long) latent period between the action of a carcinogen and the manifestation of the cancer is one of the main factors making the assessment of a possible carcinogenic effect of hormonal contraceptives so difficult.

It is known that the peak age incidence for breast cancer is around fifty-five years (Editorial, Br Med J, 1981). Epidemiological evidence suggests that initiation of breast cancer generally occurs ten to fifteen years after menarche (Royal College of General Practitioners, 1981). A latent period of at least twenty to thirty years is therefore necessary before the cancer manifests itself. This means that, for example, young women who began to take the 'pill' at the age of twenty in 1965 would probably not show any increased risk (if it indeed does exist) until the year 2 000 (Editorial, Br Med J, 1981).

Pregnancy early in life is known to protect against breast cancer (MacMahon *et al.*, 1970). 'Modern' mothers tend to delay the initial childbearing by the use of oral contraceptives.

It is not yet known what price will be paid by these young women that are using oral contraceptives, sometimes for many years, to delay the birth of their first child. No study is at present available to assess this factor.

8.4.3.1.5 Hormonal contraceptives and other tumours

Other rare tumours have been linked with the use of hormonal contraceptives.

8.4.3.1.5.1 Hormonal contraceptives and melanomas

Ellenbrock (1968) and Sadoff *et al.* (1973) drew attention to several cases of women who either presented with melanomas or metastasized

after the use of oestrogens or progesterone either for menopausal symptoms or birth control. Lerner *et al.* (1979) reported two further such cases. In one case the authors related the development of melanoma metastases to the previous use of oral contraceptives and in the other case a melanoma developed from a benign naevus after the use of oral contraceptives. Both patients died at a young age from metastases.

Seeking an association between oral contraceptive use and malignant melanoma, Beral *et al.* (1977) conducted two different studies: a prospective study in 17 942 women and a case-control study on known melanoma patients and matched controls. Although the incidence rate of malignant melanoma was higher in oral contraceptive users, the difference was not significant; a trend was, however, evident. The authors concluded that the association between oral contraceptives and malignant melanoma is suggestive.

In this context it is interesting to mention that Harlap (1978) found an excess of pigmented skin lesions in babies of former oral contraceptive users when compared with babies of controls. These pigmented lesions were particularly evident in babies from underweight mothers. The authors suggested a possible interaction of 'pill' and nutritional state and its possible relevance in benign and malignant skin conditions in adult users of oral contraceptives.

8.4.3.1.5.2 Hormonal contraceptives and pituitary adenomas

Since 1970 a significant increase in the incidence of pituitary adenomas has been noted in Minnesota, USA (Annegers *et al.*, 1978).

This increase was noted mainly in women aged fifteen to forty-four years. No change in the incidence was noted in males or in females under fifteen years or over forty-five years of age. These authors suggested an association between such tumours and the use of oral contraceptives but this hypothesis was tested in a small case - control study (only nine cases) and no association was found. Further evidence to support this suggestion came from a report by Sherman *et al.* (1978) when seventy-four per cent of forty-two women with amenorrhoea and galactorrhoea due to a prolactin secreting pituitary adenoma, gave an history of use of oral contraceptives or postpartum prior to the onset of the symptoms. The authors suggested that oestrogens either as contraceptive pills or during pregnancy stimulates the growth and clinical expression of otherwise silent tumours.

Buchanan *et al.* (1977) reported an interesting case of a fifty-five year old man who complained of gynaecomastia, galactorrhoea and impotence for two years; a pituitary adenoma was diagnosed and removed. The patient worked in a pharmaceutical plant that manufactured oral contraceptives. He had been exposed to mestranol and norethindrone by inhalation.

In none of these studies except those specified in the above discussion, is mention made of the type of hormonal contraceptive used. There is however the case report of a patient on ethynodiol diacetate with mestranol (Ovulen-21) who subsequently developed a malignant melanoma (Ellerbroek, 1968).

8.4.3.1.6 Summary

All the neoplasias discussed in the previous pages in association with

the use of hormonal contraceptives may well be interpreted as the phenotypic manifestation of the genetic damage caused by the hormonal contraceptives in the somatic cells of the women who took them.

This damage can be either in the form of an invisible point mutation or alternatively mutations in the form of visible chromosome aberrations.

(continued on page 196)

The first form of genetic damage would at present not be possible to demonstrate directly. The latter form of genetic damage cannot be proven as chromosome studies were not done in any of these solid tumours possibly related with the use of hormonal contraceptives.

However, in all solid tumours chromosomally studied so far, chromosome aberrations were found (Sandberg, 1980). It may therefore be logical to expect to have found chromosome aberrations in such tumours had they been chromosomally studied.

If the use of hormonal contraceptives are associated with an increased incidence of chromosome aberrations in the haemopoietic tissue, as a systemic drug there is the possibility that similar effects occur in other tissues as well.

If one of these so-called random chromosome aberrations happens to have a proliferative advantage, and a cytogenetically abnormal clone emerges in a particular tissue, this would be the final step in the clinical emergence of the neoplasm.

8.4.3.2 Germ cells

Any possible genetic damage occurring in the germ cells of a woman taking hormonal contraceptives would only be manifested in her offspring. The effect could be manifested in the first generation, or only several generations later.

As part of this section will overlap with the section discussing the effects of genetic damage on the foetus, the author will attempt to avoid undue repetition. The possible genetic damage to germ cells can either be in the form of detectable chromosome breakage or in the

form of single gene mutations.

Chromosome breakage in the germ cell could lead to atresia of the oocyte or to the formation of stable chromosome abnormalities either in a balanced or unbalanced form in the oocyte.

Death of the oocyte would be unimportant since the 'stock' of oocytes in a woman far exceeds the number necessary for monthly ovulation during her reproductive life; and in any case the great majority of oocytes are condemned to atresia. Although, very unlikely, the observed delay in fertility after discontinuation of use of hormonal contraceptives (Janerich and Piper, 1978) could be attributed to an excess destruction of oocytes due to extensive imbalance of genetic material.

If a chromosomally unbalanced oocyte is formed due to chromosome breakage, and this particular egg happens to be fertilized, a phenotypically abnormal live-born child or a spontaneous abortion would result in the first generation of the parent. Chromosome abnormalities in both live-borns and spontaneous abortions in former oral contraceptive users have already been discussed.

If a chromosomally balanced abnormal oocyte results due to breakage, and it happens to be fertilized, the child in the first generation will be phenotypically normal, but later generations springing from this child may well be phenotypically abnormal due to segregation of unbalanced gametes at meiosis.

To-date, the limited data available concerning chromosomal studies in offspring of women who have used hormonal contraceptives does not allow us to arrive at any firm conclusion on the incidence

of balanced chromosomal abnormalities in their children. As none of these children have yet reached reproductive age, we therefore cannot assess what the outcome will be when they have offspring.

If the genetic damage in germ cells of women taking hormonal contraceptives is in the form of a point gene mutation then the role of hormonal contraceptives is even more difficult to assess. If a dominant mutation occurs, it will be manifested in the first generation and it can be expressed in three different ways: a) if the dominant mutation is lethal the embryo will be spontaneously aborted; or b) the mutation could be manifested immediately at birth as MCA or a single abnormality; or c) the mutation may only be manifested later in life. It is virtually impossible to test the first hypothesis, but an increase in abortions would tend indirectly to point in that direction. The second alternative would be expressed at birth but most of the monogenic diseases are difficult to diagnose and rare. Therefore, even if there is an increase in such dominant diseases, the number of cases of a particular disease would be too small for a meaningful statistical analysis. The subject of congenital malformation in offspring of 'pill' users has already been discussed (see Section 8.4.1) and the mechanism of dominant mutations postulated. The third possibility of expression of a dominant mutation cannot be proved or disproved at this stage, as the first generation of children from 'pill' users is still too young.

If a X-linked mutation occurs it would be expected to manifest in the first generation as an increase in sex-linked recessive diseases in males as well as a change in sex-ratio in favour of females. As discussed in previous pages, the excess of females born

after the use of oral contraceptives noticed by some workers, and also the preponderance of males among children with congenital abnormalities born after the use of oral contraceptives, support the possibility of X-linked point mutations.

If a recessive mutation occurs it will not be manifested in the first generation but only in later generations when union between heterozygotes will lead to an increase of affected homozygotes.

In the present discussion the possible genetic damage to germ cells of a woman taking hormonal contraceptives has focused on inherited diseases or congenital abnormalities.

Cancer in offspring is another theoretical implication of genetic damage in germ cells of the mother. Several theoretical models of oncogenesis have been proposed to explain the origin of malignancy and its genetic control. Many of these are very similar with only minor modifications to reconcile some inconsistencies which could not be fully explained in the original models (Purtilo *et al.*, 1978). One of the most accepted models is the two-step mutational model of oncogenesis proposed by Knudson and colleagues (Purtilo *et al.*, 1978). Knudson and colleagues proposed that cancer results from two mutations ('hits') affecting genetic material. (Mutation in this context is used to define any change in genetic material - chromosomal, viral integration, point mutation.) The first 'hit' (considered by some as 'initiation' of the neoplastic process) can take place in the germinal tissue of the mother. The second 'hit' will be the 'promotional' event for further propagation of the neoplasm. This

could conceivably lead to childhood and young adult cancers in contrast to the older age group cancers. The latter would also be explained by this model, except that both mutational 'hits' occur in the somatic cells of the individual in postnatal life (Ohno, 1971; Purtilo *et al.*, 1978).

In conclusion, as hormonal contraceptives are a group of agents with the probable capability of damaging the genetic material it is conceivable that they can act either as an 'initiation hit' or as a 'promotional hit' to the malignant process.

8.4.4 Summary of the possible effects of hormonal contraceptives on genetic material

The debate on hormonal contraceptives and neoplasia continues; answers remain elusive. The main difficulty in this assessment is that the latent interval between exposure and development of cancer, is unknown, but long. Some estimate it at fifteen to forty years (Selikoff and Hammond, 1973). This makes the establishment of any association between cancer and hormonal contraceptive use difficult to prove since hormonal contraceptives have not been in general use for long enough.

The debate on hormonal contraceptives and possible teratogenic effects also continues; answers remain equally elusive. Until national surveys on the incidence of pregnancies spontaneously aborted due to chromosome anomalies are available, and the incidence of genetic diseases in general (either chromosomal or single gene in origin) is known, one can only speculate on the possible detrimental effects of hormonal contraceptives on the genetic pool.

These concepts not only apply to hormonal contraceptives but also to any other chemical agent in general.

In the 20th century, society has embarked on an unprecedented era of manufacture of synthetic chemicals on behalf of commodity, which have invaded all facets of our every day life. We should bear in mind that all commodities have a 'price', the value of which, as far as chemicals are concerned, is only beginning to be gauged. In this context *hormonal contraceptives* should be no exception.

APPENDIX

TABLE 4.8. Chromosome abnormalities found in the forty-four pairs presented in this study

Pair	Subject	Number of cells	Dicentrics	Acentric fragments	Rings	Rearrangments	Chromosome deletions	Chromatid deletions	Chromosome breaks	Chromatid breaks	Chromosome gaps	Chromatid gaps
01	C	30	0	0	0	0	0	0	0	4	0	1
01	HC	30	0	0	0	0	0	0	1	11	0	1
02	C	30	1	0	0	0	0	1	0	7	0	1
02	HC	30	0	1	0	0	0	0	0	12	0	0
03	C	30	0	0	0	0	0	0	0	8	0	0
03	HC	30	0	0	0	0	0	0	0	2	0	2
04	C	30	0	0	0	0	0	0	0	2	0	1
04	HC	30	0	0	0	0	0	1	0	7	0	0
05	C	30	0	0	0	0	0	0	0	2	0	0
05	HC	30	1	2	0	1	0	1	1	8	0	0
06	C	30	0	0	0	0	0	0	0	1	0	1
06	HC	30	0	0	0	0	0	4	0	3	0	0
07	C	30	0	0	0	0	0	0	0	5	0	0
07	HC	30	0	2	0	0	0	0	1	2	0	0
08	C	30	0	0	0	0	0	0	0	5	0	0
08	HC	30	2	1	0	0	0	0	0	4	0	0
09	C	30	0	0	0	0	0	0	0	2	0	1
09	HC	30	0	0	0	2	0	0	2	3	0	0
10	C	30	0	0	0	0	0	0	0	3	0	1
10	HC	30	0	0	0	0	0	1	1	4	0	0
11	C	30	0	0	0	0	0	0	2	4	0	1
11	HC	30	0	0	0	1	0	0	1	7	1	0
12	C	30	0	0	0	0	0	0	0	1	0	1
12	HC	30	0	0	0	0	0	2	2	9	0	1
13	C	30	0	0	0	0	0	0	1	4	0	1
13	HC	30	0	0	0	0	0	0	3	8	0	0
14	C	30	0	0	0	0	0	0	0	5	0	1
14	HC	29	0	1	0	1	0	0	3	9	1	3
15	C	29	0	0	0	0	0	0	1	8	0	0
15	HC	30	1	0	0	0	0	0	4	8	0	0
16	C	30	0	0	0	0	0	0	0	11	0	1
16	HC	30	0	1	0	0	0	0	0	2	0	1
17	C	30	0	0	0	0	0	0	0	4	0	1
17	HC	30	0	0	0	0	0	0	3	7	0	4
18	C	14	0	0	1	0	0	0	1	4	0	0
18	HC	30	0	2	0	0	0	0	2	13	0	1

Pair	Subject	Number of cells	Dicentrics	Acentric fragments	Rings	Rearrangements	Chromosome deletions	Chromatid deletions	Chromosome breaks	Chromatid breaks	Chromosome gaps	Chromatid gaps
19	C	30	0	0	0	0	0	0	1	7	1	0
19	HC	30	0	3	0	0	0	0	3	11	0	0
20	C	30	0	0	0	0	0	0	1	6	0	3
20	HC	30	1	0	0	0	0	0	1	3	0	2
21	C	30	0	0	0	0	0	0	2	5	0	0
21	HC	22	0	2	0	0	0	2	1	9	0	0
22	C	21	0	0	0	0	0	0	0	2	0	1
22	HC	30	0	4	0	0	0	0	2	7	0	0
23	C	30	0	0	0	0	0	0	1	5	0	1
23	HC	30	0	2	0	0	0	0	4	7	0	0
24	C	30	0	1	0	0	0	0	0	4	0	1
24	HC	30	0	0	0	0	0	0	2	7	0	0
25	C	30	0	0	0	0	0	0	0	6	0	0
25	HC	30	0	1	0	0	0	0	2	10	0	1
26	C	30	0	0	0	1	0	0	1	5	0	2
26	HC	26	0	1	0	0	0	0	3	4	0	0
27	C	30	0	0	0	0	0	0	0	4	0	0
27	HC	30	0	1	1	0	0	1	0	8	0	0
28	C	30	0	0	1	0	0	0	1	2	0	2
28	HC	30	0	1	0	1	1	0	5	5	0	0
29	C	30	0	0	0	0	0	0	0	2	0	1
29	HC	30	0	0	1	0	0	0	1	8	0	0
30	C	27	0	0	0	0	0	0	0	6	0	0
30	HC	30	0	0	0	0	0	0	2	10	0	0
31	C	30	0	0	0	0	0	0	1	7	0	1
31	HC	30	0	0	0	1	0	1	8	9	0	0
32	C	30	0	1	0	0	0	0	0	6	0	1
32	HC	30	0	1	0	0	0	0	0	3	0	0
33	C	30	0	0	0	0	0	0	0	1	0	6
33	HC	30	0	0	0	0	0	0	0	8	0	2
34	C	30	0	0	0	0	0	0	0	5	0	1
34	HC	30	0	0	0	0	0	1	0	15	0	3
35	C	27	0	0	0	0	0	0	0	4	0	2
35	HC	30	0	0	0	0	0	0	1	6	0	0
36	C	30	0	0	0	0	0	0	0	4	0	1
36	HC	30	0	0	0	0	0	1	2	9	0	0

Pair	Subject	Number of cells	Dicentrics	Acentric fragments	Rings	Rearrangements	Chromosome deletions	Chromatid deletions	Chromosome breaks	Chromatid breaks	Chromosome gaps	Chromatid gaps
37	C	24	0	0	0	0	0	0	0	3	0	3
37	HC	30	0	1	0	0	0	1	0	9	0	3
38	C	30	0	0	0	0	0	0	1	5	0	1
38	HC	30	0	2	0	0	0	1	2	12	0	4
39	C	28	0	0	0	0	0	0	0	5	0	3
39	HC	23	0	0	0	0	0	0	0	10	0	0
40	C	24	0	0	0	0	0	0	0	4	0	2
40	HC	21	0	0	0	0	0	0	1	6	0	2
41	C	26	0	0	0	0	0	0	0	2	0	3
41	HC	30	0	0	0	0	0	0	2	7	0	1
42	C	30	0	0	0	0	0	0	0	5	0	6
42	HC	30	0	0	0	0	0	0	0	15	0	2
43	C	21	1	0	0	0	0	0	0	5	0	0
43	HC	30	0	0	0	0	0	2	0	15	0	3
44	C	24	0	0	0	0	0	0	0	3	0	0
44	HC	25	0	1	0	0	1	1	3	9	1	2

Key to Table 4.9

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Pair - number	Treatment	Age	Race	Children - number	Smoking	Type of hormonal contraceptive	Time in months on hormonal contraceptives	Intervals (in months) during the treatment	Total number of cells (forty-six and less than forty-six chromosomes)	Dicentrics	Acentrics	Rings	Rearrangements	Chromosome deletions	Chromatid deletions	Chromosome breaks	Chromatid breaks	Chromosome gaps	Chromatid gaps

1 Pair number (from 1 to 44).

2 Treatment
1. Control.
2. Hormonal contraception.

3 Age.

4 Race
1. White.
2. Black.

5 Number of children.

6 Smoking habits
1. No smoking.
2. Less than average (less or equal to ten cigarettes/day).
3. Average (eleven to twenty cigarettes/day).
4. Heavy (equal or more than twenty-one cigarettes/day).
5. Unknown.

7 Type of hormonal contraceptive
1. Minovlar ED.
2. Depo-Provera.
3. Ovral or Nordiol.
4. Gynovlar.
5. Serial.
6. Lyndiol 2.5 plus Minovlar ED.
7. Minovlar ED plus Ovral.
8. Micro-Novum plus Minovlar ED.
9. Ovulen plus Minovlar ED.
10. Minovlar ED plus Nordette.
11. Depo-Provera plus Micro-Novum.
12. Ovral plus Depo-Provera.
14. Ovral plus Micro-Novum plus Depo-Provera.
15. Ovral plus Minovlar ED plus Micro-Novum.
16 Gysilon plus Unigynon plus Nordette.
17. Depo-Provera plus normovlar.

8 Time in months on hormonal contraceptives.

9 Intervals in months during the treatment.

10 Total number of cells analyzed (cells with forty-six and cells with less than forty-six chromosomes).

11 Dicentrics.

12 Acentrics.

13 Rings.

14 Rearrangements.

15 Chromosome deletions.

16 Chromatid deletions.

17 Chromosome breaks.

18 Chromatid breaks.

19 Chromosome gaps.

20 Chromatid gaps.

TABLE 4.9. Clinical and chromosomal data on the forty-four pairs presented in this study

																				Cells with forty-six chromosomes											Cells with less than forty-six chromosomes										
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	10	11	12	13	14	15	16	17	18	19	20	10	11	12	13	14	15	16	17	18	19	20
01	1	19	1	0	2	00	00	00	30	0	0	0	0	0	0	0	04	0	1	30	0	0	0	0	0	0	0	04	0	1	00	0	0	0	0	0	0	0	00	0	0
01	2	25	1	1	2	01	36	00	30	0	0	0	0	0	0	1	11	0	1	28	0	0	0	0	0	0	1	09	0	1	02	0	0	0	0	0	0	0	02	0	0
02	1	16	1	0	5	00	00	00	30	1	0	0	0	0	1	0	07	0	1	28	0	0	0	0	0	1	0	06	0	1	02	1	0	0	0	0	0	0	01	0	0
02	2	27	1	2	1	15	72	10	30	0	1	0	0	0	0	0	12	0	0	29	0	1	0	0	0	0	0	11	0	0	01	0	0	0	0	0	0	0	01	0	0
03	1	21	1	1	5	00	00	00	30	0	0	0	0	0	0	0	08	0	0	26	0	0	0	0	0	0	0	04	0	0	04	0	0	0	0	0	0	0	04	0	0
03	2	19	1	3	5	01	14	00	30	0	0	0	0	0	0	0	02	0	2	30	0	0	0	0	0	0	0	02	0	2	00	0	0	0	0	0	0	0	00	0	0
04	1	19	1	0	5	00	00	00	30	0	0	0	0	0	0	0	02	0	1	30	0	0	0	0	0	0	0	02	0	1	00	0	0	0	0	0	0	0	00	0	0
04	2	29	1	3	5	01	48	00	30	0	0	0	0	0	1	0	07	0	0	29	0	0	0	0	0	1	0	06	0	0	01	0	0	0	0	0	0	0	01	0	0
05	1	19	1	0	1	00	00	00	30	0	0	0	0	0	0	0	02	0	0	29	0	0	0	0	0	0	0	01	0	0	01	0	0	0	0	0	0	0	01	0	0
05	2	24	1	0	3	06	84	14	30	1	2	0	1	0	1	1	08	0	0	28	0	2	0	0	0	1	1	07	0	0	02	1	0	0	1	0	0	0	01	0	0
06	1	22	1	2	5	00	00	00	30	0	0	0	0	0	0	0	01	0	1	30	0	0	0	0	0	0	0	01	0	1	00	0	0	0	0	0	0	0	00	0	0
06	2	26	1	2	5	01	24	00	30	0	0	0	0	0	4	0	03	0	0	27	0	0	0	0	0	2	0	03	0	0	02	0	0	0	0	0	2	0	00	0	0
07	1	32	2	2	2	00	00	00	30	0	0	0	0	0	0	0	05	0	0	29	0	0	0	0	0	0	0	04	0	0	01	0	0	0	0	0	0	0	01	0	0
07	2	35	2	3	1	03	48	00	30	0	2	0	0	0	0	1	02	0	0	28	0	0	0	0	0	0	1	02	0	0	02	0	2	0	0	0	0	0	00	0	0
08	1	21	1	0	1	00	00	00	30	0	0	0	0	0	0	0	05	0	0	29	0	0	0	0	0	0	0	04	0	0	01	0	0	0	0	0	0	0	01	0	0
08	2	29	2	2	5	01	56	02	30	2	1	0	0	0	0	0	04	0	0	29	1	1	0	0	0	0	0	04	0	0	01	1	0	0	0	0	0	0	00	0	0
09	1	17	1	0	3	00	00	00	30	0	0	0	0	0	0	0	02	0	1	29	0	0	0	0	0	0	0	02	0	0	01	0	0	0	0	0	0	0	00	0	1
09	2	25	1	0	1	01	36	01	30	0	0	0	2	0	0	2	03	0	0	26	0	0	0	0	0	0	0	03	0	0	04	0	0	0	2	0	0	2	00	0	0
10	1	21	2	1	2	00	00	00	30	0	0	0	0	0	0	0	03	0	1	29	0	0	0	0	0	0	0	03	0	0	01	0	0	0	0	0	0	0	00	0	1
10	2	31	2	2	5	11	60	00	30	0	0	0	0	0	1	1	04	0	0	29	0	0	0	0	0	0	1	04	0	0	01	0	0	0	0	0	0	1	00	0	0
11	1	18	1	0	3	00	00	00	30	0	0	0	0	0	0	2	04	0	1	28	0	0	0	0	0	0	1	02	0	1	02	0	0	0	0	0	0	1	02	0	0
11	2	20	1	0	3	01	30	00	30	0	0	0	1	0	0	1	07	1	0	28	0	0	0	0	0	0	0	07	1	0	02	0	0	0	1	0	0	1	00	0	0
12	1	18	1	0	3	00	00	00	30	0	0	0	0	0	0	0	01	0	1	30	0	0	0	0	0	0	0	01	0	1	00	0	0	0	0	0	0	0	00	0	0
12	2	29	1	2	5	03	66	06	30	0	0	0	0	0	2	2	09	0	1	30	0	0	0	0	0	2	2	09	0	1	00	0	0	0	0	0	0	0	00	0	0
13	1	21	2	2	5	00	00	00	30	0	0	0	0	0	0	1	04	0	1	28	0	0	0	0	0	0	1	03	0	0	02	0	0	0	0	0	0	0	01	0	1
13	2	27	2	3	5	01	32	00	30	0	0	0	0	0	0	3	08	0	0	28	0	0	0	0	0	0	1	06	0	0	02	0	0	0	0	0	0	2	02	0	0
14	1	23	2	2	1	00	00	00	30	0	0	0	0	0	0	0	05	0	1	30	0	0	0	0	0	0	0	05	0	1	00	0	0	0	0	0	0	0	00	0	0
14	2	28	2	4	2	14	38	00	29	0	1	0	1	0	0	3	09	1	3	25	0	1	0	0	0	0	2	08	1	2	04	0	0	0	1	0	0	1	01	0	1

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Cells with forty-six chromosomes											Cells with less than forty-six chromosomes										
																				10	11	12	13	14	15	16	17	18	19	20	10	11	12	13	14	15	16	17	18	19	20
15	1	25	1	0	5	00	00	00	29	0	0	0	0	0	0	1	08	0	0	26	0	0	0	0	0	0	0	06	0	0	03	0	0	0	0	0	0	1	02	0	0
15	2	25	1	2	1	01	24	00	30	1	0	0	0	0	0	4	08	0	0	27	0	0	0	0	0	0	3	07	0	0	03	1	0	0	0	0	0	1	01	0	0
16	1	23	2	3	5	00	00	00	30	0	0	0	0	0	0	0	11	0	1	25	0	0	0	0	0	0	0	07	0	0	05	0	0	0	0	0	0	0	04	0	1
16	2	32	2	5	5	12	78	10	30	0	1	0	0	0	0	0	02	0	1	30	0	1	0	0	0	0	0	02	0	1	00	0	0	0	0	0	0	0	00	0	0
17	1	20	1	0	5	00	00	00	30	0	0	0	0	0	0	0	04	0	1	28	0	0	0	0	0	0	0	03	0	0	02	0	0	0	0	0	0	0	01	0	1
17	2	18	1	1	2	01	18	00	30	0	0	0	0	0	0	3	07	0	4	26	0	0	0	0	0	0	3	05	0	2	04	0	0	0	0	0	0	0	02	0	2
18	1	17	2	0	1	00	00	00	14	0	0	1	0	0	0	1	04	0	0	14	0	0	1	0	0	0	1	04	0	0	00	0	0	0	0	0	0	0	00	0	0
18	2	28	2	2	5	02	19	00	30	0	2	0	0	0	0	2	13	0	1	25	0	1	0	0	0	0	2	07	0	0	05	0	1	0	0	0	0	0	06	0	1
19	1	19	1	0	1	00	00	00	30	0	0	0	0	0	0	1	07	1	0	28	0	0	0	0	0	0	0	05	1	0	02	0	0	0	0	0	0	1	02	0	0
19	2	23	2	1	1	08	38	00	30	0	3	0	0	0	0	3	11	0	0	25	0	2	0	0	0	0	3	04	0	0	05	0	1	0	0	0	0	0	07	0	0
20	1	29	2	1	2	00	00	00	30	0	0	0	0	0	0	1	06	0	3	27	0	0	0	0	0	0	1	03	0	1	03	0	0	0	0	0	0	0	03	0	2
20	2	32	1	5	5	16	32	12	30	1	0	0	0	0	0	1	03	0	2	27	0	0	0	0	0	0	0	02	0	2	03	1	0	0	0	0	0	1	01	0	0
21	1	20	1	0	1	00	00	00	30	0	0	0	0	0	0	2	05	0	0	29	0	0	0	0	0	0	0	2	04	0	0	01	0	0	0	0	0	0	0	01	0	0
21	2	24	1	1	5	02	30	00	22	0	2	0	0	0	2	1	09	0	0	17	0	1	0	0	0	1	0	07	0	0	05	0	1	0	0	0	1	1	02	0	0
22	1	18	1	0	1	00	00	00	21	0	0	0	0	0	0	0	02	0	1	19	0	0	0	0	0	0	0	01	0	0	02	0	0	0	0	0	0	0	01	0	1
22	2	34	2	2	5	17	22	00	30	0	4	0	0	0	0	2	07	0	0	25	0	1	0	0	0	0	2	03	0	0	05	0	3	0	0	0	0	0	04	0	0
23	1	34	1	3	3	00	00	00	30	0	0	0	0	0	0	1	05	0	1	30	0	0	0	0	0	0	1	05	0	1	00	0	0	0	0	0	0	0	00	0	0
23	2	31	1	3	5	02	36	00	30	0	2	0	0	0	0	4	07	0	0	29	0	2	0	0	0	0	4	06	0	0	01	0	0	0	0	0	0	0	01	0	0
24	1	21	1	0	5	00	00	00	30	0	1	0	0	0	0	0	04	0	1	29	0	1	0	0	0	0	0	03	0	1	01	0	0	0	0	0	0	0	01	0	0
24	2	25	2	2	1	01	24	00	30	0	0	0	0	0	0	2	07	0	0	29	0	0	0	0	0	0	1	07	0	0	01	0	0	0	0	0	0	1	00	0	0
25	1	22	1	0	5	00	00	00	30	0	0	0	0	0	0	0	06	0	0	29	0	0	0	0	0	0	0	05	0	0	01	0	0	0	0	0	0	0	01	0	0
25	2	24	1	0	1	01	39	00	30	0	1	0	0	0	0	2	10	0	1	27	0	1	0	0	0	0	2	07	0	1	03	0	0	0	0	0	0	0	03	0	0
26	1	20	1	0	1	00	00	00	30	0	0	0	1	0	0	1	05	0	2	28	0	0	0	0	0	0	1	04	0	2	02	0	0	0	1	0	0	0	01	0	0
26	2	33	2	3	5	12	36	60	26	0	1	0	0	0	0	3	04	0	0	25	0	1	0	0	0	0	3	03	0	0	01	0	0	0	0	0	0	0	01	0	0
27	1	29	1	0	5	00	00	00	30	0	0	0	0	0	0	0	04	0	0	30	0	0	0	0	0	0	0	04	0	0	00	0	0	0	0	0	0	0	00	0	0
27	2	22	1	0	5	08	27	00	30	0	1	1	0	0	1	0	08	0	0	30	0	1	1	0	0	1	0	08	0	0	00	0	0	0	0	0	0	0	00	0	0
28	1	19	1	0	1	00	00	00	30	0	0	1	0	0	0	1	02	0	2	29	0	0	1	0	0	0	0	02	0	2	01	0	0	0	0	0	0	1	00	0	0
28	2	30	2	4	5	02	34	00	30	0	1	0	1	1	0	5	05	0	0	27	0	1	0	0	1	0	4	02	0	0	03	0	0	0	1	0	0	1	03	0	0

																				Cells with forty-six chromosomes										Cells with less than forty-six chromosomes									
1	2	3	4	5	6	7	8	9	10	11	12	13	13	15	16	17	18	19	20	10	11	12	13	14	15	16	17	18	19	20	10	11	12	13	14	15	16	17	18	19	20
29	1	19	1	0	1	00	00	00	30	0	0	0	0	0	0	0	02	0	1	29	0	0	0	0	0	0	0	01	0	1	01	0	0	0	0	0	0	0	01	0	0
29	2	19	1	0	5	01	40	03	30	0	0	1	0	0	0	1	08	0	0	29	0	0	1	0	0	0	0	08	0	0	01	0	0	0	0	0	0	1	00	0	0
30	1	19	1	0	1	00	00	00	27	0	0	0	0	0	0	0	06	0	0	27	0	0	0	0	0	0	0	06	0	0	00	0	0	0	0	0	0	0	00	0	0
30	2	23	1	0	2	03	24	09	30	0	0	0	0	0	0	2	10	0	0	28	0	0	0	0	0	0	2	08	0	0	02	0	0	0	0	0	0	0	02	0	0
31	1	19	1	0	1	00	00	00	30	0	0	0	0	0	0	1	07	0	1	29	0	0	0	0	0	0	1	06	0	1	01	0	0	0	0	0	0	0	01	0	0
31	2	31	1	1	5	09	98	11	30	0	0	0	1	0	1	8	09	0	0	26	0	0	0	0	0	1	6	03	0	0	04	0	0	0	1	0	0	2	06	0	0
32	1	21	2	1	5	00	00	00	30	0	1	0	0	0	0	0	06	0	1	28	0	0	0	0	0	0	0	05	0	1	02	0	1	0	0	0	0	0	01	0	0
32	2	28	2	3	5	07	40	06	30	0	1	0	0	0	0	0	03	0	0	30	0	1	0	0	0	0	0	03	0	0	00	0	0	0	0	0	0	0	00	0	0
33	1	20	1	0	1	00	00	00	30	0	0	0	0	0	0	0	01	0	6	30	0	0	0	0	0	0	0	01	0	6	00	0	0	0	0	0	0	0	00	0	0
33	2	22	1	0	5	10	36	00	30	0	0	0	0	0	0	0	08	0	2	30	0	0	0	0	0	0	0	08	0	2	00	0	0	0	0	0	0	0	00	0	0
34	1	18	1	0	1	00	00	00	30	0	0	0	0	0	0	0	05	0	1	28	0	0	0	0	0	0	0	02	0	1	02	0	0	0	0	0	0	0	03	0	0
34	2	22	1	0	3	07	40	03	30	0	0	0	0	0	1	0	15	0	3	24	0	0	0	0	0	0	0	10	0	1	06	0	0	0	0	0	1	0	05	0	2
35	1	20	1	0	1	00	00	00	27	0	0	0	0	0	0	0	04	0	2	26	0	0	0	0	0	0	0	04	0	1	01	0	0	0	0	0	0	0	00	0	1
35	2	21	1	1	1	01	24	00	30	0	0	0	0	0	0	1	06	0	0	28	0	0	0	0	0	0	0	04	0	0	02	0	0	0	0	0	0	1	02	0	0
36	1	21	1	0	1	00	00	00	30	0	0	0	0	0	0	0	04	0	1	28	0	0	0	0	0	0	0	01	0	1	02	0	0	0	0	0	0	0	03	0	0
36	2	22	1	1	3	01	07	00	30	0	0	0	0	0	1	2	09	0	0	26	0	0	0	0	0	0	1	05	0	0	04	0	0	0	0	0	1	1	04	0	0
37	1	25	1	2	5	00	00	00	24	0	0	0	0	0	0	0	03	0	3	24	0	0	0	0	0	0	0	03	0	3	00	0	0	0	0	0	0	0	00	0	0
37	2	20	1	1	5	01	15	00	30	0	1	0	0	0	1	0	09	0	3	29	0	0	0	0	0	1	0	08	0	3	01	0	1	0	0	0	0	0	01	0	0
38	1	23	1	1	5	00	00	00	30	0	0	0	0	0	0	1	05	0	1	30	0	0	0	0	0	0	1	05	0	1	00	0	0	0	0	0	0	0	00	0	0
38	2	28	1	2	1	04	84	07	30	0	2	0	0	0	1	2	12	0	4	29	0	2	0	0	0	1	1	12	0	4	01	0	0	0	0	0	0	1	00	0	0
39	1	23	1	0	1	00	00	00	28	0	0	0	0	0	0	0	05	0	3	26	0	0	0	0	0	0	0	04	0	2	02	0	0	0	0	0	0	0	01	0	1
39	2	25	1	1	3	01	24	00	23	0	0	0	0	0	0	0	10	0	0	23	0	0	0	0	0	0	0	10	0	0	00	0	0	0	0	0	0	0	00	0	0
40	1	25	1	0	1	00	00	00	24	0	0	0	0	0	0	0	04	0	2	23	0	0	0	0	0	0	0	04	0	1	01	0	0	0	0	0	0	0	00	0	1
40	2	22	1	1	5	01	24	00	21	0	0	0	0	0	0	1	06	0	2	20	0	0	0	0	0	0	1	05	0	1	01	0	0	0	0	0	0	0	01	0	1
41	1	28	1	1	1	00	00	00	26	0	0	0	0	0	0	0	02	0	3	24	0	0	0	0	0	0	0	00	0	3	02	0	0	0	0	0	0	0	02	0	0
41	2	25	1	2	2	01	24	00	30	0	0	0	0	0	0	2	07	0	1	29	0	0	0	0	0	0	1	07	0	1	01	0	0	0	0	0	0	1	00	0	0
42	1	19	1	0	1	00	00	00	30	0	0	0	0	0	0	0	05	0	6	28	0	0	0	0	0	0	0	03	0	4	02	0	0	0	0	0	0	0	02	0	2
42	2	20	1	0	1	07	18	00	30	0	0	0	0	0	0	0	15	0	2	22	0	0	0	0	0	0	0	05	0	1	08	0	0	0	0	0	0	0	10	0	1

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Cells with forty-six chromosomes											Cells with less than forty-six chromosomes										
																				10	11	12	13	14	15	16	17	18	19	20	10	11	12	13	14	15	16	17	18	19	20
43	1	19	1	1	5	00	00	00	21	1	0	0	0	0	0	0	05	0	0	20	0	0	0	0	0	0	0	05	0	0	01	1	0	0	0	0	0	0	00	0	0
43	2	28	1	1	5	05	72	12	30	0	0	0	0	0	2	0	15	0	3	25	0	0	0	0	0	2	0	11	0	1	05	0	0	0	0	0	0	0	04	0	2
44	1	33	2	2	1	00	00	00	24	0	0	0	0	0	0	0	03	0	0	23	0	0	0	0	0	0	0	02	0	0	01	0	0	0	0	0	0	0	01	0	0
44	2	31	2	4	5	12	39	00	25	0	1	0	0	1	1	3	09	1	2	20	0	0	0	0	1	0	2	05	1	2	05	0	1	0	0	0	1	1	04	0	0

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