DECLARATION

I declare that this thesis 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. It has not been submitted for a degree in any other University.

Jennifer Rosendorff

December 1985
To my parents
ACKNOWLEDGEMENTS

This study was carried out during my period of employment as a Research Assistant on the joint staff of the South African Institute for Medical Research and the University of the Witwatersrand. I am extremely grateful for the opportunity and facilities afforded me at the South African Institute for Medical Research, under the directorship of Professor Jack Metz.

My sincere thanks to Professor René Bernstein, my supervisor and mentor, who has unselfishly provided outstanding encouragement and sound advice throughout the period of these studies. It is indeed impossible to adequately express my gratitude for her unceasing patience, assistance and general influence on my career.

I thank Professor Trefor Jenkins for his guidance and support, as well as for providing the necessary facilities in his department for this project.

I would also like to extend my appreciation to my colleagues in the Department of Human Genetics for their friendship, advice and encouragement during the past few years.

I gratefully thank Professor Lorna Macdougall, Doctor Richard Cohn and numerous private practitioners for their interest in this study and for referring their patients for chromosomal analysis.

I am also indebted to and wish to thank the following individuals:

Doctor S H Reinach and staff of the Institute for Biostatistics (Transvaal Branch) for the complex statistical analysis of the results.

Mrs Lilly Battaglia and staff of the Library for their continued goodwill in obtaining numerous publications for me.
Mrs Yvonne Descy, Mrs Hazel Lenkoe, Mr Mel Anderson, Mrs Martha Ulrich and Mrs Gill Martin for photographic assistance.

Mrs Ardyn Erasmus and Mrs Janice Gallant of the Map and Illustration Unit for illustrations.

Mrs Lucette Moore, Miss Denise Anstey, Mrs Gwen Dique and Mrs Shirley Alper for their much needed help and efficiency in the typing of this thesis.

Finally, a big 'Thank You' to my family and friends for their love, tolerance and tireless encouragement during the course of my studies.
ABSTRACT

Fancioni’s anaemia (FA) is characterized by phenotypic diversity and delayed onset of pancytopenia. This clinical heterogeneity, together with the existence of other syndromes associated with one or more features of FA, make the accurate diagnosis of this rare, autosomal recessive disorder difficult in some patients. Furthermore, in vitro chromosome breakage may be variable and inconsistent, and thus non-diagnostic. Therefore, the in vitro enhancement of chromosome breakage by 2 clastogenic mutagens, Diepoxybutane (DEB) and Mitomycin C (MMC), was studied in FA homozygotes and heterozygotes for the accurate diagnosis of affected homozygotes, as well as for the identification of FA heterozygotes.

In total, 24 FA homozygotes, 28 obligatory FA heterozygotes, 42 normal controls, 6 siblings of FA homozygotes and 15 patients with some features of FA were investigated for the presence of both unstable and stable chromosome abnormalities. Peripheral blood specimens were subjected to standard short-term lymphocyte culture techniques with the in vitro addition of clastogenic concentrations of DEB and MMC. In all instances, control blood specimens from normal individuals were concurrently identically processed. One amniotic cell prenatal diagnosis of FA was also successfully performed.

The Wilcoxon signed ranks test comparing differences within genotypic/phenotypic classes showed that both MMC and DEB significantly enhance chromosome breakage in FA homozygotes. DEB had no significant effect in normal control subjects, but control cells exhibited increased susceptibility to MMC. Heterozygotes, siblings and individuals with features of FA showed no increased sensitivity to DEB or MMC clastogenicity.

A comparison between classes using the U-test of Mann-Whitney demonstrated that DEB and MMC stressing are reliable techniques for the definitive cytogenetic diagnosis of FA homozygosity. However, the present study provides no evidence that individual FA heterozygotes may be differentiated from normal individuals on the basis of spontaneous, DEB-induced or MMC-induced breakage. While DEB
induced a significantly higher number of chromosome aberrations in FA heterozygotes as a group, compared to normal controls, a number of heterozygotes had breakage rates similar or even lower than control subjects, thus limiting the certainty with which data on a single putative carrier can be interpreted. The inclusion of 'gaps' in the calculation of aberration rates and the exclusion of fathers from the heterozygote class had no effect on the statistical conclusions.

The minimum birth incidence of FA in White, Afrikaans-speaking South Africans was estimated to be 1 in 22 000, with the heterozygote prevalence being approximately 1 in 77. This relatively high incidence may be attributed to a founder effect in this population group.

The significance of chromosomal instability in the pathogenesis of FA is evaluated specifically with respect to the broader etiological and pathogenetic aspects of teratogenesis and oncogenesis.
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1. INTRODUCTION

Fanconi's anaemia (FA) is not a 'new' syndrome, having been described by Fanconi in 1927. It was however, only in 1964 that Schroeder et al. first demonstrated that this disorder is associated with abnormal susceptibility of the sufferers' somatic chromosomes to spontaneous aberrations, thus initiating the intensive cytogenetic study of FA.

The era of modern human cytogenetics is said to have dawned with the conclusive discovery by Tjio and Levan (1956) that nuclei of human embryonic lung fibroblasts contained a diploid number of 46 chromosomes - 22 paired autosomes plus one pair of sex chromosomes. Shortly thereafter, Ford and Hamerton (1956) demonstrated that human spermatocytes contain 23 bivalents. This was the starting point which was to lead to a number of spectacular developments in the study of human chromosomes (see review Hsu, 1979b).

The first example of an autosomal trisomy in man was discovered by Lejeune et al. (1959) who established that Mongolism (Down syndrome) was caused by trisomy of one of the smallest human chromosomes. This finding not only illustrated the significance of the genome in the comprehension of human disorders, but also that deviations from the normal human chromosome complement do occur, and in so doing, opened the area of human cytogenetics to intensive investigation.

Lejeune's breakthrough was followed by the discovery of further constitutional stable, numerical chromosomal abnormalities associated with specific clinical syndromes viz. Turner's syndrome (Ford et al., 1959); Klinefelter's syndrome (Jacobs and Strong, 1959); XXX females (Jacobs et al., 1959); trisomy 13 ['D,'] (Patau et al., 1960) and trisomy 18 ['E'] (Hynders et al., 1960). The chromosome complement in Turner's syndrome and Klinefelter's syndrome demonstrated that unlike the situation in Drosophila, the male sex in man is determined by the presence of a Y chromosome.

The first constitutional structural chromosomal abnormality syndrome to be noted was the 5p-, or cri-du-chat syndrome (Lejeune et al., 1963).
In 1960, Nowell and Hungerford established that chromosome abnormalities could also be acquired. They noted the presence of the so-called Philadelphia chromosome (Ph1) in the great majority of patients suffering from chronic myeloid leukaemia (CML).

Most of the above-mentioned findings coincided with an important innovation in cell culture technique - Nowell (1960) and Moorhead et al. (1960) together perfected a reliable method for the short-term culture of peripheral blood lymphocytes. (See section 2.1) The effectiveness of this technique was based primarily on the mitogenic activity of phytohaemagglutinin (PHA).

In 1964, Schroeder et al. recognized that hereditary disorders, in this case FA, may be associated with normal karyotypes, but with abnormal somatic chromosome fragility. Subsequently, similar chromosome findings were reported in two other rare autosomal recessive disorders - Bloom's syndrome (German, 1964) and ataxia telangiectasia (Hecht et al., 1966). FA, Bloom's syndrome (BS) and ataxia telangiectasia (AT), although differing in their clinical presentation, are characterized by increased spontaneous (or endogenous) chromosomal instability, a probable basic defect in DNA repair pathways and a greatly increased predisposition to malignancy (Arlett and Lehmann, 1978). (See section 1.6.5)

These findings were followed by the recognition that certain environmental agents can also cause chromosome breaks. In 1968, Cleaver reported that cultured xeroderma pigmentosum (XP) cells, although showing no spontaneous chromosome instability, are ultraviolet (UV) light sensitive and specifically defective in their ability to repair UV-induced DNA damage. Similarly, Schuler et al. (1969) showed FA chromosomes to be highly sensitive to alkylating agents. (See section 1.6.2.1)

Cleaver (1980) coined the term 'hypersensitivity' diseases to include those single gene disorders which are thought to be defective in their DNA replication and/or repair mechanisms. FA, BS, AT and XP are the best known of the hypersensitivity syndromes, but others include Cockayne's syndrome, tuberous sclerosis, neurofibromatosis,
hereditary retinoblastoma and the Aniridia - Wilm's tumour association (AWTA) (Cleaver, 1980). All these single gene disorders are associated with an increased risk of malignancy.

Clearly then, individuals with defective DNA replication/repair are cancer-prone. This leads to the prediction that un repaired, damaged DNA has a high carcinogenic potential. Swift (1976b) has indicated that AT and FA heterozygotes may comprise as many as 1% of the general population. (See section 5.3.4) It therefore follows that the study of endogenous and/or environmental factors which cause chromosomal breakage will hopefully aid in the elucidation of (a) human DNA replication and repair and (b) the aetiology of malignancy. In turn, this may help in the establishment of the as yet unknown genetic defect in FA. Furthermore, the identification of heterozygotes would allow monitoring of subjects who may be at-risk for having affected offspring and who may themselves be at-risk for the development of cancer.

1.1 FANCONI'S ANAEMIA - AN HISTORICAL PERSPECTIVE

Aplastic anaemia was first described by Ehrlich in 1888. Benjamin (1911) (see Beard et al., 1973) reported on 3 unrelated children with refractory anaemia, mental retardation, skeletal underdevelopment and hypogenitalism. By 1919, Smith was able to review 64 published cases of aplastic anaemia - the majority were adults, but his review also included 10 children. Smith noted that congenital abnormalities were present in 3 of the latter, while 1 case was characterized by hyperpigmentation. It is possible that the unusual cases of aplastic anaemia plus congenital anomalies described by Benjamin and then by Smith were examples of the syndrome subsequently reported by Fanconi in 1927.

Fanconi (1927) described a syndrome of familial aplastic anaemia with congenital defects in 3 brothers, ranging in age from 5 to 7 years. These children were of short stature and had increased skin pigmentation, microcephaly, testicular hypoplasia and convergent strabismus. Bone marrow failure developed in all 3 siblings between
the ages of 4 and 5 years. It is of interest to note that the unexplained delay in onset of pancytopenia, the familial nature and the characteristic type of congenital defects, were all carefully described in this original report. In 1931, Naegeli proposed that this condition, accurately described by Fanconi, be known as Fanconi's anaemia (FA). Subsequently, Estren and Dameshek (1947) reported the presence of familial bone marrow failure in patients with no congenital defects, and suggested that such cases were a subclass of FA. The first case of FA in South Africa was described by Kessel and Cohen (1953).

In 1964, Schroeder et al. noted a high incidence of spontaneously occurring chromosome aberrations in peripheral blood lymphocyte cultures of FA patients, so discovering an important cytogenetic concept and diagnostic tool. Further landmark findings in the delineation of FA as a distinct and separate type of marrow aplasia, included the observations by Todaro et al. (1966) that cultured FA skin fibroblasts are more easily transformed by SV40 virus than fibroblasts from normal skin. Zaizov et al. (1969) confirmed Estren and Dameshek's findings (1947) that familial bone marrow failure can occur without congenital defects, and also showed that this subclass of FA is characterized by abnormal chromosomal breakage. Schuler et al. (1969) were the first to describe the influence of an environmental agent on a genetic disease. These workers noted that chromosomes of an FA patient had an increased sensitivity to an alkylating agent, in this case, tetramethanesulphonil-d-mannit. This observation encouraged many further investigations into the enhancement of chromosome breakage in chromosome instability syndromes such as FA. (See section 1.6.2)

1.2 PREVALENCE OF THE FANCONI'S ANAEMIA GENE

As far as is known, there are no precise estimates of the incidence of FA homozygotes or alternatively, of the frequency of the FA gene. Swift (1971) calculated a minimal frequency based on the number of reported FA cases born in New York State from 1956 to 1967. He concluded that the estimated frequency of FA homozygotes in North
America is 1 in 348 000. If the normal and FA alleles follow the Hardy-Weinberg law, then the expected heterozygote frequency is about 1 in 300. Arlett and Lehmann (1978) quote Wunder (1978) of the Federal Republic of Germany as having arrived at an estimated FA homozygote frequency of 1 in 70 000 in 'mid-Europe', giving a heterozygote frequency of 1 in 125. This discrepancy in gene frequencies may be due to either the use of different criteria in the ascertainment of FA cases, or conversely, simply the result of an increased incidence of the gene in the 'mid-European' countries.

A relatively high gene frequency is also suspected in the South African Afrikaner population (See section 4.1.3), the majority of whom trace their ancestry back to Holland, Germany and France. Although Meme et al. (1975) regarded FA as being rare in individuals of 'Bantu' extraction, the present study appears to indicate that this is probably untrue. In section 3.1.3, an attempt is made to estimate the prevalence of the FA gene in South Africa.

1.3 SEX RATIO

A number of reports have indicated that males are more frequently affected than females. Garriga and Crosby (1959) found a ratio of approximately 2 males : 1 female, while Fanconi (1967) reported a proportion of 3 males to 1 female. According to Evans (1979) affected boys predominate in a ratio of 3 : 2, while Gozdasoglu et al. (1980) noted a male to female ratio of 2 : 1 in their 18 cases. Schroeder et al. (1976b) also found a shift towards males, but the latter workers noticed that the proportion of males was also increased among the healthy siblings, and therefore concluded that there was no significant sex difference between afflicted and healthy siblings. However, since almost all the reports in the literature note an unequal sex-ratio, one is forced to conclude that either there is a sex-dependent effect on the expression of this disorder or alternatively, there is an additional sex-linked form of FA, implying that FA is heterogeneous.
1.4 CLINICAL FEATURES OF FANCONI'S ANAEMIA

1.4.1 Classification of Fanconi's anaemia

Since the original description of FA (Fanconi, 1927), it has become apparent that although many typical cases exist, this disorder may present with a wide spectrum of clinical and pathological malformations and with a variable natural history and age of onset. This has resulted in the disease being considered not as a single entity, but rather as belonging to a group of 'constitutional' (a term used by Fanconi in his original report) aplastic anaemias (Visfeldt and Mortensen, 1970). Broadly speaking, the 'constitutional' aplastic anaemias may be considered as a group of diseases characterized by chronic pancytopenia, either with or without various congenital malformations.

Bloom et al. (1966) recognized 2 forms of constitutional aplastic anaemia - type I being known as 'Fanconi's aplastic anaemia' and characterized by pancytopenia associated with multiple congenital abnormalities, especially of the upper limbs and kidneys. Also included in this group were persons with pancytopenia and no evident congenital anomalies, but who were sibs of patients with the classic manifestations of FA. Patients without congenital abnormalities in whom amegakaryocytic thrombocytopenia was present at birth or early infancy, followed later in childhood by pancytopenia, were considered as type II.

Zaizov et al. (1969) did however, regard those patients without congenital anomalies but showing chromosome breakage as belonging to a subgroup of FA, i.e. a subclass of the type I described by Bloom et al. (1966).

Miller and O'Reilly (1984) have defined the constitutional aplastic anaemias in more detail, describing the following major forms:

i. Constitutional aplastic anaemia (delayed onset) with congenital anomalies and/or chromosome abnormalities (FA) (= type I Bloom et al., 1966).
ii. Constitutional aplastic anaemia (delayed onset) without congenital anomalies or chromosome breakage (Katzen Hameshek type anaemia).

iii. Delayed onset aplasia with dyskeratosis congenita.

iv. Constitutional aplastic anaemia (= type II Bloom et al., 1966) with congenital thrombocytopenia and delayed onset pancytopenia, without congenital anomalies.

Notwithstanding the above criteria, reports in the literature appear to be confused by the inclusion of diverse clinical entities under the category of FA. In this respect, it must be emphasized that FA and Fanconi's syndrome are 2 separate disorders, with Fanconi's syndrome being primarily characterized by refractory rickets and multiple renal tubular defects (Lee et al., 1972). Because of the above-mentioned difficulties in the definition and diagnosis of FA, the presently held criteria for the clinical delineation of the disease warrants a more detailed description.

1.4.2 Clinical description of 'classic' Fanconi's anaemia

True FA is a multiple congenital anomaly syndrome, characterized by physical stigmata at birth, followed by the development of severe aplastic anaemia, usually between the ages of 5 and 9 years (Bloom et al., 1966).

A wide range of congenital anomalies may be present, either singly or in combination. The more common physical findings that particularly contribute to the diagnosis of FA homozygosity are hyperpigmentation, skeletal malformations and small stature. (See Table 1.1)

Garriga and Croany (1959) and Nilsson (1960) investigated the incidence of a wide range of congenital anomalies reported in FA. More recently, Evans (1979) and Alter et al. (1981) listed the more common congenital defects. (See Table 1.1)
TABLE 1.1 The incidence of various congenital defects in Fanconi's anaemia

<table>
<thead>
<tr>
<th>Defect</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of patients</td>
<td>129</td>
<td>100</td>
</tr>
<tr>
<td>Hyperpigmentation</td>
<td>99</td>
<td>77</td>
</tr>
<tr>
<td>Skeletal anomalies</td>
<td>85</td>
<td>66</td>
</tr>
<tr>
<td>Microsomoy</td>
<td>78</td>
<td>60</td>
</tr>
<tr>
<td>Low birth weight</td>
<td>20/36</td>
<td>56</td>
</tr>
<tr>
<td>Microcephaly</td>
<td>51</td>
<td>40</td>
</tr>
<tr>
<td>Renal anomalies</td>
<td>36</td>
<td>28</td>
</tr>
<tr>
<td>Hypogenitalism</td>
<td>26 (M), 5 (F)</td>
<td>24</td>
</tr>
<tr>
<td>Strabismus</td>
<td>28</td>
<td>22</td>
</tr>
<tr>
<td>Microphthalmia</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>Ptosis</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Nystagmus</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Epicanthal folds</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Hyperreflexia</td>
<td>24</td>
<td>19</td>
</tr>
<tr>
<td>Mental retardation</td>
<td>22</td>
<td>17</td>
</tr>
<tr>
<td>Ear anomalies and deafness</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Congenital heart disease</td>
<td>8</td>
<td>6</td>
</tr>
</tbody>
</table>

(After Alter et al., 1981)

More detailed descriptions of some of the above and other FA-associated anomalies follow:

a) Skin pigmentation

Pigmentation may be generalized, but more commonly manifests as cafe-au-lait spots or skin fold pigmentation. Pigmentation is usually central, involving the neck, axillae, areolae, abdomen, umbilicus, genitalia and groin (Miller and O'Reilly, 1984).
Histologically, increased melanin is seen in the basal layer (Nelson et al., 1964).

b) Skeletal anomalies

Skeletal anomalies of the hands and forearms, usually radial in distribution, are common. (See Fig. 1.1 and Fig. 1.2) These include absence of one or both thumbs, hypoplasia of the thumb nail, hypoplasia or absence of the first metacarpal, triphalangeal thumbs and a decreased number of ossification centres in the wrist (Pochedly, 1972). Other anomalies include atrophy of the thenar eminence, immobility of one or both thumb joints, attachment of the thumb to the hand by soft tissue and thinning of phalanges (Miller and O'Reilly, 1984). The radius may be unilaterally or bilaterally absent, and in these cases, the corresponding thumb/thumbs are absent (Minagi and Steinbach, 1966). Additional reported skeletal abnormalities include congenital hip dislocation, webbing of the 2nd and 3rd toes, Klippel-Feil deformity, and Sprenkel's deformity (Miller and O'Reilly, 1984).

c) Short stature

The growth retardation seen in FA is slightly disproportionate - the trunk is usually short and the legs are characteristically long (Gmyrek and Syllm-Rapoport, 1964). Intrauterine growth retardation may account for the low birth weight which sometimes accompanies FA. Although growth retardation has long been recognized as one of the most common features of FA, the first reported case of FA with isolated growth hormone deficiency was documented in 1971 (Pochedly et al., 1971). Interestingly, Nordan et al. (1979) investigated a single FA family where growth hormone deficiency was observed in only one of the two affected sibs. Therefore, heterogeneity appears to extend to growth hormone function in selected families.
Figure 1.1 Patient 48: A nine-year-old, Afrikaans-speaking PA homozygote; a hypoplastic right thumb is clearly evident.
Figure 1.2 Patient 49: A four-year-old, Afrikaans-speaking FA homozygote (sibling of patient 48); note should be taken of the patient's bilateral absent thumbs.
d) Renal anomalies

The most common anomaly is the absence of one kidney. Horseshoe kidneys and hydronephrosis have also been reported (Minaqi and Steinbach, 1966).

e) Hypogenitalism

Hypogenitalism has been reported in affected FA males, but is difficult to evaluate in younger patients (Miller and O'Reilly, 1984).

f) Central nervous system anomalies

Abnormalities in CNS development include hyperreflexia, microcephaly and mental retardation (possibly due to intra-uterine brain growth retardation), microphthalmia and ptosis of the eyelid, strabismus and nystagmus (Miller and O'Reilly, 1984).

g) Immune deficiency

FA may be associated with a defective immune system. In a study by Pedersen et al. (1977), repeated immunologic investigations on a 6 year-old patient showed increasingly deficient T cell function, whereas T cell number and T/B cell ratio, immunoglobulins, complement factor and neutrophil functions were normal. Although it was assumed that this was indicative of a primary immune deficiency, therapy-induced immune suppression in their patient cannot definitely be excluded. Furthermore, since the patient had contracted measles and chickenpox without complications at an earlier age, the authors concluded that this deficiency in cellular immunity probably develops gradually from a certain age. However, not all FA patients have an immune defect. Zaizov et al. (1978) reported that immunoglobulins were not markedly different in the FA patients they studied, when compared to the paediatric population as a whole. In 4 of their 5 patients investigated, cell mediated immunity was also found to be normal. Thus, some degree of immunological heterogeneity is apparent in FA, in contrast to AT where immunological disturbances are a prominent feature.
In 1982, Herse et al. described an FA patient with apparent defective natural killer cell (NK) activity. In vitro experiments revealed that the absence of NK activity in this patient was secondary to a defect in interferon release from lymphocytes on exposure to tumour antigens. Clearly, this finding may be indicative of a factor predisposing these patients to the development of malignancy.

h) Other anomalies

The following have also been reported to occur in FA - atresia of the external auditory canal, deafness, lacrimal duct atresia (Miller and O'Reilly, 1984), congenital heart defects, vitiligo, spina bifida, hypoplastic spleen and scoliosis (Gozuasoglu et al., 1980).

i) Predisposition to malignancy

It is well-known that FA homozygotes are at an increased risk of developing malignancies. This predisposition will be described in detail in section 5.3.

j) Haematologic abnormalities

Although all, some or none of the above-mentioned congenital defects may occur in FA, bone marrow aplasia and the consequent haematologic abnormalities are considered to form an integral part of the disorder.

In most patients with FA, congenital anomalies (if present) are usually recognized at birth or in early infancy. The haematologic abnormalities, the basis of a definitive diagnosis (if combined with increased chromosome breakage), are however, rarely evident before 17 months of age and may not develop until as late as 22 years of age (Gmyrek and Sylln-Rajoprot, 1964). The description of 'late-onset' marrow failure, again emphasizes the heterogeneous nature of this disorder. Schroeder et al. (1979) have in fact suggested that 2 forms of this disease are evident: (a) early onset pancytopenia,
possibly accompanied by more severe malformations, and (b) the adult form which is characterized by late onset marrow failure and usually less severe congenital abnormalities.

The average age of onset of marrow aplasia in male FA patients is 6.6 years, whereas 8.8 years is the average age in female FA patients (Gmyrek and Syllm-Kapoport, 1964). There appears to be an intrafamilial correlation in age of onset (Schroeder et al., 1976b). The temporal course of the full expression of pancytopenia may vary from a period of months to years. The genetic and environmental factors contributing to the onset of pancytopenia and its progression, are at this stage unknown.

Thrombocytopenia is usually the first haematologic abnormality to develop, with the subsequent onset of granulocytopenia and anaemia (Minagi and Steinbach, 1966). With progression of the disease, compensatory marrow haemopoiesis eventually fails, leading to progressive hypoplasia. When fully developed, the pancytopenia is identical to that seen in many acquired forms of aplastic anaemia, resulting in the same spectrum and severity of clinical expression (Ibid).

According to Evans (1979), the red cells are normochromic with poikilocytosis and often, macrocytosis. At the time of diagnosis, platelets are reduced in number, the haemoglobin concentration is usually 5-7g/dl and neutrophils are usually less than 1x10⁹/litre, and may show toxic granulation. There is however, usually no reduction in lymphocytes and monocytes. As the disease progresses, reticulocytopenia, indicating marrow failure, predominates.

As in the acquired form of aplastic anaemia, there may be an increase in foetal haemoglobin (HbF), due to overproduction of erythrocytes in response to increased destruction of abnormal red cell precursors (Nathan, 1981). Zaizov et al. (1978) found that only 2 of 9 FA patients had normal HbF levels. Raised levels of HbF were found prior to the development of typical haematologic abnormalities in 2 'at-risk' sibs who had been followed since infancy, emphasizing the value of HbF estimations in suspected FA cases older than one year.
Abnormal variations in glycolysis, hexokinase, ATP and ATPase have all been reported. (See section 1.8.2) There does not however, appear to be any consistent detectable defect in the red cells. It is therefore apparent that the aplastic anaemia characterizing FA, is not attributable to any single red cell metabolic abnormality.

To sum up what is known of the clinical picture in FA homozygotes, a defect in the bone marrow is clearly combined with a bizarre assortment of congenital malformations affecting a variety of tissues and systems. The basis of the variable manifestations of FA is still unknown. Althoff (1953) pointed out that the different organ systems frequently involved, all undergo embryonic differentiation at a similar time (24th to 34th day of foetal life). The author thus suggested that a single early embryonic defect may be responsible for inducing all the later manifestations of the disease.

1.4.3 Clinical anomalies in the Fanconi's anaemia heterozygote

Heterozygous carriers of the FA gene, although usually healthy, may also be predisposed to some of the congenital malformations or developmental disabilities that are common among homozygotes (Gmyrek and Syllm-Rapoport, 1964; Altay et al., 1975; Welshimer and Swift, 1982). In 25 families studied by Welshimer and Swift (1982), there was a significant excess of individuals unaffected by 'classic' FA (i.e. possible FA heterozygotes), with multiple malformations, especially genitourinary and distal limb anomalies. These features were not found in a comparative sample of AT and XP relatives, although the latter also showed minimal expression of the disease affecting some members of their families. There was however, no excess of haematological disorders in the FA families. Since heterozygosity is estimated at a minimal frequency of 1 in 300 in North America and of 1 in 125 in mid-Europe (see sections 1.2 and 4.1.3), FA heterozygotes may therefore constitute a significant proportion of individuals at-risk for specific malformations or developmental abnormalities.
Swift et al. (1972) noted an increased prevalence of diabetes mellitus in the relatives ($n = 102$, probability of heterozygosity $>0.125$) of 8 probands homozygous for FA. It was observed that female carriers of the FA gene were approximately 6 times more likely than normal controls to be affected by or die of diabetes, while male heterozygotes were not at a significantly greater risk of developing diabetes than the general population. (In the present study, only 1 of the 18 female obligatory heterozygotes was known to be affected by diabetes). The significance and biological meaning, if any, of this difference in risk estimates between the sexes are at this stage unclear.

1.4.4 Diagnostic criteria and differential diagnosis of Fanconi's anaemia

The diagnosis of FA may often be suspected at birth when suggestive congenital anomalies prompt chromosome studies, which reveal a high incidence of spontaneous chromosomal aberrations. However, not all cases of FA are characterized by high rates of spontaneous chromosome breakage. (See section 1.6.1). Furthermore, careful physical examination cannot indicate whether marrow failure will develop, and characteristic congenital anomalies may be found in FA family members who have never developed pancytopenia. (See section 1.4.3)

If typical congenital anomalies and chromosome breakage are present, the diagnosis may be positively established with the development of bone marrow failure. If no physical defects are apparent, pancytopenia in affected siblings may suggest the correct diagnosis which should then be confirmed by chromosome analysis. The first affected member of an FA family must be subjected to chromosome studies to aid in the diagnosis. An elevated level of fetal haemoglobin (HbF) may suggest a diagnosis of FA, but is non-specific. (See section 1.4.2)

Therefore, although absolute diagnostic criteria cannot be defined, the following guidelines should be employed in the establishment of a diagnosis (Beard, 1976):
1. characteristic congenital defects associated with delayed onset bone marrow failure; or
2. bone marrow failure either familial or in only one child, without congenital malformations, but with an elevated number of chromosomal aberrations.

In the establishment of the differential diagnosis of FA, a number of different conditions must be considered. These disorders may be divided into 2 groups viz. (1) those characterized by radial and/or haematologic abnormalities and (2) those exhibiting chromosome instability.

The main conditions which must be considered in the differential diagnosis of FA are:

1. CONDITIONS DISPLAYING RADIAL AND/OR HEMATOLOGIC ABNORMALITIES
   a) Triphalangeal thumb with hypoplastic anaemia (Autosomal recessive)
   b) Thrombocytopenia and absent radii (TAR) (Autosomal recessive)
   c) Acquired aplastic anaemia
   d) Dyskeratosis congenita (Usually X-linked recessive)
   e) Constitutional aplastic anaemia without congenital anomalies (Estren-Dameshek anaemia) (See section 1.4.1)
   f) Constitutional aplastic anaemia - type II (see section 1.4.1)
   g) Blackfan-Diamond syndrome (Autosomal recessive)

2. CHROMOSOME BREAKAGE SYNDROMES (Autosomal recessive)
   a) Ataxia telangiectasia
   b) Bloom's syndrome
   c) Xeroderma pigmentosum

1. Conditions displaying radial and/or haematologic abnormalities

Triphalangeal thumb with hypoplastic anaemia was first described in 2 male sibs by Aase and Smith (1969) and subsequently, some cases have been confused with FA (Jones and Thompson, 1973). However, essentially there should be no cause for confusion as, unlike FA, the haematologic problems in triphalangeal thumb with hypoplastic anaemia develop at birth.
Thrombocytopenia and absent radii (TAR) is characterized by clinical features similar to those found in FA, but it has been concluded that these 2 disorders are probably unrelated (Hall et al., 1969). Table 1.2 summarizes the points of difference.

**TABLE 1.2** Comparison between the most frequent clinical features of Fanconi's anaemia and thrombocytopenia with absent radii

<table>
<thead>
<tr>
<th>FEATURE</th>
<th>FA</th>
<th>TAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at onset of haematological symptoms</td>
<td>Usually 5-10 years</td>
<td>Infancy</td>
</tr>
<tr>
<td>Absent radii but thumbs present</td>
<td>Rare</td>
<td>In all patients</td>
</tr>
<tr>
<td>Cardiovascular defects</td>
<td>Uncommon</td>
<td>Common</td>
</tr>
<tr>
<td>Hyperpigmentation</td>
<td>Very common</td>
<td>Rare</td>
</tr>
<tr>
<td>Other congenital defects</td>
<td>Common</td>
<td>Rare</td>
</tr>
<tr>
<td>Bone marrow findings</td>
<td>Generalized aplasia</td>
<td>Reduced mega-karyocytes, but white and red blood cells normal</td>
</tr>
<tr>
<td>Fetal haemoglobin</td>
<td>Often increased</td>
<td>Normal</td>
</tr>
<tr>
<td>Lymphocyte chromosomes</td>
<td>Increased breaks</td>
<td>Normal</td>
</tr>
<tr>
<td>Prognosis</td>
<td>Poor</td>
<td>Good if survival exceeds 1 year</td>
</tr>
</tbody>
</table>

(after Beard, 1976)

It is of interest that skeletal defects involving the lateral radial aspect of the forearm and hand are either common or an integral part of FA, TAR and triphalangeal thumb with hypoplastic anaemia. There is at present no known explanation for this association of bone marrow dysfunction and congenital skeletal defects in these particular sites.
<table>
<thead>
<tr>
<th><strong>Mode of inheritance</strong></th>
<th><strong>Dyskeratosis congenita</strong></th>
<th><strong>Fanconi’s anaemia</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Usually X-linked recessive, can be autosomal recessive or dominant</td>
<td>Autosomal recessive</td>
</tr>
<tr>
<td><strong>M:F sex ratio</strong></td>
<td>10:1</td>
<td>2:1 (approximate)</td>
</tr>
<tr>
<td><strong>Birth weight</strong></td>
<td>Usually normal</td>
<td>Low</td>
</tr>
<tr>
<td><strong>Microcephaly</strong></td>
<td>Absent</td>
<td>Present (40%)</td>
</tr>
<tr>
<td><strong>Eye abnormalities</strong></td>
<td>Puncta or/and stricture strabismus (22%)</td>
<td>Microphthalmia (16%)</td>
</tr>
<tr>
<td></td>
<td>(78%)</td>
<td></td>
</tr>
<tr>
<td><strong>Leukoplakia</strong></td>
<td>Present (87%)</td>
<td>Absent</td>
</tr>
<tr>
<td><strong>Alopecia</strong></td>
<td>Present (51%)</td>
<td>Absent</td>
</tr>
<tr>
<td><strong>Hyperpigmentation</strong></td>
<td>Present (100%)</td>
<td>Present (77%)</td>
</tr>
<tr>
<td><strong>Nail dystrophy</strong></td>
<td>Present (98%)</td>
<td>Absent</td>
</tr>
<tr>
<td><strong>Skeletal malformations</strong></td>
<td>Rare</td>
<td>Present (66%)</td>
</tr>
<tr>
<td><strong>Internal abnormalities</strong></td>
<td>Oesophageal diverticulum or stricture; dysphagia (59%)</td>
<td>Renal anomalies (28%)</td>
</tr>
<tr>
<td><strong>Stature</strong></td>
<td>Hyposthenic build (54%)</td>
<td>Short (60%)</td>
</tr>
<tr>
<td><strong>Mental deficiency</strong></td>
<td>Present (42%)</td>
<td>Present (17%)</td>
</tr>
<tr>
<td><strong>Marrow aplasia</strong></td>
<td>Present (52%)</td>
<td>Present (100%)</td>
</tr>
<tr>
<td></td>
<td>(delayed onset)</td>
<td>(delayed onset)</td>
</tr>
<tr>
<td><strong>Malignancy</strong></td>
<td>No leukaemias reported</td>
<td>Leukaemias reported</td>
</tr>
<tr>
<td><strong>Chromosomal aberrations</strong></td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td><strong>Immunological defect</strong></td>
<td>Present</td>
<td>Variable</td>
</tr>
</tbody>
</table>

*(based on Sirinavin and Trumbidge 1975 and - FA incidences based on Alter et al., 1981)*
Dyskeratosis congenita is a rare genodermatosis characterized by skin hyperpigmentation, nail dystrophy and leukoplakia of the oral mucosa (Sirinavin and Trowbridge, 1975). This disorder shares many features with FA, but differences are also clearly evident (Steier et al., 1972), as summarized in Table 1.3.

Therefore, dyskeratosis congenita and FA have among other features, delayed bone marrow failure, abnormal skin pigmentation and poor growth in common. More important, skeletal and renal abnormalities are present in FA and absent in dyskeratosis congenita, and in addition, chromosomal findings in dyskeratosis congenita are normal, even following DEB-stressing (Auerbach et al., 1979b) (See section 1.6.2.1.2)

Constitutional aplastic anaemia (Type II) in contrast with FA, consists of a megakaryocytic thrombocytopenia that is present at birth, followed by the subsequent development of pancytopenia (Bloom et al., 1966).

Blackfan - Diamond syndrome is an autosomal recessive disorder which may be encountered in the newborn and is progressive and non-regenerative (McKusick, 1978). There is no erythroblastosis, haemolysis or hepatosplenomegaly and the leukocytes and platelets are usually normal (Ibid). This condition may be confused with cases of FA without associated congenital anomalies. (Hirschman et al., 1969; Alter et al., 1981).

2. Chromosome breakage syndromes

Fanconi's anaemia should not be regarded as being unique with respect to chromosome instability. It is therefore deemed necessary to include brief, and by no means comprehensive, outlines of the other classic chromosome breakage syndromes. It is hoped that these overviews will aid in enabling the reader to view FA in the context of being one of a number of disorders characterized by chromosomal instability.
The term 'hypersensitivity' diseases describes those disorders characterized by increased chromosomal instability, presumably due to defective DNA replication and/or repair (Cleaver, 1980). These include diseases such as Cockayne’s syndrome; progeria; Chediak-Higashi syndrome; tuberous sclerosis and neurofibromatosis; incontinentia pigmenti; scleroderma (progressive systemic sclerosis); porokeratosis of Mibelli; Kostmann’s agranulocytosis, and basal cell nevus syndrome (Arlett and Lehmann, 1978; Sandberg, 1980; Lehmann, 1981). Most of the latter diseases are the result of single gene mutations and many of them, but not all, have been found to be associated with an increased risk of malignancy (Sandberg, 1980).

Best characterized among the 'hypersensitivity diseases' are the well-known classic 'chromosome breakage syndromes' viz. Fanconi’s anaemia (FA), ataxia telangiectasia (AT), Bloom’s syndrome and xeroderma pigmentosum (XP). It is of interest to note that although pathologically and clinically distinct, FA, AT, BS and XP do have a number of features in common (Arlett and Lehmann, 1978; Sandberg, 1980; Bernstein, 1981), among which are:

i. autosomal recessive mode of inheritance
ii. rare incidence
iii. stunted growth
iv. pigmentary changes
v. altered immunological responses
vi. probable defects in DNA repair
vii. spontaneous chromosomal instability in FA, BS and AT; and only induced instability in XP
viii. increased predisposition to the development of malignancy

The major clinical features of AT, BS and XP will now be discussed. The cytogenetic abnormalities and postulated repair defects associated with these disorders are discussed in sections 1.6.5 and 1.8 respectively.

Ataxia telangiectasia (AT) (Louis-Bar syndrome) is a rare genetic
condition which is transmitted as an autosomal recessive trait (Tadjoein and Fraser, 1965). This syndrome is relatively common in Moroccan Jews (an incidence of approximately 1:8000), presumably due to a founder effect (Levin and Perlov, 1971). The frequency of the homozygous condition in the general population has been estimated to be 1 in 40,000 (Swift, 1976b).

Patients usually appear normal at birth, but begin to exhibit cerebellar ataxia after a few months of life. The ataxia is progressive and leads to degeneration of the central nervous system. Telangiectasias appear on the bulbar conjunctivae between the third and sixth years of life and then usually spread to the face and sometimes the body (Paterson and Smith, 1979; Sandberg, 1980). Often, but not always, immunological defects are present. These defects involve both cell-mediated immunity, resulting in poor graft rejection and humoral immunity, giving rise to low levels of IgA and occasionally IgE (Lehmann, 1981).

Death often results before the age of 20 either from sinopulmonary infections due to the immune deficiency or from malignancies in the third decade of life (Ibid). The incidence of cancer is increased approximately 1200-fold over age-matched controls (Lehmann, 1981). Cancer, which accounts for 10% of AT deaths (Paterson and Smith, 1979), primarily affects lymphoreticular tissues, but solid tumours of the gastrointestinal tracts, ovaries and central nervous system have also been described (Ray and German, 1981). Swift (1976b) reported that AT heterozygotes are also more prone to develop malignancies than the normal population. (See section 5.3.4)

Patients with AT show hypersensitivity to ionizing radiations at the clinical and cellular levels (Morgan et al., 1968; Higurashi and Conen, 1973; Taylor et al., 1975, 1976; Natarajan and Meyers, 1979). The clinical manifestation of this sensitivity is an often fatal reaction to X-ray therapy in the treatment of solid tumours (Morgan et al., 1968; Cunliffe et al., 1975).
Bloom's syndrome (BS) is inherited as an autosomal recessive disorder (German et al., 1965). This condition is the rarest of the chromosome breakage syndromes (Gianelli, 1980), but is found more frequently among Ashkenazi Jews living in the United States of America (German, 1979a). To date, no case of BS has been documented in Ashkenazi Jews of South Africa. The incidence of BS is higher in males than in females, with a ratio of 1.61 to 1 (German, 1974). There is no known reason for this altered sex ratio, but interestingly, a similar shift is seen in FA. (See section 1.3)

The major clinical features of BS are intrauterine growth retardation leading to stunted growth and proportional dwarfism. Facial and conjunctival telangiectases are present and these are usually exaggerated on exposure to sunlight (German, 1973a). The levels of one or more of the circulating immunoglobulins (usually IgA and IgM) are decreased (German, 1973b). The latter finding probably accounts for the predisposition to infection seen during childhood (Ibid). This susceptibility to infections subsequently subsides, but a severe disturbance in immunity remains demonstrable. The incidence of cancer is much higher among BS patients than in the general population (German, 1960, 1974; German et al., 1977b). The predominant cancer seen is leukemia, but lymphomas and tumours of the respiratory and alimentary tracts and of the kidney are also known to occur (Ray and German, 1981).

Xeroderma pigmentosum (XP), like the other classic chromosome breakage syndromes, is also transmitted as an autosomal recessive trait (Ariett and Lehmann, 1978; Sandberg, 1980). XP has been found in all racial groups and the incidence of homozygotes is estimated to be 1 in 250,000 (Robbins et al., 1974).

Clinically, XP is primarily characterized by acute skin sensitivity to sunlight. This manifests as sunburn, freckling, hypo- and hyperpigmentation, keratosis and finally multiple skin carcinomas and melanomas. These skin cancers are the usual cause of death, which mostly occurs before the age of 30 years (Robbins et al., 1974). The
skin lesions are seen predominantly on areas of the skin exposed to sunlight. Some patients also suffer from neurological abnormalities, mental retardation and growth-retardation (Ariett and Lehmann, 1978).

Genetic heterogeneity was first suspected on clinical grounds since the characteristic skin lesions are accompanied by neurological abnormalities in some patients and families, but not in others (Gianelli, 1980). This prediction was conclusively confirmed by the results of cell hybridization and complementation studies using fibroblasts from different XP patients. It is now well-known that there are at least 8 distinct XP complementation groups, namely types 'A' to 'G' and 'variant' XP (Cleaver and Bootsma, 1975; Cleaver et al., 1975; Kraemer et al., 1975; Lehmann et al., 1975; Arase et al., 1979; Keijzer et al., 1979; Robbins and Moskell, 1979;).

Complementation groups A to G comprise 80 to 90 percent of XP patients, while the remaining 10 to 20 percent of XP patients are termed 'variants' (Gianelli, 1980). The frequency and distribution of the different XP mutations vary according to ethnic groups (Takebe et al., 1977).

In conclusion, it is apparent that a number of widely differing conditions should be considered in the differential diagnosis of FA. However, in most cases, FA is clearly recognizable and may be regarded as a clinically distinct disorder.

1.4.5 Treatment of Fanconi's anaemia

Drug Therapy

The treatment regimen adopted for pancytopenic FA patients is similar to that given to patients with acquired aplastic anaemia viz. supportive care, androgen and steroids (Miller and O'Reilly, 1964). More specifically, the best treatment remains a combination of androgenic steroids and a low dose of prednisolone, as originally proposed by Diamond and Shahidi (1967). Although most FA patients generally show a good haematological response to this therapy, and fairly long-term remission may occur (McDonald and Mirshahi, 1968),
treatment must usually be given continuously, as relapses are in most instances the rule after discontinuance (Ibid).

The complications of androgen therapy may be most distressing for the patient, particularly virilization in females, and acne. Liver toxicity remains one of the more serious complications of the 17-alkylated androgenic steroids used in therapy (Beard, 1976). Obeid et al. (1980) reported that jaundice and hepatomegaly developed in a boy with FA, after he had undergone treatment with oxymetholone for 9 years. The discontinuance of this treatment resolved the jaundice and led to a decrease in liver size.

The risks of long-term androgen therapy are difficult to assess because of the relative paucity of FA cases and the small number of long-term survivors. The patient described by Obeid et al. (1980) developed an hepatic tumour and leukaemia following long-term oxymetholone therapy. This raises the possibility of oxymetholone exerting a teratogenic effect. Increased cell proliferation occurs when marrow hypoplasia is treated with androgens and moreover, this increased proliferative rate would in FA be accompanied by an increased mutation rate due to the inherent chromosome instability in FA (Meisner et al., 1978). Thus, androgen therapy may in fact be instrumental in creating conditions favourable for the development of malignant clones - even normal cells subjected to increased proliferation demands, have been shown to be at a high risk for the development of leukaemia (Dameshek, 1967). However, it must be emphasized that not all leukaemias occurring in FA patients are treatment-related: of 8 reported cases of leukaemia in FA homozygotes, only 4 patients had received oxymetholone therapy prior to the development of leukaemia (Obeid et al., 1980). (The possible oncogenic effects of androgens is further discussed in section 5.3.5.1.)

In growth hormone deficient cases, combined treatment with growth hormone plus androgens results in a greater growth acceleration than does the use of growth hormone alone (Nordan et al., 1979). Pancytopenia is not however influenced by growth hormone therapy.
Bone Marrow Transplantation

At the time of writing, bone marrow transplantation remains a difficult procedure with a high mortality rate and doubtful efficacy. In 1977, Barret et al. reported one patient who was ostensibly 'cured' following a successful bone marrow transplant. Gluckman et al. (1980) noted that of 5 FA patients treated by bone marrow transplantation from HLA identical donors, only 1 patient survived for more than 3 years. The other 4 patients died of acute severe Graft-versus Host disease (GVHD) soon after grafting. To the writer's knowledge, there are no reports of increased breakage in donor cells, indicating the probable absence of a 'breakage factor' in the serum of FA patients. (See section 1.7.3)

Severe cyclophosphamide toxicity was also encountered following bone marrow transplantation (Gluckman et al., 1980). The rate of chromosome aberrations in FA lymphocytes is significantly increased when the cells are incubated with the serum of a patient treated with high dose cyclophosphamide (Berger et al., 1980b). This finding probably indicates the need to modify the transplantation conditioning regimen in FA patients.

In an effort to devise a less toxic immunosuppressive regimen, Auerbach et al. (1983) compared the clastogenic effect of cyclophosphamide with that of procarbazine in cells from FA homozygotes and normal controls. It was noted that FA cells were not more sensitive than normal cells to procarbazine-induced chromosome breakage. Procarbazine may therefore be a safer drug than cyclophosphamide for immunosuppression of FA patients in preparation for bone marrow transplantation.

Generally, because of the relatively good short-term outlook for FA patients, bone marrow transplantation is not recommended until androgen and steroid therapy have failed. However, the results of a study by Deeg et al. (1983) are extremely promising and indicate that successful allogeneic bone marrow transplantation may in fact be feasible in FA homozygotes.
1.4.6 Prognosis

Prior to the use of steroid and androgen therapy, FA was usually fatal within 2 years of diagnosis (Miller and O'Reilly, 1984). At present, the overall median survival for FA patients is 5 years from the onset of aplastic anaemia (Alter and Potter, 1981). Death is usually due to haemorrhage or infection. The survivors of bone marrow failure do however, appear to be more susceptible to the development of malignancy than unaffected individuals, either as a result of treatment or more likely, a genetic predisposition to neoplasia. (See section 5.3.5)

1.5 FORMAL GENETICS OF FANCONI’S ANAEMIA

Fanconi (1927) described a syndrome of familial aplastic anaemia and discussed the possible presence of a 'constitutional factor'. The analysis of 14 FA families described in the literature, led Reinhold (1952) to the conclusion that FA is characterized by an autosomal recessive mode of inheritance. Most authors agreed with this conclusion, and McKusick (1966), in the first edition of 'Mendelian inheritance in man', regarded it as confirmed. Disagreements were based primarily on those heterozygotes who may show some congenital anomalies typical of the syndrome. (See section 1.4.3) These atypical cases suggested a dominant or intermediate mode of inheritance (Gmyrek and Sylis-Rapoport, 1966; Fanconi, 1967).

Swift and Hirschhorn (1966) pointed out that, based on the usual criteria for the determination of an autosomal recessive mode of inheritance, there is good evidence that FA is an autosomal recessive condition:

i. Many families with affected siblings have been reported, but none showed vertical transmission from parents to child;

ii. In rare autosomal recessive traits, an increased prevalence of parental consanguinity is expected - in a compiled series, 20% of the parents of FA patients were related, whereas the frequency of consanguinous marriages in the general population studied was 0.05% (Fanconi, 1964).
The results of an extensive analysis of 21 families plus 69 sibships were fully compatible with a simple autosomal recessive mode of inheritance (Schroeder et al., 1976b). Two of the latter affected homozygotes had offspring who were still unaffected at the time of publication - the one child, an 8 year-old boy had had diabetes mellitus since the age of 2 years (see section 1.4.3); the other child, a 7 year-old girl, was healthy. In the latter study, the number of sporadic cases was not greater than expected for an autosomal recessive disorder. Although affected males were somewhat more frequent than females, this sex difference was also found among the unaffected siblings, and is not statistically significant. (See section 1.3)

Weicker (1959) noted that affected siblings had an increased tendency to occur 'in runs', and Gmyrek and Syllim-Sapoport (1964) mentioned an advanced maternal age effect. However, Schroeder et al. (1976b) found no evidence for clustering of affected individuals in the sequence of siblings and no maternal age or birth order effect, but a completely random sequence of healthy and affected siblings within their sibships, as expected.

This clinically heterogeneous disorder (see section 1.4.2) is associated with a high intrafamilial correlation for age of onset of marrow aplasia, and probably the number and severity of malformations (Schroeder et al., 1976b). However, apart from the classic form of FA characterized by early onset, several malformations and a malignant course, an unusually mild, late onset type, with few malformations and a relatively benign course, also seems to exist (Ibid).

This 'clinical heterogeneity' seen in FA has been confirmed by somatic cell hybridization experiments (Krzewski and Sperling, 1980a). Tetraploid hybrid fibroblast strains were cytogenetically analyzed with respect to their Mitomycin C (MMC) - sensitivity. (See section 1.6.2.1.3) Complementation is considered to be an indication of genetic heterogeneity. Therefore, these hybrids, if complementary, should result in a normal frequency of MMC-induced chromosomal damage. Hybrid between cells of a classic FA patient
and one without skeletal malformations did not lead to MMC complementation. However, hybrids of FA fibroblasts between early and late onset FA types, had a substantially reduced aberration rate. The latter finding confirms the presence of at least 2 different FA complementation groups. These results have been confirmed by additional similar investigations which also led to the detection of only 2 complementation groups (Yoshida, 1982; Zakrzewski and Sperling, 1983; Zakrzewski et al., 1983). It was therefore concluded by these authors that intergenic heterogeneity plays a much smaller role in FA than in XP or AT. (See section 1.4.4)

Schroeder (1982) proposed that until the basic genetic defect is defined and can be assayed directly, the term 'FA variant' should be reserved for those disorders which are symptomatically and cytogenetically identical, but differ in complementation tests in cell fusion experiments.

To sum up, FA appears to be a clinically and cytogenetically heterogeneous autosomal recessive disorder characterized by at least 2 'variant types', the childhood and adult types. It should however, be stressed that although heterogeneous in expression, FA follows an autosomal recessive mode of inheritance, indicating the probable presence of different alleles at the same or different loci.

1.6 CYTOGENETIC FINDINGS IN FANCONI'S ANAEMIA

1.6.1 Spontaneous chromosomal aberrations in Fanconi's anaemia

1.6.1.1 Historical background

An inherent cytogenetic disturbance in FA was first reported 37 years after the clinical syndrome was originally described (Fanconi, 1927; Schroeder et al., 1964). Schroeder et al. (1964) reported that about 25% of the cultured lymphocytes from 2 affected brothers showed...
chromosome aberrations. These anomalies were not found in the parents nor the youngest brother of the patients. This was the first human case report in which abnormal susceptibility of somatic chromosomes to spontaneous breakage was observed in association with a hereditary trait. Subsequently, similar chromosomal findings were described in 2 other rare autosomal recessive disorders, viz. Bloom's syndrome (BS) (German, 1964) and ataxia telangiectasia (AT) (Hecht et al., 1966). A number of other 'chromosome instability syndromes' have since been described in which the cytogenetic changes (either spontaneous or induced) are similar to those in FA, BS and XP. (See section 1.6.5)

1.6.1.2 Spontaneous chromosomal aberrations in peripheral blood lymphocyte cultures from Fanconi's anaemia homozygotes

1.6.1.2.1 Quantitative assessment

Cytogenetic analyses of lymphocytes from more than 100 patients with FA have now been published. The number of chromosome breaks and resulting abnormal configurations, varies from case to case. Usually between 10 and 50% of peripheral blood lymphocytes contain one or more breaks after 72 hour culture (Rushkell et al., 1976). However, breakage rates as high as 74% have been documented (Swift and Hirschhorn, 1966). Sasaki (1978) discussed the possibility of 2 groups of FA patients, both of which are characterized by growth retardation, occasional mental deficiency and congenital malformations. His group 'A' consists of all patients with a mean spontaneous breakage rate of 55.7%, whereas the mean spontaneous breakage rate in group 'B' is only 5.9%.

Since FA presents with broad phenotypic diversity, often rendering clinical diagnosis difficult, spontaneous chromosome breakage has been suggested as a laboratory diagnostic tool to detect affected individuals (Bloom et al., 1966; Swift and Hirschhorn, 1966). Although typical chromosome changes are present in the overwhelming
majority of cases, a few individuals have been found who do not exhibit significantly increased aberration frequencies. At least 10 such cases have been reported in whom the haematologic and phenotypic constitutions suggested FA, but the incidence of spontaneous chromosome breakage was not remarkable enough to establish a definitive diagnosis in the absence of typical phenotypic anomalies (Bloom et al., 1966; Nathanson et al., 1968; Vowels et al., 1970; Bernstein et al., 1971; von Koskull and Aula, 1973; Bushkell et al., 1976; Dosik et al., 1979; Gozdasoglu et al., 1980; Cohen et al., 1982a).

Lubs and Samuelson (1967) concluded that variation in chromosomal aberrations seen in cultured normal lymphocytes exists both between and within individuals and between sexes and that seasonal variation is also possible.

Clearly, the frequency of aberrant cells detected, varies widely among FA individuals (Bloom et al., 1966; Perkins et al., 1969; Beard et al., 1973; Sasaki and Tonomura, 1973; Schmid and Fanconi, 1978; Zaizov et al., 1978). Cohen et al. (1982a) pointed out that differing breakage rates may represent actual individual differences, as indicated by the consistency of the breakage frequency obtained in a repeat study on each of three patients. This postulate does not always however, hold true, since in addition to interpatient variation, intrapatient variation was also evident in some studies when repeated cultures were performed on the same patient. Berger et al. (1977) subjected a single FA individual to 8 cytogenetic analyses on FIA-stimulated lymphocytes over a 3 year period; the aberration frequency at different times varied from as low as 5% to as high as 42%. Similarly, Schroeder et al. (1976a) reported a wide range in chromosome breakage frequencies (11% to 52%) in 19 blood cultures from a male FA patient who was studied over a 6 year period. Several authors have confirmed that intrapatient variation is evident in FA, and that this variation is probably random (Bloom et al., 1966; Crossen et al., 1972; Bourgeois and Hill 1977; Zaizov et al., 1978). However, several breakage studies on a male African FA patient over a period of 5 years, showed successively increasing frequencies of chromatid and chromosome breaks, exchange
configurations and endoreduplications, rising from 36% to 50% and later 60%, as the illness progressed (Meme et al., 1980). Contrary to this finding, Zaizov et al. (1978) followed up 9 children, ranging in age from 3 and a half years to 9 years, for up to 14 years, and concluded that the number of cultured lymphocytes with chromosome aberrations tend to decrease with age and/or remission.

In general, cytogenetic aberrations remain, even when the patient is in spontaneous or therapy-induced hematological remission (Beird, 1976; Meisner et al., 1978). There is however, some evidence that steroid therapy may in some cases tend to normalize the chromosomal findings in cultured lymphocytes (Crosen et al., 1972).

Bushkell et al. (1976) found no significant difference between the proportion of breakages in separated T and B FA lymphocyte subpopulations. An unexplained finding was that the separated T and B lymphocytes showed much higher breakage rates than did the unseparated lymphocytes from the same patients. This difference was not evident in the normal control cultures.

In summary, interpatient plus intrapatient variation characterizes the high incidence of endogenous chromosomal breakage noted in cultured lymphocytes from most FA homozygotes.

1.6.1.2.2 Qualitative assessment

The aberrations to be discussed in this section refer to differing anomalies of structure affecting a variety of chromosomes in isolated FA cells derived from in vitro cultures, and do not include any stable structural or numerical changes of the karyotype. As previously mentioned, these simple cells may represent more than 50% of the total mitoses screened (Schroeder and Kurth, 1971).

The aberrations found in cultured FA cells may be roughly classified under the following headings:

1. chromatid and isocho-
ii. chromatic and isochromatid breaks
iii. acentric fragments
iv. chromatic and isochromatid deletions
v. reunion figures which include rings, dicentrics, triradials, quadriradials and complex exchange configurations

(The reader is referred to section 2.6.2 for definitions of these aberration types.)

The most characteristic finding in FA consists of a large proportion of cells exhibiting chromatic-type gaps and breaks, with a smaller fraction of cells exhibiting chromosome-type gaps and breaks (Schmid, 1967; Polani, 1976). Schmid (1967) found that about 60% of the metaphases showed chromatic anomalies of which 38% had breaks and 12.5% of cells had more than one chromatic break per cell. Only 1 to 2% of cells contained chromosome-type exchanges confirming the observations of Schroeder et al. (1964). Bloom et al. (1966) also found no changes of the latter type in their cases.

Although chromosome exchange figures are rarely present, a significant number of chromatic exchange configurations may be observed in conjunction with breaks and gaps. The number of chromatic exchanges, which lead to the formation of triradial and quadriradial reunion figures, is not large, but is nevertheless in excess of that found in normal control cultures. The incidence of reported chromatic exchange figures ranges from a few percent to more than 25%, and appears to bear no correlation to the observed incidence of breaks (Schmid, 1967). For instance, von Koskula and Aula (1973) noted a range of 1.0% to 2.3% of rearrangement-bearing FA cells in 5 cases, compared to a complete absence of chromatic exchanges in their controls. Lupu and Samuelson (1967) reported an incidence of chromatic interchanges less than 0.002 per cell in normal controls, i.e., less than 1 per 1000 cells. The presence of exchange figures in FA strongly suggests that the observed breaks and probably gaps in FA are not merely postfixation artefacts.

In contrast to cultured Bloom's syndrome lymphocytes, the majority of
which show symmetrical quadriradial configurations between homologous chromosomes, 95% of the exchanges in FA are asymmetrical and occur between non-homologous chromosomes (German, 1972). Such exchanges are potentially more damaging than those that occur between homologues at homologous sites, since there is potential for the deletion or duplication of genetic material at mitosis if non-homologous exchange occurs.

In addition to the above-mentioned common chromatid and less frequent chromosome-type aberrations found in cultured lymphocyte chromosomes, a tendency to chromosome endoreduplications seems to exist in FA lymphocytes. Schroeder et al. (1964) and Bloom et al. (1966) noted an endoreduplication frequency of 10% and more, as compared with the normal incidence in blood cultures which is 1 endoreduplicated cell per 1000 mitoses, and rarely exceeds 5 per 1000 (Schmid, 1967). However, the presence of endoreduplications appears to be an inconsistent finding and even in cultures from the same patient, gross variation exists (case 1 of Bloom et al., 1966). Dosik et al. (1979) reported a form of inherited aplastic anaemia in 2 sisters with no typical congenital anomalies of FA and no unstable chromosome aberrations, but a significantly increased number of endoreduplications. They proposed that these patients may represent a variant form of FA.

1.6.1.2.3 Distribution of spontaneous chromosomal aberrations in Fanconi's anaemia lymphocytes

The breakpoints in FA chromosomes have been reported as being non-randomly distributed. Von Koskull and Aula (1973) noted a clear clustering of breaks at specific chromosomal loci, such as 3q27 and 13q32, and the distal regions of the A-group chromosomes showed an excess of breaks whereas the centromeric areas had far fewer breaks. The sex chromosomes showed a deficit of breaks. Dutrillaux et al. (1977) reported that 29% of breaks were localized to positions of sister chromatid exchanges (SCEs) in FA, thus indicating a possible but, as yet, unexplained correlation between chromosomal breakage and sister chromatid exchanges. However, the underlying causative mechanisms are thought to be different (see section 1.6.3).
The non-randomness of breaks in the control group of an experiment is very difficult to demonstrate because of the low number of breaks in normal control cultures. Cumulative studies on a large number of normal individuals and cells have shown that the breaks in normal human chromosomes are also non-randomly distributed, with distinct clustering at certain loci (Aula and von Koskull, 1976; Aymer et al., 1976; von Koskull and Aula, 1977; Mattei et al., 1979). Mattei et al. (1979) have for example, demonstrated that in normal lymphocytes, chromosomes 1, 2, 3 and 16 have 'hot spots' for chromatid breaks, while chromosomes 7 and 14 have 'hot spots' for breaks resulting in rearrangements. Similar non-random patterns were found in lymphocytes from normal individuals and from patients who had measles at the time of investigation (von Koskull and Aula, 1977). These patterns were however, different to those found in FA.

A number of studies have shown chromosomal breakpoints in both normal and FA patients to be localized almost exclusively in the darkly stained areas of G-banded chromosomes (von Koskull and Aula, 1973 and 1977; Aula and von Koskull, 1976). However, Dutrillaux et al. (1977), using three consecutive banding techniques, G-, Q- and R-banding, concluded that almost all the breaks seem to take place in the negative bands when R-banding is used, and in the negative bands when Q-banding is used. They therefore proposed that the breaks in FA occur in the interband regions between the R- and Q-bands. Mattei et al. (1979) noted that the breakpoints in their studies on normal human chromosomes were approximately equally distributed in both dark and light G-bands. They postulated that 'the transition zone from one type of band to another might be the weakest point in the chromatid, and that the observer subjectively interprets the localization on one or the other band types according to the technique used'. This hypothesis would be in agreement with the results of Dutrillaux et al. (1977).

Dutrillaux et al. 1977 have suggested that the distribution of breakpoints in cultured FA lymphocytes is probably:

i. non-random in relation to banding patterns, with a large excess of breaks being localized in the interbands;

ii. non-random in relation to the length of the chromosomes,
with an excess in the longer chromosomes; (as would be expected) and

iii. random in relation to centromeric and telomeric regions of the chromosomes.

1.6.1.3 Spontaneous chromosomal aberrations in fibroblast cultures from Fanconi's anaemia homozygotes

Cytogenetic investigations on FA homozygous fibroblasts have yielded varied results. Schroeder et al. (1964) repeatedly tried to culture fibroblasts from one of their patients, but were unable to obtain analyzable metaphases - the fragility of the chromosomes was tentatively blamed. Beard et al. (1973) claimed normal breakage rates in FA fibroblast cultures, while many other investigators have noted an increased number of chromosomal aberrations in dermal fibroblast cultures (Schmid et al., 1965; Swift and Hirschhorn, 1966; Varela and Sternberg, 1967; McDougall, 1971; Wolman and Swift, 1972; Auerbach and Wolman, 1976; Meisner et al., 1978 and Auerbach et al., 1980).

The general impression obtained from the numerous published studies is that although the aberration frequency in fibroblasts is usually lower than that in lymphocytes, there is a wide range of variation in the proportion of aberrant cells both between and within patients. (cf. similar findings in FA lymphocytes, see section 1.6.1.2) FA fibroblast chromosomal aberrations consist primarily of chromatid and isochromatid breaks and gaps, but exchange configurations may also be found.

1.6.1.4 Spontaneous chromosomal aberrations in bone marrow cells from Fanconi's anaemia homozygotes

An obvious drawback of in vitro studies is the difficulty of extrapolation to a meaningful in vivo comparison. Direct bone marrow preparations should therefore be examined to determine if chromosome breakage is also an in vivo characteristic of FA homozygotes.
In a review by Ray and German (1981), it was noted that cytogenetic results on only 28 bone marrow specimens have been published. Considering that more than 100 documented FA cases have been chromosomally analyzed, this figure is surprisingly low. This may however, be due to the very nature of the disease - in the presence of marrow hypoplasia, yields of analyzable metaphases from marrow aspirates may be very scanty.

Ray and German (1981) summarized the available data on direct preparations from FA marrows and noted that of the 28 marrows chromosomally investigated, 8 had no breakage, 13 had breakage in 1 to 10% of the cells, and 7 had breakage in more than 10% of the cells. Varela and Sternberg (1987) noted chromosomal aberrations simultaneously in blood, skin and direct bone marrow cultures from a pre-anaemic FA patient who was yet to undergo therapy.

Polani (1976) pooled the direct bone marrow chromosome results on 22 FA patients and found that approximately 2.5% of about 1000 metaphases displayed chromatid lesions (excluding gaps), and less than 3.0% showed chromosome-type aberrations. By comparison, O'Riordan et al. (1970) obtained comparative results on marrow aspirates from 32 'normal' males, none of whom had haematological disorders; they noted the presence of chromatid aberrations (including gaps) in 3.19% of metaphases - 2 of these 'normal' subjects had exceptionally high values of 10% and 24% respectively. Chromosome-type changes were observed in 0.55% of metaphases. The latter study is not however, a true comparison because there were no simultaneous FA, BM studies done by the same observers.

In contrast to direct marrow cultures, FA marrows cultured for 48 hours revealed a large proportion of chromatid aberrations, including exchanges and some chromosome-type anomalies (Varela and Sternberg, 1967; Germain et al., 1968). 'Long-term' bone marrow cultures thus appear to 'behave' similarly to peripheral blood lymphocyte and fibroblast cultures, in this respect.

Morphological studies of the various stages of dividing cells seen in bone marrow smears also represent a direct view of in vivo events.
Reports of anaphase bridges (Schroeder 1966a and 1966b; Shahid et al., 1972) and micronuclei (Schroeder 1966a and 1966b, Gmyrek et al., 1969) in marrow smears favour the existence of in vivo chromosomal instability in FA. The observations of clones of cells with aberrant chromosomal complements in freshly aspirated FA marrow, also support the other evidence of in vivo chromosomal fragility (Hirschman et al., 1969; Crossen et al., 1972; Berger et al., 1977; Bourgeois and Hill 1977; Meisner et al., 1978; Zaizov et al., 1978).

To sum up, from the available data, it is apparent that chromosome breakage in direct preparations from FA bone marrow occurs distinctly less frequently than in those cells subjected to in vitro culture conditions. In the majority of marrow studies, the chromosomal aberration rate in FA homozygotes does not appear to be higher than that found in normal subjects. However, definitive conclusions as to the possible in vivo existence of increased chromosomal instability in FA are as yet not possible, since so much variability in chromosome breakage exists between different FA marrows, and even in those cases where chromosomal instability is increased, the observed breakage rate may be low.

Swift and Hirschhorn (1966) pointed out that the particularly high aberration rates seen in peripheral blood lymphocytes are probably primarily the result of the mitogenic activity of phytohaemagglutinin (PHA). (See section 2.1.5.3) The mitogenic activity of PHA on slowly dividing lymphocytes artificially forces the simultaneous display of aberrations which have gradually accumulated, since those cells that were transformed by PHA may very well have never divided in vivo. Thus, the observed differences between aberration frequencies in FA bone marrow and lymphocyte preparations may reflect not only the difference in intermitotic intervals between these two tissues, but also the fact that the cultured lymphocytes are artificially forced into mitosis (Ibid).
1.6.1.5 **Spontaneous chromosomal aberrations in lymphoblastoid cell-lines from Fanconi's anaemia homozygotes**

Cohen et al. (1982b) investigated 3 different FA long-term lymphoid cell-lines (LCLs). All 3 FA LCLs had significantly increased spontaneous aberration rates compared to the aberration rates found in normal, XP and AT lines. Considerable variation was evident between the 3 FA LCLs viz. 0.30, 0.52, and 1.50 breaks/cell.

1.6.1.6 **Spontaneous chromosomal aberrations in amniotic fluid cells from Fanconi's anaemia homozygotes**

Amniotic fluid cultures from 3 'at-risk' foetuses exhibited increased spontaneous aberration rates compared to amniotic fluid cultures from normal control foetuses (Auerbach et al., 1981). The one pregnancy was carried to term and chromosome breakage characteristic of FA was found in peripheral blood cultures from the infant. The other 2 foetuses were aborted and FA homozygosity was confirmed on the basis of anatomical features plus increased peripheral blood lymphocyte or fibroblast chromosomal breakage rates. A high endogenous breakage rate was also reported by Voss et al. (1981) in a foetus later demonstrated to be homozygous for FA. Further studies on FA amniotic fluid cells have since been reported and their significance for the prenatal diagnosis of affected homozygotes is discussed in section 4.5.

1.6.1.7 **Spontaneous chromosomal aberrations in peripheral blood lymphocyte cultures from Fanconi's anaemia heterozygotes**

A number of cytogenetic investigations on healthy relatives of FA individuals have revealed essentially normal spontaneous chromosome breakage rates (Schroeder et al., 1964; Schmid et al., 1965; Konnagel et al., 1969; Dosik et al., 1970; Bernd et al., 1973; Cohen et al., 1982a). Both Guanti et al. (1971) and De Grouchy et
al. (1972) found that the breakage rates in FA heterozygotes were intermediate between those of affected children and normal controls. Only Mene et al. (1980), observed significantly increased endogenous chromosomal aberration rates, in a set of unrelated obligatory heterozygous parents. Bloom et al. (1966) found a normal chromosome breakage rate in parents of FA patients, but observed a numerical chromosomal anomaly in one of these mothers (viz. a 47,XXX cell-line) and Dosik et al. (1970) found a few trisomic cells in the cultured lymphocytes of both parents of a FA patient. Thus, the overall view is that heterozygotes cannot be reliably detected by spontaneous chromosome breakage rates.

1.6.1.8 Chromosomal aberrations and the stage and severity of the disease

Varela and Sternberg (1967) were probably the first to describe the presence of typical FA aberrations together with congenital malformations in a pre-anaemic infant. A similar case was described by Perkins et al. (1969). Schroeder and Kurth (1971) observed chromosome breakage in a pre-anaemic child both in vivo and in vitro. Schroeder et al. (1979) investigated the adult sister of a 'classic' FA patient. This sibling had several malformations characteristic of FA, but showed no haematological disorder or signs of bone marrow insufficiency, but typical FA chromosomal aberrations were present. The authors consequently suggested that, in addition to early and late onset forms of FA (see section 1.4.2), there may also be a 'forme fruste' with mild malformations and no bone marrow aplasia in both adults and children. These patients may conceivably escape clinical detection.

Although Mene et al. (1990) postulated that chromosome breakage in FA may be progressive, a continuous but variable presence of breakage with little or no increase with time, is usually the situation in FA (Bloom et al., 1966). It has therefore been concluded that no general correlation exists between clinical severity, radiation exposure, drug therapy and the frequency of aberrations in FA (Ibid). This contrasts with studies on one Bloom's syndrome patient which
indicated that there may be an in vitro increase in the frequency of breakage during the course of the disease (no abnormal cells were concurrently found in bone marrow) (Schoen and Shearn, 1967). Schroeder and Kurth (1971) have therefore speculated that chromosome breakage in BS is an accelerating process which may be initiated by external factors.

1.6.2 The enhancement of chromosomal aberrations in Fanconi's anaemia by clastogenic stress

Since FA presents with broad phenotypic heterogeneity, the clinical diagnosis is often difficult; therefore spontaneous chromosome breakage has been suggested as a diagnostic laboratory test (Schroeder et al., 1964; Swift and Hirshhorn, 1966). However, at least 16 patients have been reported in the literature in whom the haematologic and phenotypic constitution suggested FA, but the degree of chromosome breakage was unconvincing. (See section 1.6.1.2.1) Moreover, when chromosomal aberrations are present, intrapatient and interpatient variability is apparent. (See section 1.6.1.2.1) Consequently, the presence of endogenous or spontaneous chromosomal breakage cannot be used as an absolute diagnostic criterion in FA.

Some of the clastogenic agents employed to stress FA cells will now be discussed.

1.6.2.1 Alkylating agents

1.6.2.1.1 Tetramethanesulphonil-d-mannit

The first alkylating agent (i.e. a substance which binds to DNA by cross-linking DNA helices) shown to have an increased clastogenic effect in FA chromosomes compared to normal chromosomes, was tetramethanesulphonil-d-mannit (Schuler et al., 1969). (No further references to this clastogen with respect to FA, are made in the
Diepoxynutane (DEB) is a difunctional alkylating agent (i.e., both inter- and intrastrand DNA cross-linking can occur) and requires no metabolic activation in vitro (Singer and Kusmerek, 1982). The CA registry number of DEB is 1464535 and it has the following chemical structure: (Fishbein et al., 1970)

\[
\begin{align*}
\text{H}_2\text{C} & \quad \text{CH} \quad \text{CH} \quad \text{CH}_2
\end{align*}
\]

\[
\begin{align*}
\text{O} & \quad \text{O}
\end{align*}
\]

The cyclic alkylating agents (all have a similar structure to DEB) comprise a number of unrelated mutagens (e.g., epoxides, lactones, S-mustards, N-mustards, glyceraldehyde) and owe their alkylating activity to a reactive unstable ring structure (Singer and Kusmerek, 1982). The epoxides, including DEB, react with DNA and RNA at the N-7 position of guanosine and the N-1 and N-3 positions of adenosine, so forming hydroxyethyl or hydroxypropyl derivatives (Lawley and Jarman, 1972). Difunctional alkylating agents have 2 reactive sites which form 'bridges' i.e., cross-links between complementary DNA strands.

Experimentally, DEB is an active radiomimetic substance producing skin cancers, and sarcomas and depression of the haemopoietic system (see review in Fishbein et al., 1970). Furthermore, its mutagenicity has been proven in a number of organisms (Ibid). For example, the chemical was shown to be mutagenic in Neurospora and Drosophila (Kolmark and Westergaard, 1953; Bird and Fahmy, 1953) and carcinogenic in mice and rats (van Duuren, 1969). It is clastogenic in rat fibroblasts treated in vitro (Wolman and Sivak, 1975) and is a potent inducer of sister chromatid exchanges in Chinese hamster fibroblasts (Perry and Evans, 1975). Since DEB is not the only
alkylating agent reacting with DNA at the N-7 position of quanosine, one cannot specifically equate this N-7 reaction with carcinogenesis and/or mutagenesis.

### 1.6.2.1.2.1 Fanconi's anaemia fibroblasts plus DEB

Auerbach and Wolman (1976) were the first to report a statistically significant increase in sensitivity of FA fibroblasts to chromosome damage induced by DEB. DEB-induced [0.01 μg/ml] clastogenic stress increased the aberration rates of the treated FA cell strains 3- to 5-fold compared to the spontaneous breakage rates. Qualitatively, the breakage caused by DEB was similar to that occurring spontaneously in FA cells: both before and after DEB treatment, chromatid breaks were fairly homogeneously distributed, affecting the majority of cells. In sharp contrast to its effect on cultured FA fibroblasts, comparative fibroblast cultures treated with similar non-toxic concentrations of DEB, from xeroderma pigmentosum and trisomy 18 patients and normal individuals, did not show any increased breakage. Neither the growth nor viability of cells were reduced after the addition of non-toxic concentrations of the chemical to the latter cultures. Auerbach and Wolman (1976) therefore concluded that cell selection during chronic exposure to DEB (6 days) was probably minimal and furthermore, the clastogenic effects of the chemical can be separated from its toxic effects.

In 1978, Auerbach and Wolman again reported on the usefulness of DEB as a diagnostic tool, but this time DEB was applied to 3 FA heterozygous fibroblast cultures. DEB-exposure resulted in a highly significant, approximately 5-fold increase in breakage in the FA heterozygous cells, whereas no increase was noted in normal cells, even though the 1 normal cell-line had a higher intrinsic (spontaneous) breakage rate than the FA heterozygous cell-lines. As in the homozygous fibroblast cultures, drug treatment resulted in a poisson distribution of breaks, i.e. breakage was not concentrated in only a few cells. Qualitatively, the DEB-induced aberrations were similar to those found in FA homozygotes. FA heterozygous fibroblasts are not normally prone to endogenous chromosomal
breakage, but do show increased induced breakage proportional to that observed in FA homozygotes. It was therefore concluded that susceptibility to the enhancement of breakage was not related to the incidence of spontaneous breakage in the cell strains being tested (Ibid). Auerbach and Wolman (1978) hypothesized that FA heterozygotes may have a reduced capacity to repair DNA, but that this is not apparent except under the stress of increased damage caused by chronic exposure to a chemical such as an alkylating agent.

A comparative study of susceptibility to the clastogenic effect of DEB revealed that as in FA, chronic exposure of ataxia telangiectasia (AT) fibroblasts to a low concentration of DEB induced extensive damage (approximately a 2-fold increase) without reduction in cell viability (Auerbach and Wolman, 1979). FA chromosomes are however, more sensitive to DEB than AT chromosomes. Fibroblasts from normal subjects, Bloom's syndrome and xeroderma pigmentosum patients did not display susceptibility to the same concentration of DEB (Ibid). The exposure of normal fibroblasts to a higher dose of the same chemical resulted in significantly decreased viability with little increase in chromosomal aberrations. The different 'chromosome breakage syndromes' therefore appear to be characterized by genetic differences in susceptibility to carcinogen-induced chromosome damage.

1.6.2.1.2.2 Fanconi's anaemia lymphocytes plus DEB

Due to the significant enhancement of breakage observed in DEB-treated FA fibroblasts, the technique was subsequently applied to peripheral blood lymphocyte cultures.

1) FA homozygote detection

PHA-stimulated lymphocytes from each of the 5 FA patients investigated by Auerbach et al., (1991) displayed both elevated spontaneous and DEB-induced chromosome breakage. However, compared with their inherent aberration rates, FA lymphocytes showed an approximately 5-fold and 35-fold mean increase in breakage when
treated with DEB at concentrations of 0.01 µg/ml and 0.1 µg/ml respectively, but a wide range of induced breakage rates was found. Characteristic complex chromatin rearrangements were observed after exposure to 0.01 µg/ml DEB; such configurations were present in at least 75% of the metaphases from 4 of 5 patients, and almost all the cells had some type of chromosomal aberration. The fifth patient exhibited similar chromosomal abnormalities to those seen in the other patients, but only 20% of cells were affected, indicating that an intrinsic lymphocyte dimorphism (i.e., 2 cell populations, one of which is completely unresponsive to DEB) may have been present in this patient. Similarly different populations of unstressed Bloom's syndrome lymphocytes showing both normal and abnormally high levels of sister chromatid exchanges have been reported (German et al., 1977a).

Cohen et al. (1982a) reported a mean group aberration rate for lymphocyte cultures from 5 FA patients that differed significantly from both the control and heterozygous groups; concentrations of DEB ranging from 0.01 µg/ml to 1.00 µg/ml were used. Contrary to expectations, 2 of the 5 FA patients did not exhibit DEB sensitivity characteristic of FA homozygotes—they induced breakage values were within the ranges of the normal and FA heterozygous groups. Furthermore, 3 of the 4 'non-FA' individuals exhibited DEB-induced breakage rates equivalent to that expected for FA homozygotes. FA patients did however, show a tendency towards higher numbers of structural rearrangements, when compared with controls and heterozygotes, but a degree of overlap was present (Ibid).

ii) FA heterozygote detection

Although Auertbach et al. (1981) did not find an increase in spontaneous chromosome breakage in lymphocytes of FA family members compared with normal control subjects, a highly significant difference in the mean chromosome breakage rates of DEB-treated lymphocytes between these 2 groups was seen. Certain of the heterozygotes and normal individuals however, showed similar DEB-induced chromosome breakage, thus limiting the usefulness of this
technique in the cytogenetic detection of individual putative heterozygotes.

Cohen et al. (1982a) reported that no discrimination was possible between heterozygotes and normal controls subjected to DEB stress; the mean frequency of DEB-induced rearrangements was not significantly different between FA carriers and controls. Cervenka and Hirsch (1983) also concluded that heterozygote detection by DEB stress of cultured lymphocytes yielded equivocal results. These findings have been substantiated by Duckworth-Rysiecki et al. (1984) who also noted that it is not possible to demonstrate consistently raised levels of induced chromosome breakage in obligate FA carriers.

Contrary to all of the above-mentioned findings, Marx et al. (1983) recorded clear-cut differences between their FA heterozygote and normal control DEB-induced chromosome breakage ranges. The mean breakage rates were found to differ significantly.

DEB may however, be useful in distinguishing between dyskeratosis congenita and FA (see section 1.4.4). In contrast to FA lymphocytes, the DEB-induced breakage rate in dyskeratosis congenita lymphocytes is equivalent to that found in normal controls. (Auerbach et al., 1979b).

1.6.2.1.2.3 Fanconi's anaemia lymphoblastoid cell-lines plus DEB

At varying concentrations of DEB, FA long-term lymphoblastoid cell-lines (LCLs) exhibited greater chromosomal hypersensitivity than did normal, AT and XP LCLs (Cohen et al., 1982b; Cohen et al., 1983). For each individual FA line, a dose-response relationship was obvious: greater damage occurred with increasing doses. Again, FA heterozygotes could not be reliably detected with DEB (Cohen et al., 1982b).
1.6.2.1.2.4 Fanconi's anaemia amniotic fluid cells

plus DEB

FA homozygous and heterozygous amniotic fluid cells have also been shown to be sensitive to the clastogenic effect of DEB (Auerbach et al., 1979a; Auerbach et al., 1981; Voss et al., 1981; Marx et al., 1982). Since the difference between FA homozygote and FA heterozygote cells was clear-cut, this technique provides an important diagnostic tool for prenatal diagnosis. Cultured amniotic cells from normal foetuses did not exhibit increased chromosomal aberrations in response to DEB (Ibid).

In summary, the enhanced in vitro response of cells to the clastogenic effects of DEB provides a potentially useful diagnostic test for the prenatal and postnatal detection of the FA gene. Unfortunately, this test does not appear to be reliable for the identification of individual FA heterozygotes and some FA homozygotes.

1.6.2.1.3 Mitomycin C (MMC)

Mitomycin C (MMC) is an antibiotic which was first isolated in 1956 from the broth of Steptomyces caesiporis (see review in Fishbein et al., 1970). It is distinguished from other mitomycin fractions by its thermal stability, high melting point, ultraviolet absorption peak and solubility in organic solvents. MMC was used primarily as an antineoplastic agent in the treatment of Hodgkins disease (Ibid).

MMC is biologically inactive in its natural state, but upon chemical or enzymic reduction, it becomes a mono- and/or bifunctional alkylating agent (Ibid; Evans, 1977). Basically, MMC is metabolically reduced in the cell to hydroquinone derivatives by a quinone reductase (diaphorase) and these components are then able to alkylate and extensively cross-link DNA (Reich et al., 1961; Iyer and Szybalski, 1963). A report by Kodama (1967) suggests that MMC may in addition have intercalating capabilities.
The CA registry number of MMC is 50077 and it has the following chemical structure:

![Chemical Structure of MMC](image)

MMMC was first shown to be especially deleterious to FA lymphocytes by Sasaki and Tonomura in 1973 and to FA fibroblasts by Finkelberg et al. in 1974. Sasaki and Tonomura (1973) noted that MMC-sensitivity varied between different FA homozygote lymphocyte cultures: MMC at a concentration of 0.01 μg/ml resulted in 33- to 100-fold increases in chromosomal breakage compared to similarly stressed normal control cells. The findings of Finkelberg et al. (1974) in MMC stressed FA fibroblasts have been confirmed by Latt et al. (1975) who noted that normal fibroblasts from 2 different FA patients reacted to MMC with an increase in chromatid breaks.

A study by Cervenka et al. (1981) revealed a 50-fold increase in MMC (80 ng/ml)-induced multiradial figures in 92- to 96-hour FA homozygote lymphocyte cultures, when compared to the average MMC-induced breakage rates in cells from patients with aplastic anaemia or cells of healthy control subjects. The high incidence of asymmetrical exchange figures in the FA cultures probably accounted for the diminished mitotic yield obtained at all MMC concentrations studied. Of importance to the present study, the authors noted variability in individual sensitivity: FA patients with relatively low spontaneous breakage rates responded to MMC with lower sensitivity than did patients with high spontaneous aberration rates. This was not obvious in the frequency of radial exchange figures. Cohen et al. (1982a) have confirmed the above observations. Higher DSB responders were also found to be very sensitive to MMC and vice versa (ibid).
In a subsequent study by Cervenka and Hirsch (1983), the authors observed a mean 60-fold increase in chromosomal breakage in FA patients compared to patients with aplastic anaemia, regardless of clinical severity.

Schroeder and Stahl-Mauge (1979) pointed out that even low MMC concentrations (e.g. 0.16μg/ml) induce statistically significant increases in aberrations in normal control cells as well as in FA cells. Comparative diagrams of FA and normal cells showing dose-related increases in the percentage of aberrations, demonstrate that the slopes of the ascending lines run parallel to each other and that the angles are very similar. Thus, the conclusion of a generally higher susceptibility of FA cells to MMC is perhaps questionable.

In spite of the aforesaid reservations, the MMC-stress test is clearly useful in distinguishing most FA homozygotes from FA heterozygotes as well as normal individuals and other cases of aplastic anaemia, all of whom show comparatively low rates of MMC-induced aberrations.

1.6.2.1.4 Nitrogen mustard

Nitrogen mustard, a bifunctional alkylating agent like DEB and MMC, also selectively enhances chromosomal breakage in PHA-stimulated FA homozygous lymphocytes (Sasaki and Tonomura, 1973). At a concentration of 0.01μg nitrogen mustard/ml, about 0.2 chromatid aberrations per cell were produced in normal control cells, while an extensive number of aberrations, mostly of the chromatid type, was induced in the FA cells. These findings were confirmed by Berger et al. (1980a), who found that low doses of nitrogen mustard had a significant clastogenic effect on FA cells, whereas much higher doses were required to increase the aberration rate in normal control cultures. FA heterozygotes could not be distinguished from normals as both groups responded similarly to nitrogen mustard (Ibid).
1.6.2.1.5 Cyclophosphamide

Cyclophosphamide is also an alkylating agent which has cytotoxic and immunodepressive activity (Berger et al., 1980b). In the parent form, cyclophosphamide is inactive and requires metabolic transformation to become biologically active (Ibid).

Berger et al. (1980b) noted a high susceptibility of chromosomes from PHA-stimulated FA lymphocyte cultures to low concentrations of serum from a cyclophosphamide-treated patient. Chromosome breakage was not significantly increased in cultured lymphocytes from FA heterozygotes and normal controls subjected to comparable concentrations of cyclophosphamide-treated sera. The parent form of cyclophosphamide had no effect on the chromosomes of FA homozygotes, FA heterozygotes and normal controls (Ibid).

The high sensitivity of FA cells to cyclophosphamide metabolites, can be compared with the cyclophosphamide toxicity often seen in FA patients when cyclophosphamide is used as a conditioning treatment for none marrow transplantation (Gluckman et al., 1980). (See section 1.4.5)

1.6.2.1.6 Ethylmethanesulphonate (EMS)

Ethylmethanesulphonate (EMS) is an alkylating agent which requires no metabolic activation in vitro and has mutagenic and weak carcinogenic properties (Auerbach and Wolman, 1976).

Auerbach and Wolman (1976) observed a significant increase in chromosomal aberrations in EMS-stressed cultured fibroblasts, when compared to untreated cells. No breaks were seen in normal cells at the same non-toxic concentrations of EMS. At DEB and EMS concentrations equivalent in their cytotoxic effects, DEB was found to be a significantly more potent clastogen. In contrast to the above EMS findings, Latt et al. (1975) and Hedde et al. (1978) have reported that EMS is not capable of causing extensive DNA cross-linking and is not unduly toxic to FA cells. (See section 1.6.4)
1.6.2.2 Non-alkylating agents

1.6.2.2.1 Isonicotinic acid hydrazide (INH)

Isonicotinic acid hydrazide (INH) is an antifolate drug normally used in the treatment of tuberculosis. Unlike DEB, MMC, nitrogen mustard, cyclophosphamide metabolites and EMS, INH does not induce alkylation or cross-links in DNA (Schroeder and Stahl-Mauge, 1973). However, INH substantially increases chromosomal instability in FA homozygous lymphocytes but has no significant clastogenic effect on chromosomes in FA heterozygous and normal cells (ibid).

1.6.2.2.2 Bleomycin

Bleomycin possesses a number of interesting biochemical properties, including the ability to chelate heavy metals, and to cause in vitro scission and fragmentation of DNA and other polydeoxynucleotide strands (Haidle et al., 1972). This agent is known to also inhibit incorporation of thymidine into DNA (Cohen et al., 1972).

Cohen et al. (1982b) demonstrated that long-term lymphoid cell-lines derived from patients with FA and patients with AT are clastogenically sensitive to the non-alkylating, anti-tumour glycopeptide, bleomycin. AT cells however, appear to be specifically sensitive to bleomycin (Cohen et al., 1981), whereas FA homozygous cells are sensitive to a host of clastogenic agents.

1.6.2.3 Antimetabolites

Sasaki and Tonomura (1973) evaluated the effect of actinomycin D, caffeine and chloramphenicol on the production of chromosomal aberrations in cultured FA lymphocytes and normal controls.
1.6.2.3.1 Actinomycin D

Actinomycin D, although primarily an RNA inhibitor, in very high doses cross-links DNA and blocks DNA replication (Yarbro, 1970). This antimetabolite binds to DNA and also blocks the action of RNA polymerase, thus inhibiting DNA-directed RNA synthesis (Ibid).

Actinomycin D [0.02μg/ml] resulted in slightly elevated breakage levels in the control and FA cells. There was however, no significant difference in response to this antimetabolite.

1.6.2.3.2 Caffeine

Caffeine at concentrations of 50 and 500μg/ml enhanced the number of chromosomal aberrations in FA lymphocytes by approximately 5-fold, compared to the control cells. (Sasaki and Tonomura, 1973). Caffeine at a much lower final concentration of 1mM [1.9μg/ml] does not however, significantly alter chromosomal instability in FA lymphocytes (Schroeder and Stahl-Mauge, 1979). Interestingly, although INH enhances FA breakage rates, INH plus caffeine [1mM] fails to increase chromosomal breakage when compared with non-treated cells (Ibid).

1.6.2.3.3 Chloramphenicol

Chloramphenicol binds to the 50S ribosomal unit and blocks the transfer of the amino acid from the tRNA-aa complex to the peptide chain so that peptide synthesis ceases (Yarbro, 1970).

Sasaki and Tonomura (1973) noted that FA lymphocytes were 12 times more clastogenically sensitive to chloramphenicol than control cells [0.1μg/ml]. Chloramphenicol is an antibiotic in common usage, especially in Europe, and may occasionally cause an irreversible and often fatal marrow aplasia. Clearly then, this drug should never be prescribed for FA patients.
In contrast to the cytogenetic findings in DEX-treated cultures (see section 1.6.2.1.2), a conspicuous feature of the antimetabolite-treated FA cells was a significantly decreased number of exchange configurations - approximately 10% of the chromatid aberrations in the untreated FA cells were of the exchange type, whereas such exchange aberrations constituted only 1.3 to 4.2% of chromatid aberrations in the treated cells (Sasaki and Tonomura, 1973).

1.6.2.4 Other chemical agents

According to Sasaki and Tonomura (1973), PHA-stimulated FA lymphocytes are not abnormally sensitive to methylmethanesulphonate (MMS), N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and 4-nitroquinoline 1-oxide (4NQO). The susceptibility of FA cells to octamethylnitromycin C (DMMC - a monofunctional derivative of MMC), was approximately 6-fold higher than that of normal control cells following treatment with 2µg DMMC/ml. However, the cells were shown to be far more sensitive to MMC (see section 1.6.2.1.3).

Wunder and Fleischer-Reischmann (1983) performed FA lymphocyte cloning survival tests following treatment with several doses of 8-methoxypsoralen and exhaustive activation with UV-light. Sensitivity of the FA homozygous lymphocytes was found to be greatly increased. Rather surprisingly, the degree of the cellular defects corresponded to the clinical course. However, no such sensitivity was noted by Sasaki and Tonomura (1973).

1.6.2.5 DNA viruses

Adenovirus type 12 produces tumors when inoculated into newborn hamsters, rats and mice (McDougall, 1971). Interestingly, this DNA virus results in a smaller overall increase in chromosomal breakage in FA cells compared to normal cells infected with the virus (ibid). In contrast to FA cells, normal cells showed a significant increase in damage to chromosome 1, while chromosome 2 appeared to be specifically damaged in the FA cells. Although chromosome 17 was
highly sensitive to adenovirus type 12 damage in FA cells, normal cells appeared to be even more sensitive to chromosome 17 damage. McDougall (1971) has thus proposed that the cell–virus relationship is different in FA and normal cells and that FA cells are less permissive to adenovirus type 12 infection than are normal human cells. [It is interesting to note that chromosomes 1 and 17 appear to be the most highly sensitive chromosomes to adenovirus type 12 in normal cells and the integration sites for adenovirus type 12 have been mapped to chromosomes 1 and 17 (McDougall et al., 1975)].

1.6.2.6 Physical mutagens

1.6.2.6.1 Gamma radiation

Higurashi and Cohen (1971; 1973) demonstrated that cultured lymphocyte chromosomes from FA patients were at least 4 times more radiosensitive than those of controls, and cultured fibroblasts were about twice as radiosensitive as controls. This significantly high in vitro radiosensitivity exhibited by FA homozygous cells was evidenced by an increased frequency of dicentrics and rings per cell, especially following 100 rads, gamma irradiation. However, Sasaki and Tonomura (1973) found that FA lymphocytes responded normally to gamma-ray irradiation. In fact, following G2 and S phase irradiation, fewer chromosome aberrations were observed in the FA cells than in the irradiated normal cells. The conflicting findings of Higurashi and Cohen (1971; 1973) and Sasaki and Tonomura (1973) have not been substantiated, but interestingly, Ramon and Omutsi (1976) found 2 of 4 FA fibroblast strains be be deficient in the ability to excise gamma-ray products. The latter finding indicates a degree of heterogeneity with respect to gamma-ray sensitivity in different FA cell strains.

1.6.2.6.2 X-Irradiation

Beard et al., (1973) exposed peripheral blood lymphocytes from
patients with FA and matched controls to graded doses of X-irradiation. They concluded that there was no apparent difference between the 2 sets of lymphocytes in their ability to repair X-ray-induced damage in the dose range 50 to 300 rads.; nor was any difference detected in their susceptibility to chromosome damage induced by X-irradiation. These conclusions were confirmed by Poon et al. (1974) who demonstrated that FA cells were capable of rejoining X-ray-induced DNA breaks. Further confirmation of the lack of X-irradiation-induced clastogenicity was provided by clonagenic survival experiments which showed that FA fibroblasts were no more sensitive to X-irradiation than normal cells (Fornace et al., 1979). However, Heddle et al. (1978) found a slight increase in post-irradiation chromosomal breakage in PHA-stimulated FA lymphocytes, as compared with normals and FA heterozygotes. Ataxia telangiectasia cells on the other hand, are extremely sensitive to X-rays. (See section 1.6.5.1)

1.6.2.6.3 Ultraviolet irradiation (UV)

Sasaki and Tonomura (1973) found FA lymphocytes to be 'slightly' susceptible to short-wavelength UV irradiation. Following a dose of 75 ergs/mm² of 254nm UV, approximately 7 times more chromatid aberrations were produced in the FA cells than in irradiated control cells. Neither the treatment with 8-methoxypsoralen alone nor 355 nm UV (i.e. long-wavelength) irradiation alone was effective in producing chromosomal aberrations in the FA and control cells (ibid). However, 2x10⁵ ergs/mm² of 355 nm UV in the presence of 8-methoxypsoralen [0.1μg/ml] resulted in extensive chromatid breakage; the FA lymphocytes were about 44 times more susceptible to chromatid aberrations than the control cells; the latter breakage rate was comparable to that obtained by the same authors, for MMC and nitrogen mustard sensitivity. This finding is not unexpected since it is known that 8-methoxypsoralen induces interstrand cross-links in DNA when treatment is followed by long-wavelength UV irradiation (Cole, 1970). In a later study by Fornace et al. (1979), FA fibroblasts were no more sensitive to UV irradiation than normal fibroblasts.
Regan et al. (1973) have stated that FA cells can excise UV-induced pyrimidine dimers normally, whereas Poon et al. (1974) found these cells to be deficient in an exonuclease function. (See section 1.8)

1.6.2.7 Summary

Direct comparisons of the various studies to date, is difficult since vastly different protocols have been employed. However, FA cells do appear to be specifically susceptible to chromosomal breakage by difunctional (or polyfunctional) alkylating agents such as DEB, MMC, nitrogen mustard, cyclophosphamide metabolites and EMS. They are also unduly sensitive to long-wavelength UV light in the presence of 8-methoxypsoralen. Susceptibility to chromosomal damage by monofunctionally reacting agents such as MMS, MNNG, 4NQO and DCMMC is within normal limits. These findings have beeninterpreted as an indication that FA cells are defective in the repair mechanism that excises interstrand DNA cross-links (Sasaki and Tonomura 1973; Sasaki, 1975). (See section 1.8.1.3.4)

1.6.3 Sister chromatid exchanges in Fanconi's anaemia

Sister chromatid exchanges (SCEs) represent the interchange of chromatids at apparently homologous loci. These exchanges are thought to involve DNA breakage and reunion, but little is known about the molecular basis of SCE formation. Consequently, the biological significance of SCEs is obscure and all information in this respect is largely circumstantial. However, it appears that SCEs are in some way associated with DNA damage and the repair of DNA (Wolff, 1977; Arlett and Lehmann, 1978; Latt et al., 1980). A few SCEs are usually found in normal individuals (+3 - 11/cell), but increased numbers of SCEs are produced in normal cultured cells by irradiation and a wide range of chemical mutagens, often at very low non-toxic doses (Perry and Evans, 1975).
1.6.3.1 **Spontaneous sister chromatid exchanges**

Available evidence indicates that in FA homozygotes, the baseline (or spontaneous) SCE frequencies are equivalent to those found in normal control cells. (Hayashi and Schmid, 1975; Latt et al., 1975; Sperling et al., 1975; Novotna et al., 1979; Berger et al., 1980d; Cervenka et al., 1981; Kano and Fujiwara, 1981; 1982). Porfirio et al. (1983) have in fact noted slightly, but significantly, reduced spontaneous levels of SCEs in cultures from 3 FA patients and 5 FA heterozygotes as compared with normal controls. However, Miura et al. (1983) reported FA cells to have slightly higher spontaneous SCE levels than normal cells. This is in stark contrast to Bloom's syndrome which is cytogenetically characterized by a high frequency of spontaneous breaks plus a distinctly elevated frequency of SCEs (Chaganti et al., 1974).

The inter- and intrachromosomal distribution of SCEs in FA was shown not to be significantly different from normal controls (Sperling et al., 1975). Generally, the frequency of SCEs per chromosome increased with chromosome length, but the smaller chromosomes exhibited fewer SCEs than expected (Ibid; Latt, 1974).

1.6.3.2 **Induced sister chromatid exchanges**

Reduced SCE induction (i.e., less than in normal controls) has been noted in cultured peripheral blood lymphocytes exposed to MMC during the pre-harvest replication cycle (Latt et al., 1975; Cervenka et al., 1981). Thus, after in vitro stressing with MMC, FA lymphocytes exhibit increased sensitivity to the induction of chromosomal breakage (see section 1.6.2.1.3), but show less than the normal number of SCEs. However, Novotna et al. (1979) found the very opposite, namely that even after very low doses of MMC or TEPA (a polyfunctional alkylating agent), i.e., below the concentrations required to induce chromosome breakage, the number of SCEs in both normal and FA lymphocytes was significantly increased. In the latter breakage study, the FA and control cells did not differ significantly in their response to MMC or TEPA. These studies thus tend to
indicate that the mechanisms operating to produce SCEs are different to those producing chromosome breakage.

Kano and Fujiwara (1981) also found that exposure of FA fibroblasts to MMC for approximately 1 cycle resulted in the induction of SCEs equivalent to that found in normal cells, but if MMC exposure lasted for only 1 hour, a larger increase in SCEs was evident in FA fibroblasts compared to normal fibroblasts. In agreement with these findings, Miura et al. (1983) reported that MMC exposed FA cells exhibit about 1.4 times higher SCE frequencies than normal cells. SCE rates are not enhanced in FA homozygous or heterozygous lymphocytes grown in the presence of DEB, irrespective of the time at which it is added to the culture (Porfirio et al., 1983). This is in contrast with SCE rates in control cells, which show increases positively related to the length of DEB treatment.

Hyperinduction of SCEs by nitrogen mustard in FA was reported by Berger et al. (1980d). Based on nitrogen mustard-induced SCE frequencies, they found that FA heterozygotes could be distinguished from normal controls. FA homozygous cells were in turn more sensitive to nitrogen mustard than FA heterozygotes and controls (Ibid).

Clearly, the available data on SCE induction in FA are mixed and often contradictory. These findings are also difficult to interpret because of the present lack of knowledge on the basic mechanisms producing SCEs. The production of SCEs by MMC for example, has been reported as being suppressed, normal or elevated, depending on the experimental protocols used. A factor which may have contributed to these conflicting results is that an inverse relationship exists between the length of storage of MMC and the number of induced SCEs (Oervenka and Hirsch, 1983).

1.6.4 Micronucleus formation in Fanconi's anemia

Chromosomal fragments lacking centromeres are not incorporated into
daughter nuclei and consequently give rise to so-called 'micronuclei'. The production of micronuclei quantitatively reflects the extent of spontaneous chromosome breakage in human lymphocyte cultures. The shape of the clastogenic dose-response curve is however distorted, presumably because of cell death at higher mutagenic concentrations (Countryman and Heddle, 1976).

Heddle et al. (1978) demonstrated that FA patients may easily be identified by the micronucleus technique on the basis of (1) an elevated spontaneous frequency of micronuclei, corresponding to the elevated inherent aberration rate in these patients; and (2) an increased production of micronuclei following MMC treatment. At relatively high doses of MMC (e.g., 30ng/ml), fewer micronuclei were present than at lower doses (e.g., 10ng/ml), presumably due to extensive cell death - in the absence of cell division, the opportunity for chromosomal fragment loss will not arise and thus no micronuclei will be formed. The same effect was noted in the controls, but at higher MMC concentrations.

Using the micronucleus method of assaying chromosomal breakage in PHA-stimulated lymphocytes, Heddle et al. (1978) noted that susceptibility to EMS-induced chromosomal damage was within normal limits. These results are in contrast to the chromosome breakage results of Auerbach and Wolman (1976) who found FA cells to be abnormally sensitive to EMS. (See section 1.6.2.1.6) Furthermore, Heddle et al. (1978) found no increase in the number of micronuclei after the exposure of FA amoqynote lymphocytes to methylmethanesulphonate (MMS) or to 5-bromodeoxyuridine (BrdU).

1.6.5 Comparison of cytogenetic findings in Fanconi's anaemia with findings in ataxia telangiectasia, Bloom's syndrome and xeroderma pigmentosum

As pointed out in section 1.4.4, FA is not unique with respect to its associated chromosomal fragility, and therefore other disorders should also be considered in the differential cytogenetic diagnosis
of FA (Lopes, 1982). The cytogenetic abnormalities associated with 3
other well-known chromosome breakage disorders are therefore
discussed and compared with the findings in FA. (The clinical
features associated with these disorders are discussed in section
1.4.4)

1.6.5.1 Ataxia telangiectasia (AT)

Cells from the majority of AT patients examined, show abnormally high
frequencies of gaps and breaks, whereas the SCE rate is normal
(Hatcher et al., 1976; Arlett and Lehmann, 1978). Some AT
heterozygotes also have an increased aberration frequency in cultured
lymphocytes and fibroblasts (Cohen et al., 1975; Oxford et al.,
1975). In addition to chromosomal breakage, lymphocytes from many
patients show a stable chromosomal rearrangement involving the long
arm of chromosome 14 (Hecht et al., 1973; McCaw et al., 1975). This
finding is of interest as chromosome 14 is often a marker for
lymphoproliferative diseases of both B and T cell-type (Klein, 1979;

At the cellular level, an increased number of chromosomal aberrations
are seen in response to ionizing radiation (Taylor et al., 1976;
Taylor, 1978). However, based on the clastogenic effects of
X-irradiation, AT heterozygotes cannot be cytogenetically delineated
from normal controls (Natarajan et al., 1982). AT cells are also
very susceptible to the induction of chromosomal breakage by certain
chemicals such as actinomycin D and mitomycin C (MMC) (Kraemer, 1977;
Natarajan and Meyers, 1979). AT cells, (like FA cells) are however,
in contrast to XP cells, proficient in the repair of UV-induced
damage. (Paterson et al., 1977; Kraemer 1977). Some degree of
genetic heterogeneity has been reported to be associated with AT
(Jaspers and Bootsma, 1982, Murnane and Painter, 1982). (See
sections 1.4.2 and 1.6 for similar findings in FA)

1.6.5.2 Bloom’s syndrome (BS)

German (1964) was the first to describe the cytogenetic findings in
BS. In contrast to FA in which chromatid abnormalities are the most numerous, the majority of abnormalities in BS are of the chromosomal variety (Schroeder and German, 1974). A striking feature of the chromosomal aberrations seen in BS are quadriradials, found in both fibroblasts and lymphocytes. These quadriradials are the result of exchanges between homologous chromosomes, usually at the centromere (Comings, 1975; Schroeder and German, 1974). Such exchanges are asymmetrical in appearance and are nonrandomly distributed among the chromosomes (German, 1972; Shiraishi and Sandberg, 1977). Between 0.5% and 14% of all dividing PHA-stimulated lymphocytes display these quadriradial figures which often involve chromosome 1 and the chromosomes of the C and F groups (Sandberg, 1900). Quadriradials are rarely seen in the other chromosome breakage syndromes, and when present, are usually of the asymmetrical type involving non-homologous chromosomes. (Triradial figures are more common than quadriradials in FA).

Chaganti et al. (1974) were the first to observe a strikingly high incidence of spontaneously occurring SCEs in cultured BS lymphocytes. BS is the only inherited disorder in which a spontaneous increase in SCEs has been recorded (Shiraishi et al., 1976). In contrast to most of the reported findings on cells from FA patients (see section 6.3.2), MMC enhances the incidence of SCEs in BS cells (Shiraishi et al., 1976; Sandberg 1980). The frequency of SCEs in BS lymphocytes is approximately 80 per cell compared to a base value of approximately 6.4 SCEs/cell in normal cells (Sandberg, 1980). This dramatic increase in SCEs is now known to occur in all BS cell types (Bartram et al., 1976; Shiraishi et al., 1976; Henderson and German, 1978; Latt, 1979). Although a positive diagnosis of BS was previously usually only made after the in vitro detection of one or more quadriradial figures, a dramatically high frequency of SCEs is today considered pathognomonic in suspected BS cases.

BS cases have been reported to be moderately sensitive to UV light (Gianelli et al., 1977) and to X-rays (Higurashi and Conen, 1973). However, UV-induced pyrimidine dimer excision, daughter strand repair and the rejoining of single strand breaks following X-irradiation
appear to be normal (Gianelli et al., 1977; Ahmed and Setlow 1978; Vincent et al., 1978). Hypersensitivity to the alkylating agent ethylmethanesulphonate (EMS) was noted by Arlett and Lehmann (1978) and by Kreplinski et al. (1978).

1.6.5.3 Xeroderma pigmentosum (XP)

In contrast to the increased spontaneous chromosomal aberrations in FA, BS, and AT, a tendency to spontaneous chromosome breakage is not evident in XP cells (refer to review in Sandberg, 1980). However, there are isolated reports of clones of cells with pseudodiploid complements (German 1973b). The latter findings suggest that at some earlier stage i.e. prior to culturing, a tendency to chromosomal fragility and rearrangement may have existed in vivo.

The acute sun-sensitivity of XP patients is reflected at the cellular level: cultured XP cells (with the exception of the XP variants), are hypersensitive to UV light, which induces a high incidence of chromosomal aberrations and SCEs in fibroblasts and PHA-stimulated lymphocytes (Robbins et al., 1974; Cleaver and Bootsma, 1975; Bartram et al., 1976; Schonwald and Passarge, 1977; Pawsey et al., 1979). In addition, XP cells are extremely sensitive to the cell-killing and clastogenic actions of certain chemicals such as derivatives of acetylaminofluorene (AAF) and nitroquinoline oxide (Cleaver and Bootsma, 1975). XP cells however, respond normally to ionizing radiation and to alkylating agents such as MMC (San et al., 1977). As pointed out in section 1.6.2.1.3, FA cells are extremely vulnerable to the clastogenic effects of MMC. Therefore, although XP cells do not have an abnormally high incidence of spontaneous chromosomal aberrations, they are particularly hypersensitive to UV-induced damage.

1.7 CELLULAR ABNORMALITIES IN FANCONI'S ANAEMIA

In addition to inherent chromosomal instability, FA cells are characterized by a variety of cellular defects.
1.7.1 Cell cycle analysis

Cultured FA homozygous fibroblasts have a significantly longer mean population doubling time (30.3 hours) than normal control cultures (22.9 hours) grown and tested under the same standard conditions (Elmore and Swift, 1975; Swift, 1976a). Weksberg et al. (1979) also noted that FA fibroblasts have a decreased plating efficiency and a lower rate of accumulation of mitotic cells in culture. Auerbach et al. (1980) confirmed these observations.

The rate of progression through the cell cycle is reduced in FA lymphocytes, and this is further retarded by the addition of MMC to the cultures (Sasaki, 1975). Thus, exposure to DNA cross-linking agents accentuates the low mitotic index of FA cells (German et al., 1978; Weksberg et al., 1979). Flow cytometric studies of FA fibroblasts reflect this low mitotic index as a far greater accumulation of FA cells than normal cells in the G2 phase of the cell cycle, following exposure to MMC (Latt et al., 1982). It should however, be noted that following treatment with MMC the percentage of metaphase cells in the G2 + M peak decreased in all lines, but this effect was most marked in cells from patients with FA, where virtually no metaphases were found. These findings have been substantiated by Miura et al. (1983). The latter workers also noted that FA cells proliferate much more slowly than normal cells. MMC-stressed FA and normal cells were characterized by dose-related delays in cell turnover times, with the duration of delay being much longer in FA than in normal cells. In contrast to the effect of MMC, EMS (see section 1.6.2.1.6) did not effect the normal differential accumulation of FA fibroblasts in late S, G2 or M (Heddle et al., 1978).

Dutrillaux et al. (1982) observed a significant slowing of the cell cycle in vitro in all FA patients and possibly in FA carriers, although to a lesser degree. This slow cell cycle appears to be due mostly to a very long G2 phase, which would result in an apparent accumulation of cells in G2. The reason for this accumulation of FA cells in the G2 phase, remains to be determined.
In accordance with previous studies, Kubbois et al. (1985) also reported that the G2 phase of the FA cell cycle is severely prolonged, but this delay appears to be compensated in part by a subsequent GI phase duration that is unusually short. The latter authors have proposed that some FA cells may, as a consequence of this aberrant cell cycle, enter that second growth phase without prior completion of the delayed cell cycle. It is postulated that renewed replication would ensue in such cells without then passing through mitosis and cytokinesis, thus leading to endoreplication. (See section 1.6.1.2.2)

The rate of DNA chain elongation in FA cells corresponds to that of control cultures, whereas there is a significant difference between controls and cultured Bloom's syndrome cells. (Hand and German, 1975; Hand, 1977). Furthermore, the size of FA and AT replicon units was found to be the same as in normal cells (Hand, 1977).

DNA damage may alter normal DNA replication in different ways. For example, non-repaired aberrations inhibit the initiation or progression of DNA synthesis and promote exchanges between homologous DNA strands (Gianelli, 1980). Cleaver (1980) proposed that there may in fact be an active process whereby replication is 'switched-off' whilst repair is occurring. In the absence of such a mechanism, one would expect the chromosome breakage syndromes to have altered replication patterns. However, as discussed above, skin fibroblasts from FA and AT individuals were found to have autoradiographic patterns of DNA replication similar to those of normal controls (Hand, 1977). This finding suggests that despite abnormal susceptibility to DNA damage, S-phase DNA synthesis is normal in FA and AT. In fact, a normal S-phase duration has been reported by Kubbois et al. (1985). However, fibroblasts and lymphocytes from BS patients have a retarded rate of replication fork movement compared to normal adult controls (Hand and German, 1975; 1977). It is thought that the latter finding may be a specific manifestation of defective DNA synthesis in BS cells. It follows that the defect in BS may not necessarily involve one of the DNA repair mechanisms (see section 1.8.1), but rather affect, either directly or indirectly, DNA replication.
In vitro FA bone marrow cultures also show a marked decrease in colony growth in the pre-anaemic (Daneshbod-Skibba et al., 1980), anaemic (Ibid, Lui et al., 1977; Saunders and Freedman, 1978; Chu, 1979) and leukaemic (Prindull et al., 1979) phases of the syndrome. These results suggest an intrinsic stem cell defect in FA patients (Auerbach et al., 1982).

No specific nutritional supplement has yet been shown to restore a normal growth rate in FA cells. If a compound or class of compounds was found to specifically enhance the growth of FA cells, this would provide much-needed insight into the metabolic abnormality producing this in vitro growth retardation and resulting in the diverse clinical and laboratory expression of this genetic disorder (Elmore and Swift, 1975). Furthermore, and most importantly, such knowledge could be accompanied by much-needed therapeutic benefits.

1.7.2 Viral transformation

Numerous viral transformation studies have been performed in an attempt to elucidate the association between FA and leukaemia. Todaro et al. (1966) developed a quantitative system for the study of transformed cultured human diploid fibroblasts. Simian virus, SV40, a papovavirus related to the human wart virus, was used to infect FA homozygous and FA heterozygous fibroblasts cell-lines. Monolayers of FA homozygous and heterozygous cells showed a greater number of localized areas undergoing rapid and disarrayed proliferation compared with normal control cultures; while the control cultures showed 1.6 to 5.1 'transformed' areas for every 10,000 cells, the range increased to 20.1 to 28.2 per 10,000 heterozygous cells and 41.4 to 79.7 per 10,000 homozygous cells. These proliferative responses, termed 'transformation', are therefore also seen in normal individuals, but occur more readily in cells of affected homozygotes and carriers of the FA gene. The heterozygote response is intermediate between that of the homozygotes and control. German (1973b) pointed out that the difference in transformation susceptibility between the three cell-lines infected with SV40 is not
in the type of interaction between the virus and the cell, but rather
in the frequency with which it occurs.

Dosik et al. (1970) also found an increased number of SV40
transformed areas in cell cultures from a sibling and the obligate
carrier parents of 2 affected children, but both Young (1971) and
Beard et al. (1973) were unable to detect a difference between
heterozygotes and normal individuals. Fibroblasts derived from FA
homozygotes did however, show a 10-fold increase in susceptibility to
SV40 transformation (Beard et al., 1973).

The expression of SV40-T-antigen was significantly elevated over
normal controls in 10 patients, including 2 young pre-anaemic
patients (Lubiniecki et al., 1977). In the latter study, expression
of T-antigen did not appear to correlate with the incidence or
severity of clinical symptoms characteristic of FA, but did correlate
with the incidence of in vitro colony transformation. It is not
known whether there was any correlation with subsequent possible
leukaemia development in these patients.

Interestingly, 'naked' SV40 DNA (i.e. purified viral DNA) does not
transform FA cells more readily than controls (Aaronson, 1970). It
is possible that the latter observation may reflect an FA cell
surface abnormality. Other indications that the membranes of FA
cells may be altered, come from the findings by Pochedly et al.
(1972) that FA red blood cells have reduced osmotic fragility and
acrylamide gel electrophoresis of FA membrane proteins yields an
abnormal band. FA fibroblasts are also insensitive to osmotic lysis
(Swift, 1976a).

The SV40 integration sites in the human genome have been mapped to
chromosomes 7 and 17 (Khoury and Croce, 1976; Croce, 1977). It is
not known whether these chromosomes are unduly fragile in
SV40-transformed FA cells compared to transformed normal control
cells.
Clearly then, there may well be a close relationship in FA between inherent chromosomal instability, cell membrane abnormalities, increased susceptibility to transformation by oncogenic viruses (e.g. SV40) and an increased predisposition to malignancy. (See section 5.3.5.2).

1.7.3 Endogenous breakage factors

Fibroblasts from BS patients release a low molecular weight component into the culture medium which breaks chromosomes and induces SCEs in FAMA-stimulated lymphocytes from normal donors (Emerit and Cerutti, 1981). By contrast, it has been demonstrated that SCE frequencies in BS fibroblasts are reduced following cocultivation with normal cells (Bartram et al., 1979). Plasma of BS patients also contains a clastogenic factor, thus indicating that this factor is a bona fide characteristic of BS rather than merely a property of BS fibroblasts in culture (Emerit et al., 1982).

A clastogenic factor has also been detected in the culture media of fibroblasts, in the plasma and in the supernatant of amniotic cultures of ataxia telangiectasia patients (Shaham et al., 1980).

However, evidence for the presence of an endogenous clastogenic factor in FA is less conclusive. Bloom et al. (1966) attempted to demonstrate a humoral breakage substance in FA by culturing a patient's plasma with blood from a normal donor for 72 hours; no increase in the number of aberrations was found in the normal cells, nor did normal plasma lessen the chromosomal defect in FA lymphocytes. Perkins et al. (1969) substantiated the latter results and concluded that the spontaneous chromosomal aberrations in FA are not likely to be caused by a factor circulating in the plasma of FA patients. Germain and Requin (1970) also cocultured FA lymphocytes with normal cells and did not find a decrease in the frequency of breaks in the FA cells.
Yoshida (1980) noted however, that the high rate of spontaneous and MMC-induced chromosomal aberrations in FA fibroblasts from a female patient was fully corrected after euploid somatic cell hybridization with normal male embryonic fibroblasts. Unfused diploid FA cells in the mixed culture were not corrected. There was no detectable effect on the aberration frequency in unfused normal fibroblasts in the mixed culture. The author concluded that spontaneous chromosomal fragility probably reflects an intrinsic genetic defect in the FA cells themselves, rather than the effect of a circulating clastogenic factor.

Results of experiments by Nordenson et al. (1980) conflict with those of Bloom et al. (1966) and Perkins et al. (1969). The former researchers showed cocultivation with normal lymphocytes significantly reduces the aberration frequency of FA lymphocyte chromosomes - the frequency of FA chromosomal aberrations was lowered by about 80%. However, the residual aberration rate in the cocultivated FA cells was significantly higher than the rate in normal cells and in normal cocultured cells. There was no difference in the frequency of chromosomal breakage between normal cells and cocultivated normal cells.

In support of the latter results, Zakrzewski and Sperling (1980b) also noted a partial reduction in the spontaneous chromosomal aberration rate in FA fibroblasts following cocultivation with Chinese hamster ovary (CHO) cells. After MMC stressing, a significant reduction in induced chromosomal damage was observed in the cocultivated FA cells, but a significant increase in chromosomal aberrations was found in the CHO cells. This antagonistic effect is possibly attributable to some diffusible clastogenic factor(s). The authors proposed that such a factor could decrease the aberration rate in FA cells either directly, by protecting them from primary damage, or indirectly, by stimulating their supposedly defective DNA repair system.

Since the results are conflicting, the effect of cocultivated normal cells on FA cells should be considered an open question.
1.8 DNA REPAIR PATHWAYS AND THE POSSIBLE DEFECT IN FANONI'S ANAEMIA

In normal cells most chromosome breakage is the result of post-replicative events and is repaired by various DNA repair mechanisms. It should however be noted that chromosome breakage may also be due to defective de novo DNA synthesis, as in pernicious anaemia and vitamin B12 and folate deficiency. (Kiossoglou et al., 1965; Heath, 1966). Since there is no evidence that the defect in FA is one of DNA synthesis (see section 1.7), the discussion that follows will centre on DNA repair mechanisms.

Prokaryotic and eukaryotic cells are endowed with many different pathways for the repair of damaged DNA. Corrective processes are also an integral part of normal DNA replication. Chromosome and/or chromatid breaks and/or gaps seen at metaphase may be regarded as the end result of an imbalance between chromosomal breakage events and repair processes. It should be emphasized that breaks in the continuity of one DNA strand occur not only when DNA is subjected to abnormal clastogenic stress. On the contrary, breaks are thought to occur fairly often in the course of normal DNA replication, but cells have evolved very effective 'sealing' mechanisms which ensure the integrity of the DNA.

Xeroderma pigmentosum (XP) is a rare genodermatosis with hypersensitivity to UV irradiation and a propensity to skin cancer. (See section 1.4.4) In 1968, XP was found to be associated with a defect in the repair of UV-induced damage (Cleaver, 1968). This, the first DNA repair defect discovered in man, has led not only to considerable activity in the investigation of this condition, but has also stimulated interest in the aetiology of all the 'chromosome breakage disorders'.

1.8.1 DNA repair pathways

Three major systems of DNA repair characterize eukaryotic cells.
These systems (originally discovered and defined in bacteria) differ in their action and importance. Short discussions of these mechanisms follow since, by implication, defective DNA repair is probably associated with the pathogenesis of all or some of the disorders showing undue chromosomal fragility.

1.8.1.1 Photoreactivation

![PHOTOREACTIVATION](image)

**Figure 1.3** Photoreactivation (after Bertram, 1980)

Enzymatic photoreactivation (Fig. 1.3) is specific for the repair of UV-induced aberrations (Bertram, 1980). This system is however, thought to play at the most, a very minor role in man (Cleaver, 1978). Photoreactivation is man's simplest repair pathway since it relies on only one enzyme and it does not require the presence of a complementary, undamaged DNA strand. DNA photolyase binds to cyclobutane pyrimidine dimers (Fig. 1.3), the principal lesions caused by UV radiation, and monomerizes them in the presence of visible light, without removing any DNA bases (Cook and McGrath, 1967; Cleaver, 1977; 1978).
Sutherland et al. (1975) noted that XP cells have significantly less than normal photolyase activity. Since photoreactivation is thought to play only a minor role in the repair of UV-induced damage, compared to the role of excision repair, the above-mentioned photolyase deficiency is probably not the primary defect in XP cells. There appear to be no comparable reports of a photoreactivation defect in FA cells.

1.8.1.2 Postreplication repair

![Postreplication Repair Diagram](image)

**Figure 1.4** Postreplication repair (after Bartram, 1980)

If damaged DNA replicates before the activation of DNA repair mechanisms, the newly synthesized strand opposite the damaged DNA molecule will contain gaps. Following replication, these gaps are filled by *de novo* synthesis (Lehmann, 1972). Hence postreplication
repair (Fig. 1.4) may be defined as the ability of, for example, UV-irradiated cells 'to achieve the eventual synthesis of high molecular weight daughter strands of DNA despite the presence of unexcised damage in the template strands.' (Arlett and Lehmann, 1978). However, since the damaged segment is not removed from the template, this type of repair is error-prone, with a consequent high mutation yield. [As the damaged segment of DNA is not removed by this mechanism the term 'repair' is not altogether satisfactory for this system (Park and Cleaver, 1979).] Caffeine and theophylline inhibit this repair system because these purine analogues are introduced into the gaps in the single-stranded DNA (Cleaver, 1978).

Whereas FA cells have no detectable defect in postreplication repair (Arlett and Lehmann, 1978), one class of XP cells, the 'XP variants', are deficient in this system (Lehmann et al., 1975). These XP variants, although showing the classic XP stigmata have normal or near normal sensitivity to UV light and normal excision repair (Cleaver and Bootsma, 1975). (See section 1.6.5.3)

1.8.1.3 Excision repair

The excision repair system (Figs. 1.5 to 1.9) is the most important DNA repair mechanism operating in mammalian cells. This system is based on many different enzymes which together are able to remove different kinds of DNA damage (Cleaver, 1978). Excision repair involves several sequential steps (Gianelli, 1980; Bartram, 1980):

i. recognition of the site of DNA damage;

ii. distortion of the nucleoproteins;

iii. incision of the damaged DNA strand by an endonuclease at a site adjacent to the error;

iv. removal of the damaged strand by an exonuclease;

v. synthesis of a new DNA strand, to replace the excised damaged section of DNA, by using the undamaged complementary strand as a template; and

vi. ligation of the newly polymerized piece of DNA to the existing, undamaged segments.
The roles of the major enzymes involved in the above-mentioned steps are schematically represented in Fig. 1.5.

Excision repair forms the basis of the so-called nucleotide, base, single strand, and DNA-DNA cross-link repair mechanisms.

**Figure 1.5** Schematic representation of the actions of enzymes essential to excision repair pathways (after Watson, 1977).
1.8.1.3.1 Nucleotide excision repair

This repair mechanism (Fig. 1.6) is initiated by excision of the damaged DNA strand and requires site-specific endonucleases which identify the lesions and incise the DNA, so allowing subsequent excision of the lesions and degradation and resynthesis of the damaged segments (Friedberg et al., 1977). This system is the principle repair mechanism acting on dimers or carcinogen-DNA adducts (Arlett and Lehmann, 1978; Cleaver, 1978).

Poon et al. (1974) reported that the exonucleolytic step in excision repair of UV-induced thymine photodimers may be deficient in FA. However, no such deficiency was previously noted by Regan et al. (1973). This type of repair defect in FA is therefore questionable.

At the molecular level it is well established that the cells cultured from most XP patients i.e. those belonging to complementation groups A to G, but not the 'XP variants', are partially or totally defective in the excision of UV-induced damage or damage produced by carcinogens such as acetylaminofluorene or benzo(a)pyrene derivatives (Lehmann, 1981). At least five, and probably all seven of the excision-defective complementation groups, are characterized by an apparent defect in the first step of the excision repair pathway, i.e. the recognition of the site of damage in the DNA, followed by the nicking of the DNA by an endonuclease in a position adjacent to the damaged region (Ibid). These findings clearly indicate that the first step of incision repair is probably highly complex, requiring the interaction of the products of multiple gene loci for its operation.
1.8.1.3.2 Base excision repair

Base excision repair (Fig. 1.7) is initiated by a family of enzymes known as the N-glycosidases or glycosylases. These enzymes remove damaged bases by cleaving the bond between the base and the deoxyribose, without breaking the DNA strand (Wist et al., 1978; Kuhnlein et al., 1978). Subsequently, the base-free site is attacked by specific apurinic/apyrimidinic endonucleases which incise the DNA and allow degradation and resynthesis to take place (Laval, 1977; Friedberg et al., 1977). Normal levels of apurinic endonuclease activity were found in FA cell extracts (Teebor and Duker, 1975; Moses and Beaudet, 1978). (See section 1.8.2.3)

1.8.1.3.3 Single strand repair

Single strand interruptions are probably repaired by modification (or 'cleaning') of the termini surrounding the breaks through the action of a special exonuclease, followed by polymerization of a small piece
of new DNA, and finally ligation (Fig. 1.8). (Gianelli, 1980; Bartram, 1980). No reports of single strand repair in FA cells are available.

Hypersensitivity to ionizing radiation suggests that AT cells are defective in the repair of DNA damage produced specifically in response to ionizing radiation, but not by UV radiation. The exact nature of the associated molecular defect is yet to be unequivocally elucidated (Lehmann, 1981; Natarajan and Meyers, 1979), but is thought to involve defective single strand repair (Bartram, 1980).

1.8.1.3.4 DNA-DNA cross-link repair

![Diagram of DNA-DNA cross-link repair](image)

**Figure 1.9** DNA-DNA cross-link repair (after Bartram, 1980)

A number of clastogenic agents (e.g., bi- and polyfunctional alkylating agents; various psoralens in the presence of near UV light and ionizing radiation) may damage both strands of the DNA molecule in
closely opposed regions. This damage results in either double strand breakage or the formation of abnormally strong bonds between the two complementary strands of DNA (Gianelli, 1980). These interstrand cross-links (Fig. 1.9) are basically due to the fact that difunctional alkylating agents for example, have two reactive sites which form 'bridges' between the opposite DNA strands. (See section 1.6.2.1) Unlike single stranded damage, interstrand cross-links in a single DNA duplex present an immediate barrier to normal replication.

FA cells are particularly sensitive to the clastogenic effects of cross-linking agents. (See section 1.6.2.1) This points to a specific defect in the ability of these cells to remove such lesions. An in-depth exploration of mammalian cross-link repair mechanisms is thus necessary for the elucidation of the aetiology of FA.

Interstrand cross-links and monoadducts (i.e. single base/nucleotide modifications) are produced in bacterial DNA by exposure to psoralen plus light, MMC, nitrogen mustard and sulphur mustard (Fujiwara et al., 1977). The cross-link repair mechanism in E. coli is believed to be a two-step process (Cole, 1973; Howard-Flanders and Lin, 1973; Cole et al., 1976).

Mammalian cells, like bacterial cells, probably also possess a two-step mechanism for the repair of DNA-DNA cross-links. (Fig. 1.9) Cross-linked eukaryotic DNA is initially subjected to 'half-excision', or 'one-arm unhooking', followed by nucleotide excision repair of half-excised mono-adducts (Reid and Walker, 1969; Fujiwara and Tateumi, 1975; Fujiwara et al., 1977). Fujiwara et al. (1977) described 'half-excision' as being 'the double nick or tandem single-strand break in the same strand, one from each side of the cross-link' (Fig. 1.9). In normal human cells the first unhooking step is relatively rapid (half-life, 2 hours), while subsequent excision repair is slow (half-life, 14 to 18 hours) (Fujiwara et al., 1977; Kano and Fujiwara, 1981).
Nucleotide, base and single strand repair are error-free since the undamaged complementary DNA strand is used as a template for repair synthesis (Witkin, 1969). By contrast, DNA cross-link repair may be error-prone if complementary bases (i.e. opposite) rather than adjacent bases are intercalated (Bertram, 1980).

Although cross-link repair in bacteria is associated with the uvr system of repair of UV-induced DNA damage (Cole, 1973), this does not appear to be the situation in human cells. This conclusion is primarily based on the following findings:

i. uvr^-like XP cells (i.e. cells unable to excise pyrimidine dimers) remove uv^-induced cross-links by the same kinetics as normal cells (Fujiwara et al., 1977); and

ii. FA cell strains are hypersensitive to cross-linking agents, but appear normal in both UV survival and UV excision repair (Told).

The above-mentioned evidence strongly implies that the cross-link repair pathway in mammalian cells probably differs from the uvr-dependent cross-link repair pathway in bacterial cells. Furthermore, these findings suggest that FA cells may be deficient in a pathway specific for the repair of DNA cross-links, while being proficient in all other repair mechanisms.

Fujiwara et al. (1977) found three FA cell-lines to have 2- to 8-fold reductions in cross-link removal rates when compared with normal and XP cells. These results indicate that different strains are not uniformly deficient in DNA cross-link repair. Furthermore, some FA strains appear to have normal cross-link repair mechanisms: Kaye et al. (1980) noted a normal removal of 8-methoxypsoralen plus light-induced cross-links in normal, XP variant and FA fibroblasts, but XP group A cells were defective. It follows that there is heterogeneity in FA, as well as in XP, since not all FA cells exhibit a generalized defect in cross-link removal and different levels of deficiency are evident. However, the latter authors' findings have not as yet been confirmed for other clastogenic agents.
FA cell strains exhibiting impaired cross-link repair appear to be
detector in the first half-excision step of cross-link removal (i.e.
in unhooking of cross-links) and not in the subsequent
mono-adduct-excision repair step (Fujiwara and Tatsumi, 1975;
Fujiwara et al., 1977; Sasaki, 1977, 1979; Kano and Fujiwara,
1981). The reverse situation is evident in XP group A cells
(Fujiwara et al., 1977: Kano and Fujiwara, 1981). It is thus at
present generally accepted that the specific defect in FA is a
reduced ability for unhooking cross-links between complementary DNA
strands (Fig. 1.9), while the other repair pathways appear to be
functioning adequately.

In contrast to the above-mentioned findings, Shafer (1977) has
proposed that FA is characterized by a defect in the SCE mechanism
rather than by a repair defect. Shafer and Palek (see Shafer, 1977)
noted that SCEs are frequently located at the juncure between
replication segments. Furthermore, cross-links are known to
interfere with replication. Consequently, Shafer (1977) postulated
that SCEs represent a cellular mechanism for repairing or
bypassing cross-links. This proposed bypass mechanism allows
replication to continue passed a cross-link, but leaves the
cross-link intact. Therefore, this SCE mechanism cannot be
considered a true repair process. The model further specifies
interchanging of parental strands at the site of the cross-link, so
producing an exchange between sister chromatids. Shafer's
replication bypass mechanism thus predicts that in normal cells, any
DNA cross-link always produces an SCE and that the cross-link
persists in one of the replicated segments.

Shafer (1977) postulated that the observed chromatic aberrations in
FA may be representative of a defect in the second step of the
replication bypass mechanism i.e. in the ligation or rejoining of
displaced parental segments. This hypothesis could account for the
finding that most FA cases do not exhibit an increase in the number
of SCEs in response to the alkylating agent MMC, for example. (See
section 1.6.3.2) However, it should be pointed out that the
selection effect due to the continuous exposure of cultured FA cells
to cross-linking agents may seriously affect the observed SCE
Although Shafer's model is theoretically plausible, both Stekta (1979) and Kano and Fujiwara (1982) noted that experimentally, not all the predictions of this model are adequately fulfilled. Kano and Fujiwara (1982) proposed three further theoretical models which postulate an association between the induction of SCEs and DNA cross-links. These tentative models are yet to be practically investigated and thus speculation as to their validity is not possible.

1.8.2 Enzymological studies

DNA repair (see section 1.8.1) is a complex, multistep enzyme-mediated process; impairment may therefore be expected to occur at any step. Defective repair could result either from interference with the synthesis of gene products required for repair, or from decreased access of the required enzymes to the site of damage, or a combination of both. The enzymes associated with the proposed DNA repair/replication defect(s) in FA are yet to be conclusively elucidated. However, alterations in levels of several enzymes involved in DNA replication and repair have been reported.

1.8.2.1 Exonuclease

Poon et al. (1974) demonstrated that FA cells possess most of the functions required for the repair of UV-induced damage. In response to pyrimidine dimer induction, FA fibroblasts were able to produce single strand scission and polymerize nucleotides into a new complementary strand to replace the damaged portion of the DNA molecule. However, as discussed in section 1.8.1.3.1, the authors noted a deficiency in an exonuclease function (Fig. 1.5) which removes the damaged strand of DNA.

In contrast to the observations of Poon et al. (1974), Regan et al. (1973) found that FA cells can excise UV-induced pyrimidine dimers in a normal fashion. (See section 1.8.1.3.1) The reason for this
discrepancy is unknown, but may relate to the use of lower doses of UV irradiation by the latter workers, or alternatively, to the fact that different FA cell-lines were studied. [Poon et al. (1974) noted that a dose of at least 150 ergs/mm² is required to demonstrate an exonucleolytic difference between normal and FA cells.]

1.8.2.2 Ligase

DNA ligase is an essential enzyme for all types of DNA repair and is also required for normal DNA replication (Okazaki et al., 1973). Ligase is needed to join the replicative intermediates and replacement segments to the continuous DNA strand (Gefter, 1975). The primary effect of a DNA ligase deficiency is the accumulation of single strand breaks in the DNA.

Normal ligase function was postulated by the capacity of FA cells to rejoin radiation-induced DNA strand breaks (Pedrini et al., 1971; Foon et al., 1974; Sheridan and Huang, 1977). However, Hirach-Kauflmann et al. (1970) found FA cells to be deficient in DNA ligase activity, whereas endonucleolytic incision of DNA, repair DNA synthesis and exonucleolytic removal of the irradiation products were normal. The latter study showed DNA ligase activity to be reduced in both the FA homozygous patient and the heterozygote mother of this patient. The authors pointed out that a DNA ligase deficiency could result in a low cell propagation rate and a prolonged cell cycle due to the delayed joining of replicative intermediates. In this respect, it should be remembered that FA cells are often characterized by a poor mitotic index and a longer than normal cell cycle. (See section 1.7.1)

1.8.2.3 Endonuclease

Since alkylating agents and gamma-radiation are capable of producing apurinic/apyrimidinic sites in DNA, an apurinic/apyrimidinic endonuclease defect is possible in FA (Moses and Beaudet, 1978). Results of studies by Teebor and Duker (1975) indicate that there are
at least two putative human repair endonuclease activities — one directed against UV-induced damage and the other against apurinic sites. Endonucleases which incise double-stranded DNA at apurinic sites have been purified from all human cell-lines tested (Teebor and Duker, 1975).

Normal levels of apurinic endonuclease activity were found in extracts of cell-lines derived from patients with FA, AT, XP (complementation group D), Cockayne dwarfism, BS and progeria (Teebor and Duker, 1975; Moses and Beaudet, 1978). Moses and Beaudet (1978) have pointed out that failure to demonstrate an endonucleolytic defect does not exclude the possibility that multiple apurinic endonucleases may be present (as is the case in E. coli) and that the experimental protocol used was unable to detect a single apurinic endonuclease deficiency in FA.

1.8.2.4 DNA polymerases

A number of investigations have revealed no significant alterations in the levels of DNA polymerase alpha, beta and gamma in FA cells (Pedrini et al., 1971; Parker and Lieberman, 1977; Bertazzoni et al., 1978; Moses and Beaudet, 1978). It is however, possible that qualitative, rather than quantitative, properties of the DNA polymerases may be altered in the affected cells (Bertazzoni et al., 1978).

The activity of the chromatin-bound enzyme poly (ADP-ribose) polymerase is markedly stimulated by DNA strand breaks (refer to Berger et al., 1982). This enzyme requires the substrate NAD\(^+\) for the synthesis of poly (ADP-ribose), which is associated with the repair of DNA damage (Ibid). Berger et al. (1982) demonstrated that FA fibroblasts have lower mean NAD\(^+\) levels than cells from normal individuals. It has not been determined whether the decreased NAD\(^+\) pools in these patients are the result of underproduction or over-utilization. Nevertheless, it follows that the low NAD\(^+\) level in cells from some FA homozygotes may contribute to their decreased ability to recover from DNA damage.
Nicotinamide analogues that interfere with NAD+ synthesis have been reported to prevent normal differentiation and result in congenital anomalies in developing embryos (Caplan, 1972). Some of the congenital anomalies associated with FA may therefore possibly be due to such interference.

1. 2.5 Topoisomerase


The intracellular distribution of enzymes usually corresponds to their site of action. Topoisomerases are typical nuclear enzymes which are synthesized in the cytoplasm and consequently need to pass through the nuclear membrane in order to reach the intra-nuclear chromatin, their main substrate. Wunder et al. (1981) assayed the distribution of topoisomerases in normal and FA placental cells and, as expected, found topoisomerase to be almost exclusively confined to the nuclei of normal placental cells. However, the cytoplasmic fraction of three FA-placentae showed markedly increased levels of topoisomerase, whereas the nuclear sap extracts showed decreased topoisomerase levels compared with normal placentae. Other repair enzymes such as DNA polymerase-alpha, DNA polymerase-beta, and exonucleases acting on single and double stranded DNA were similarly distributed in the FA and control placental cells (Ibid). Due to the altered distribution of DNA topoisomerase in FA subcellular fractions, the authors concluded that the passage of the enzyme through the nuclear membrane is probably defective, causing its accumulation in the cytoplasm, where it is synthesized.

A further study by Wunder (1984) showed enzyme activity in the cytoplasmic fractions of FA fibroblast cultures, but not in the normal controls. However, in spite of the increased cytoplasmic activity found in the FA cell cultures investigated, most of the
enzyme activity was still located inside the nucleus. Auer et al. (1982) also found most enzyme activity in the nuclear fraction (about 88%), but in their experiments the cytoplasmic activity varied between 0% and 12%, in both FA and normal fibroblasts.

Whether the increased cytoplasmic DNA-topoisomerase I activity in FA cell fractions originates during laboratory preparation or reflects the situation in living cells, is unknown at present (Wunder, 1984). However, the observed topoisomerase distribution anomaly in FA cells could be the result of a structural defect in the enzyme itself, which impedes its passage through the nuclear membrane, but does not affect its catalytic activity at the site of action. A mutation leading to a complete deficiency in topoisomerase activity would be lethal, whereas a reduced nuclear level of topoisomerase activity might be adequate to enable most cells to survive under normal, non-stressed conditions. The abnormal distribution of topoisomerase in FA cells may alternatively be caused by a defect in the nuclear membrane, which selectively interferes with the passage of this and perhaps other enzymes. Conceivably, FA may be characterized by a defective membrane-bound protein which facilitates the passage of topoisomerase and/or other enzymes into the nucleus.

The above findings and conclusions are of great interest and indicate that further experiments on FA repair enzymes should include investigations of sub-cellular distributions as well as enzyme activity in the total cell extract, because the latter alone may not be a true reflection of the catalytic availability of an enzyme.

### 1.8.2.6 Hexokinase and ATPase

Lohr et al. (1965) demonstrated a defect in hexokinase, the rate limiting glycolytic enzyme in red blood cells. In four FA patients, the enzyme was found to be altered in its biophysical properties and activity. These alterations resulted in a lowering of the cellular ATP level. The fifth patient investigated by these workers showed normal carbohydrate metabolism. However, no further cases of hexokinase deficiency have been recorded since the latter report and
normal hexokinase levels have been found in at least twenty cases of FA (Beard, 1976).

Gmyrek et al. (1968) described a case of FA in which an increase in ATPase activity was noted. The consequence of such an anomaly, like hexokinase deficiency, is a decrease in the level of ATP. Schroeder (1966a) reported that cells with diminished ATP activity have fewer reunion figures than those with normal ATP activity. This finding substantiates that of Wolff (1960) who noted that ATP is necessary for the functioning of the 'chromosomal reunion mechanism' in normal cells. It should however, be remembered that FA is generally associated with an increased number of rearrangements, indicating a normal 'chromosomal reunion mechanism' and thus presumably normal ATP levels.

As just discussed, low cellular ATP levels may be the result of a hexokinase deficiency or an increase in ATPase activity. Alternatively, low adenine levels would also lead to low ATP levels. In this respect, it is of interest that the survival of FA fibroblasts treated with MMC, an agent known to be unusually toxic to FA cells (see section 1.6.2.1.3) significantly increased when adenine was added (Frazelle et al., 1981). This purine base did not enhance the survival of MMC-treated control cells. It follows from these observations that adenine can be utilized by FA cells to partially overcome the genetic defect which makes such cells hypersensitive to the action of MMC. This finding is compatible with the proposal that FA cells may be defective in DNA repair (see section 1.8.1), since purine nucleotides are substrates and energy sources (e.g. ATP) at a number of steps in the repair process. On the other hand, this postulated DNA repair defect could well be explained by an alteration in another biochemical pathway.

1.8.2.7 Superoxide dismutase and catalase

In addition to the postulated defect in the DNA cross-link repair mechanism in FA, it has also been suggested that the condition may be associated with a deficiency in superoxide dismutase (Raj and Heddle, 1980).
Enzymatic processes in biological systems, especially oxidation-reduction reactions, involve the production of free radicals i.e. chemical compounds with an odd number of electrons, which are highly reactive and unstable (Pryor, 1970). Living organisms have therefore evolved different systems for radical 'scavenging'.

Oxygen metabolizing cells generate the superoxide free radical $O_2^-$ and hydroxyl radicals. These radicals could conceivably act as continuous chromosome damaging agents, to which all aerobic cells would be exposed. The enzyme superoxide dismutase (SOD) is therefore essential for the survival of aerobic cells - SOD acts as a scavenger of the superoxide radical, catalyzing the reaction:

$$O_2^- + H_2O_2 + 2H^+ \rightarrow H_2O_2 + O_2$$ (Fridovich, 1975).

From the above reaction, it is clear that any process generating free $O_2^-$ radicals will by virtue of the spontaneous dismutase reaction, also be generating hydrogen peroxide ($H_2O_2$). The superoxide radical can also react with hydrogen peroxide to produce the highly reactive hydroxyl radical i.e.:

$$O_2^- + H_2O_2 + OH^- + OH^- + O_2$$ (Haber and Weiss, 1934).

Since the hydroxyl radical is an extraordinarily powerful oxidant, the Haber and Weiss reaction could vastly amplify the potential dangers of $O_2^-$. In aerobic cells, to counteract this effect, hydrogen peroxide is decomposed by the enzyme catalase to oxygen and water i.e. $2H_2O_2 \rightarrow 2H_2O + O_2$ (ORDENSON, 1977). Thus, the combined actions of antioxidative enzymes such as SOD and catalase maintain the very low steady state concentrations of $O_2^-$ and $H_2O_2$, therefore minimizing the Haber and Weiss reaction and making aerobic life possible.

The last step of the cellular antioxidative defence system presumably consists of DNA repair mechanisms which would remove damage due to $O_2^-$ and OH$^-$ radicals which have slipped through the above 'defence system'.
The enzymes SOD and catalase were shown to decrease the frequency of chromosomal aberrations in normal human lymphocytes exposed to ionizing radiation in vitro (Norденсон et al., 1976). (The chief radicals produced in the radiolysis of water are hydrogen atoms and hydroxyl radicals. Thus it may be expected that the number of radiation induced chromosomal aberrations would be reduced after the addition of SOD and catalase to the cell cultures.)

Addition of SOD to normal cell cultures treated with concentrates of the Bloom syndrome breakage factor (see section 1.7.3) also prevented chromosomal breakage (Emerit and Cerutti, 1981). FA lymphocytes cultured in the presence of SOD and catalase, separately and combined, showed a significant decrease in the frequency of spontaneous gaps and breaks (Norденсон, 1977). With respect to chromatid aberrations, the effects of catalase and the combination of enzymes were greater than the effect of SOD alone. Only the combination of SOD and catalase caused a significant decrease in chromosomal aberrations.

Cysteine readily reacts with free radicals and therefore has a radioprotective effect. The addition of this amino acid was found to significantly diminish the number of chromosomal breaks in FA lymphocytes. The effect of cysteine was equivalent to that of SOD (Norденсон, 1977).

Joenje et al. (1978) noted a significant reduction in SOD activity in 2 FA patients. This finding lends support to the proposal that FA might be characterized by decreased levels of cellular SOD. Unlike normal lymphocytes, the rate of chromosomal aberrations in cultured FA lymphocytes is positively related to O_2 tension (Joenje et al., 1981; Joenje and Oostra, 1983). In normal cells, the antioxidative defence mechanism is capable of coping with mildly hypoxic conditions; whereas in FA cells, this mechanism seems to fail even under normal culture conditions. Joenje et al. (1981) suggested that this finding may have clinical relevance; the possibility exists that some FA symptoms could be relieved by treatment with dietary antioxidants.
Dallapiccola et al. (1985) investigated the effect of 5 antioxidants in standard FA cultures and in cultures stressed either with DEB, or with butylhydroperoxide (BHP) or with hydrogen peroxide (H2O2). All 3 clastogens increased the chromosomal breakage levels in homozygous and heterozygous FA cells. A partial correction of spontaneous chromosomal instability was noted following the addition of antioxidants to the lymphocyte cultures. A 'protective' effect was also seen in the DEB- or peroxide-stressed lymphocytes of patients and heterozygotes, grown in the presence of antioxidants.

The locus for SOD has been mapped to 21q22 and shows a dosage effect in cells aneuploid for chromosome 21 (Feaster et al., 1977).

Auernach et al. (1980) noted an FA clone which was trisomic for most of the long arm of chromosome 21. This clone would thus be expected to possess increased SOD activity (at the time of publication, the SOD activity level had not been measured). However, those cells with the 21q+ abnormality still maintained a high rate of spontaneous chromosomal instability in spite of the postulated increased levels of SOD they should contain. This indicates that chromosomal fragility in FA may well be the result of a deficient repair mechanism, or a combination of a repair defect and a decreased potential for radical scavenging (or an increased production of free radicals). However, a combination of these deficiencies would not be expected in a single patient if the basic defect is the result of a single recessive gene mutation.

Using the micronucleus technique for screening chromosomal damage, Raj and Heddle (1980) concluded that the effect of SOD, catalase and L-cysteine is not specific to MMC-sensitive FA cell strains. The proportional effect of SOD was similar in the normal and FA fibroblast cell strains for both spontaneous and MMC-induced breakage. Furthermore, catalase was as effective as SOD in reducing the frequency of micronuclei. These findings indicate that the primary defect in FA is probably not an SOD deficiency, but rather a DNA repair deficiency. A defective free radical scavenging system cannot explain the high susceptibility of FA cells to difunctional and polyfunctional alkylating agents, in contrast to a proficient
response to monofunctional drugs and irradiation. (See section 1.6.2) However, the concentration of free oxygen radicals may be a critical parameter for the incidence of spontaneous chromosomal breakage in FA - lower than normal levels of SOD and/or catalase may cause an excess of chromosomal aberrations and this may in turn overload an already defective DNA cross-link repair mechanism.

1.8.3 Conclusion

Since FA shows Mendelian inheritance, the manifold stigmata should, at the molecular level, be a chain of different consequences of one defective gene in each FA complementation group. (See section 1.5) Therefore, possible FA gene products are probably either proteins involved in DNA replication and repair, or alternatively, molecules which alter the access of DNA-related enzymes to the chromatin.

Although the molecular nature of the FA defect remains controversial and the enzymological abnormality awaits characterization, it is generally accepted that FA cells are defective in the first half-excision step of cross-link repair. To date, it is not known whether this DNA repair defect is primary or the result of an alteration in another biochemical pathway. Finally, it must be emphasized that there is at present no known relationship between this defect in DNA cross-link repair and the clinical stigmata displayed by FA homozygotes. It is essential to determine whether these symptoms are indeed the result of visible chromosome aberrations and, if so, whether this damage is intrinsically produced or caused by extrinsic agents.

1.9 AIMS

Fanconi's anaemia homozygotes are clinically characterized by a variety of congenital malformations and a defect in the bone marrow which is typically delayed in onset. This heterogeneity makes the accurate diagnosis of FA difficult in some patients. Furthermore, spontaneous chromosome breakage may be variable and inconsistent, and
thus non-diagnostic. Therefore, in view of the suspected increase in frequency of the FA gene in South Africa, the aims of the present study were:

a) **Identification of FA homozygotes**

*In vitro* clastogenic stressing of FA homozygotes was evaluated with a view to increasing the diagnostic reliability of FA in liveborns. In addition, the prenatal detection of affected homozygotes was also considered since this would be of great practical assistance to obligatory carrier parents of an affected child.

b) **Identification of FA heterozygotes**

The second aim of this study was the establishment of an accurate and reliable technique for the identification of FA heterozygotes to achieve the dual purpose of counselling individual families and possibly determining the frequency of the FA gene in the various South African population groups.
Section 2
2. MATERIALS AND METHODS

2.1 SHORT-TERM PERIPHERAL BLOOD CULTURES

In 1932, Haldane predicted that the study of human chromosomes would be revolutionized if peripheral blood leukocytes could be cultured (Marchilli et al., 1980). Haldane's 'revolution' began in 1960 when Nowell noted that phytohaemagglutinin, a extract from red kidney beans, which had been used for its blood agglutinating properties, was a mitotic stimulant (Nowell, 1960b). That same year, Moorhead et al. (1960) combined the peripheral blood culture technique and air-drying method of slide preparation into a technique that could easily and routinely be performed. Hungerford's (1965) microculture method followed - no longer was it necessary for leukocytes to be separated from whole blood, and in addition, very small amounts of blood were required. This technique, with some modifications, was primarily used in the present study.

2.1.1 Individuals studied

The paediatric haematology units of the Baragwanath and Johannesburg hospitals were requested to refer their confirmed and suspected cases of FA for this chromosome study. Additional blood specimens were referred by a few private practitioners from the Transvaal Province of South Africa (see Fig. 3.1).

In view of the broad phenotypic diversity apparent in FA, stringent guidelines were necessary in order to minimize possible diagnostic difficulties. Patients were considered homozygous for the FA gene if the following criteria were fulfilled:

1. pancytopenia - not evident at birth, but developing in the first few years of life (not applicable to very young patients)
2. growth retardation - manifested by low birth weight or small stature
3. demonstrable dysmorphic features (refer to Table 1.1)
Those individuals not fulfilling the necessary criteria for FA, but exhibiting some clinical features, and thus suggesting possible partial expression of the FA gene, were also studied, but were separately grouped.

In view of the proven autosomal recessive mode of inheritance of FA (see section 1.5), parents of diagnosed FA cases were classed as obligate heterozygotes. It should however, be noted that in no cases was laboratory confirmation obtained that the fathers were in fact the true, biological fathers of the affected children.

As it is not known whether the apparently healthy sibs of homozygotes are FA heterozygotes or normal, the few sibs that were studied were also separately classed.

Phenotypically normal Black and White individuals of both sexes, ranging in age from 6 months to 65 years, served as control subjects. The controls consisted of healthy laboratory personnel as well as referred patients whose blood specimens had routinely been sent into the laboratory for chromosome analysis and had subsequently been shown to be chromosomally normal. It is realized that ideally, all control subjects should have been questioned as to possible family history of FA, or any features of the syndrome. However, in view of an estimated heterozygote frequency of 1 in 300 in the North American population (Swift, 1971), or 1 in 80 in South Africa (see section 3.1.3), at the most, one undetected heterozygote is likely to have been included in this control group, consisting of 41 individuals. Afrikaners were largely excluded as controls for the present study, as the frequency of the FA gene is thought to be higher in this population (see section 3.1.3): it is therefore unlikely that more than one heterozygote was per chance included in the control group.

Prior to cytogenetic analysis, all subjects were assigned to one of five groups, viz.

i. homozygotes

ii. obligatory heterozygotes
2.1.2 Collection of blood samples

Blood samples (5-10 ml) were obtained by venipuncture from all subjects under study. Sterility of the specimens was maintained by collecting blood directly into commercially prepared, sterile evacuating tubes (Vacutainer) containing the anticoagulant, lithium heparin without preservative. Directly after the withdrawal of the blood, the tubes were inverted several times so as to ensure the prevention of coagulation.

2.1.3 Storage of the blood samples

On receipt of the whole blood specimen, it was stored at 4°C for a maximum of 2 days before culturing. (The storage temperature was not allowed to fall below 4°C or exceed 38°C).

2.1.4 Procedure

The reader is referred to Appendix D for the sources of all reagents and chemicals used.

2.1.4.1 Materials used in the culture process

1. Tissue culture medium - Hams F10 supplemented with L-glutamine: pH 7.2-7.4 or M150 medium (Phenol red, a pH indicator, was added to the medium)
2. Antibiotics - penicillin ('Crystapen': 0.12mg/ml) and streptomycin ('Novostrep': 0.24mg/ml) were added to the medium
3. Serum - pooled human AB serum
iv. Mitotic stimulant - phytohaemagglutinin
v. Mitotic spindle inhibitor - colchicine
vi. Hypotonic solution - potassium chloride; 0.075M solution
vii. Culture vessels - sterile, screw-cap, plastic disposable 15ml conical centrifuge tubes

2.1.4.2 Method of culture

2.1.4.2.1 Initiation of culture

'Sterility' was maintained throughout the planting procedure. Hands were initially washed in 'hibiscrub', and all planting took place in a 70% alcohol-swabbed, 'UV/fluorescent light box' (the UV light should be switched off whilst working in the 'box'). In addition, sterile disposable needles and syringes were used and 'flaming' was employed.

The following were added to each sterile culture tube:

i. 3.5ml Hams F10 or M150 culture medium
ii. 1ml FCS serum
iii. 0.05ml PHA
iv. 0.5ml whole blood

2.1.4.2.2 Incubation

The tightly sealed cultures were incubated at an angle of approximately 30° to obtain a maximum surface area for 66 hours at 37°C.

Temperature fluctuations of the incubator were monitored daily to ensure a constant temperature, fluctuating less than 1°C from 37°C.
2.1.4.2.3 Metaphase arrest

From this stage onwards, the cultures were no longer handled in a sterile manner.

0.5μg Colchicine/ml culture = 0.01ml colchicine working solution (see Appendix C) was added to each culture 30 minutes prior to harvesting. Cultures were then reincubated at 37°C for the following 30 minutes.

2.1.4.2.4 Harvesting

2.1.4.2.4.1 Hypotonic pretreatment

i. The cultures were centrifuged at 1600 rpm for 8 minutes.

ii. The supernatant was carefully decanted, taking care not to disturb the cell pellet. About 0.5ml supernatant was left in the centrifuge tube to prevent the cell-pellet from clotting on addition of KCl.

iii. 5ml of 0.075 M KCl prewarmed to 37°C was added, using a low speed ‘whirlimixer’, to ensure gentle but thorough mixing, during and after the addition of the hypotonic solution.

iv. The cultures were reincubated at 37°C for 20 minutes.

2.1.4.2.4.2 Fixation

Fresh fixative (4 parts methanol:1 part glacial acetic acid) was prepared at the beginning of the harvest and then stored at less than 0°C.

i. The cultures were again centrifuged at 1600 rpm for 8 minutes.

ii. The hypotonic supernatant was carefully removed with a fine bore pipette or a 10ml syringe.

iii. First fixation: 5ml fixative was added very slowly i.e. drop
by drop for at least the first 3ml, while the culture was agitated on a 'Whirlimix'.

iv. The cultures were recentrifuged at 1600 rpm for 8 minutes.
v. The supernatant was decanted.
vi. Second and third fixations were processed in the same manner as described above in steps iii to v.

vii. The cultures were again centrifuged at 1600 rpm for 8 minutes.
viii. The supernatant was decanted.

By this stage, the supernatant fixative was usually clear, but if not, repeated changes of fixative were continued until the supernatant was completely clear.

2.1.4.2.4.3 Slide preparation

Commercially precleaned slides were soaked in absolute alcohol for at least 24 hours prior to use so as to ensure that they were oil- and grit-free.

The slides were prepared in most cases immediately after harvesting. If the slides were made the following day, the cultures were stored at 4°C.

In all cases, the following procedure for slide preparation was adopted:

i. The culture was centrifuged at 1600 rpm for 8 minutes.

ii. As much supernatant fixative as possible was decanted without disturbing the cell pellet.

iii. The pellet was resuspended in approximately 0.5ml fresh fixative; the amount of fixative used was directly proportional to the size of the final cell pellet - a 'milky' consistency was required.

iv. Alcohol-soaked slides were polished with lint-free tissues.
v. The slide was flooded with fixative and any excess fixative was removed by tilting the slide.

vi. A thin bore pasteur pipette plus rubber bulb was used to drop + 5 drops of the cell suspension from a height of + 40cm on to
each wet slide, held in a horizontal position.

vii. As soon as most of the fixative had evaporated from the slide surface, the slide was gently flooded with fixative and allowed to lie flat for 45 seconds.

viii. Any excess fixative was removed by tilting the slide on to blotting paper.

ix. The slides were air dried in a vertical position in front of a warm fan.

2.1.4.3 Drug treatment

Modified PHA-stimulated lymphocyte cultures were initiated in a manner identical to that already described in sections 2.1.4.2.1 and 2.1.4.2.2 but were exposed to either of the two bifunctional alkylating agents under study, viz. DEB or MMC, for the final 42 hours of culture.

The DEB was added in a range of dilutions to a series of different cultures established from each case 24 hours after the initiation of culture. The DEB was diluted in commercially available sterile distilled water to final concentrations of 0.01; 0.1; 0.2; 0.4 or 1μg/ml culture. Similarly, MMC was diluted in sterile saline to final concentrations of 10 and 50 ng/ml and added to cultures 24 hours after their initiation.

Thereafter, the drug treated cultures were processed identically to the non-induced cultures. (See sections 2.1.4.2.3 and 2.1.4.2.4)

2.1.5 Discussion

2.1.5.1 Peripheral blood cultures

Dividing somatic cells are required for the examination of mitotic chromosomes. This can be accomplished in 3 main ways (Priest, 1977):

a) tissues already dividing rapidly in vivo, such as bone marrow,
may be examined without in vitro culture;

b) short-term in vitro culture of peripheral blood; and
c) long-term in vitro tissue culture.

Peripheral blood is however, the most common source for the analysis of human chromosomes as it is, in most cases, easily and painlessly obtainable. By contrast, bone marrow and fibroblast culture studies may cause considerable discomfort to the patient, and are thus only recommended when clinically indicated. Although the mitotic index (rate of cell division) is low in circulating lymphocytes, various mitogenic agents can induce them to undergo blastoid transformation and division (Robbins, 1964). (See section 2.1.5.3)

In the original peripheral blood culture technique of Moorhead et al. (1960), the plasma was separated from the red cells and thereafter added to the culture medium. This technique required relatively large quantities of blood. In the so-called 'microculture' techniques of Hungerford (1965) and later, Poulding and Allen (1968), techniques which form the basis of the methods employed in the present study, small quantities of whole blood are added to the culture medium. This microtechnique has the advantages that the whole blood need not be centrifuged and handling of the blood is kept to a minimum.

2.1.5.2 Choice of procedure

Commercially available Hams F10 medium was used for almost all the cases, because it contains correctly balanced ingredients necessary for cell growth, viz., essential amino acids, vitamins and co-factors, mineral salts and an energy source. In 16 cases, the number of chromosome aberrations produced in the presence of the medium M150, as opposed to F10, was investigated. Like TC199, M150 is a folate-deficient medium known to enhance the expression of some heritable fragile sites. (The reader is referred to the GIBCO catalogue for example, for differences in composition between Hams F10 and TC199.)

Although 48-hour cultures are usually recommended for chromosome
breakage studies (see section 2.6.4), a 66-hour culture period (equivalent to 72-hour conventional cultures) was chosen to enable direct comparisons with previously published data. (See Table 3.11)

2.1.5.3 Lymphocyte transformation

In normal circulating peripheral blood, about 36% (1 - 4 x 10^9/l in healthy adults) of the white cells are lymphocytes, and almost all of these are small untransformed lymphocytes (Marchilli et al., 1980). In the presence of various mitogens, the small lymphocytes undergo transformation. These transformed 'blastoid' cells are then capable of division.

The most commonly used mitogen for cyto genetic studies is phytohaemagglutinin (PHA). PHA is isolated from the seeds of Phaseolus vulgaris or P. communis by salt extraction (Rigas and Osgood, 1955). PHA has 2 components, a mucoprotein (PHA-M) and a protein (PHA-P), both of which are good mitogens (Marchilli et al., 1980). PHA, like Concanavalin-A, is thought to affect primarily the T-cell population of lymphocytes, while other mitogens such as pokeweed mitogen affect primarily the B-cells (Ibid).

Elves and Wilkinson (1962) showed that PHA causes lymphocytes to first undergo regressive changes. They suggested that PHA produced its mitogenic effect by its ability to cause rejuvenation of the intermediate and small lymphocytes, which transform to blast cells and hence to cells capable of division. This mitotic effect of PHA, which is believed to be antigenically mediated, thus appears to be secondary to transformation (Hamerton, 1971). The action of PHA is therefore said to be 'premitotic'.

Within the first 24 hours after the addition of PHA to the culture, there is a marked increase in RNA synthesis (Marchilli et al., 1980). This is followed in the next 24 hours by enlargement of the cell nucleus and the initiation of DNA synthesis (Ibid). Once DNA synthesis begins, the cells are 'committed' to divide and PHA is no longer required in the culture.
Besides having the property of inducing division in lymphocytes, PHA also selectively agglutinates and sediments mature erythrocytes (Rigas and Osgood, 1955).

2.1.5.4 Mitotic arrest

Mitotic inhibitors prevent the formation of the spindle apparatus and thereby promote dispersion of the chromosomes and the accumulation of metaphases by blocking further mitosis (Lawee and Brown, 1980). Antimitotic agents in use include velban (vincristine), colchicine and its derivative, colcemid. Colchicine and colcemid are widely used, while velban is used to a limited extent.

Bosman et al. (1975) showed that the length of the chromosomes is indirectly proportional to the concentration of colchicine and the time of exposure of the cells to this mitotic inhibitor - the higher the concentration and the longer the time of mitotic arrest, the more condensed the chromosomes will be. Colchicine at a concentration of 0.5μg/ml culture added to the cultures for 30 minutes was found to give an adequate number of metaphases of relatively good morphology. Used at low dosages, colchicine appears to have no deleterious effect on the entrance of cells into division, nor is there any evidence of it causing chromosome aberrations (Hamerton, 1971).

2.1.5.5 Hypotonic pretreatment

Early attempts to discover the chromosome number in man were severely hampered by the fact that the chromosomes were usually markedly clumped and it was thus difficult to distinguish one chromosome from another. Improved visualization of individual chromosomes followed the independent discoveries of Hsu (1952) and Hughes (1952) that hypotonic treatment causes swelling of cells and thereby enhances chromosome spreading. Hungertord (1965) introduced the use of 0.075 M KCl as a hypotonic pretreatment. It was noted that this procedure, compared to using distilled H2O,
for example (Moorhead et al., 1960) appears to be less damaging to the structural integrity of the chromosomes. Hence, hypotonic KCl solutions are today preferred for most banding protocols.

The time required to allow the hypotonic solution to swell the cells, does not appear to be critical with normal, non-leukaemic cells. However, in order to maintain uniformity, the hypotonic solution was always prewarmed to 37°C and added to the cultures for 20 minutes. The volume of hypotonic solution is however, critical for blood samples, as too little hypotonic will result in non-lysis of red cells and poor chromosome spreading and morphology. Five ml 0.075M KCl was in all cases found to be more than adequate.

2.1.5.6 Fixation

Fixation may be defined as 'the process by which tissues or their components are fixed selectively at a particular stage to a desired extent' (Sharma and Sharma, pg 30, 1980). Until this stage, the cells are alive, metabolizing and continuing to be affected by the mitotic arresting solution. The purpose of fixation is thus to kill the cells without causing any distortion of the components to be studied i.e. the chromosomes, as far as is practicable.

The fixative used should ideally have the following properties (Sharma and Sharma, 1980):

i. precipitation of chromatin
ii. immediate cell killing
iii. checking the autolysis of proteins
iv. prevention of decomposition
v. enhancement of the basophilia of the chromosomes

Fixatives fulfilling the above-mentioned criteria to a greater or lesser extent and therefore in common cytogenetic usage, include (Sharma and Talukder, 1976):

i. Acetic alcohol (1:3) - 3 parts of absolute ethyl or methyl alcohol to 1 part of glacial acetic acid.
ii. Acetic acid - 45% to 60% solution in distilled water.
iii. Carnoy's fixative - absolute ethyl alcohol 100 parts; glacial acetic acid 16 parts; chloroform 50 parts.

iv. Tjio's fixative - 95% ethyl alcohol 6 parts; glacial acetic acid 2 parts and 40% formaldehyde 1 part.

An acetic alcohol fixative (4 parts methanol : 1 part glacial acetic acid) was used throughout the present study. This fixative proved to be satisfactory in giving clear chromosome morphology and good chromosome spreading.

The fixative must be freshly made and kept tightly sealed while in use, as it can absorb water upon standing and in addition, the pH changes with time.

2.1.5.7 Slide preparation

Slide making is probably the most important step in the preparation of chromosomes. Chromosome spreading and morphology, and therefore banding quality, are in large part dependent upon how slides are prepared.

The many variables which affect slide quality can all be shown to affect the drying of the cells and thus the chromosomes, by changing the evaporation of the fixative. Given the proper harvest procedure, most technical problems in slide preparation are drying problems. Air drying is probably the simplest procedure for spreading chromosomes on a microscopic slide from a suspension of cells in fixative. Air drying is today the method of choice in most banding protocols. However, as humidity and air temperature vary from time to time, a major problem is the achievement of consistency.

Knowledge of the following variables is also helpful in obtaining consistently good slides (Lawee and Brown, 1980):

i. slide temperature

ii. fixation
iii. cell pellet dilution factor
iv. wet versus dry slides
v. pipette bore size
vi. cleanliness of slides
vii. application pressure to pipette rubber bulb
viii. air movement

In a chromosome breakage study, it is imperative that the above-mentioned variables be kept to an absolute minimum so as to avoid possible bias through non-uniform techniques. Consistency is also more likely if the same individual prepares slides on all the samples.

2.2 AMNIOTIC FLUID CELL CULTURES

Developments in 3 major areas of technology have made the prenatal detection of chromosome abnormalities possible:

i. reliable obstetrical procedures for collecting amniotic fluid;

ii. successful systems for the cultivation of amniotic fluid cells; and

iii. chromosome identification methods for the evaluation of amniotic fluid cell metaphases.

Steele and Breg (1966) were the first to describe a reliable 'closed culture' system for amniotic fluid cells. An adaptation of this technique has been employed in the present study.

2.2.1 Individuals studies

A 25 year old para 2, gravida 1, patient was referred for amniocentesis because her son is unequivocally homozygous for the FA gene. The 25% recurrence risk, the general implications of amniocentesis and the experimental nature of FA prenatal detection, were explained to the patient prior to amniocentesis by her medical attendants in another city.
Control amniotic fluid specimens from the writer's laboratory were chosen on the basis of gestational age at the time of amniocentesis and date of initiation of amniotic fluid cell culture; they were processed on the same day as the amniotic fluid of the obligatory FA heterozygote. The control amniotic fluids had been referred for cytogenetic analysis because of advanced maternal age (>35 years) and were subsequently found to be chromosomally normal.

2.2.2 Collection of amniotic fluid

Transabdominal amniocentesis was performed at 16 weeks gestation with simultaneous 'real-time' ultrasound visualization. Approximately 20ml of amniotic fluid was aseptically withdrawn into 2 x 25cm³ sterile culture flasks.

As the amniocentesis on the patient 'at-risk' was not performed in Johannesburg, this specimen was initially sent to a laboratory closer to the patient's home, where the cultures were established in a manner very similar, if not identical, to the one here described. Subcultures were transferred to Johannesburg 2 weeks later and further processed.

2.2.3 Culture procedure

2.2.3.1 Materials used in the culture process

The reader is referred to Appendix D for sources of reagents, chemicals and equipment used.

i. Tissue culture medium - Ham's F10 medium supplemented with l-glutamine: pH 7.2-7.4 (Phenol red, a pH indicator, was added to the medium)

ii. Antibiotics - Penicillin ('Crystanen' - 0.12mg/ml)

and streptomycin ('Novostrep' - 0.24mg/ml) were added to the medium

iii. Serum - foetal calf serum (PCS)
iv. Spindle inhibitor - colchicine

v. Hypotonic solution - potassium chloride; 0.05M (see section 2.2.4.2.2)

vi. Centrifuge tubes - sterile, screw-cap, plastic 15ml conical tubes

vii. Culture vessels - sterile, screw-cap, plastic 25cm³ culture flasks

viii. Pipettes - sterile, disposable 1ml plastic pipettes

ix. Gas - 5% CO₂ in air (to regulate the pH of the culture medium)

2.2.3.2 Initiation of amniotic fluid cell culture

A 'closed' culture system was used. Prior to initiation of the culture, the following were first noted -

i. Dr who performed the amniocentesis;

ii. Date and time of fluid withdrawal;

iii. Amount of fluid received;

iv. Appearance of fluid eg. clear or blood-stained; and

v. The size of the cell pellet (a viable cell count was not performed).

In addition, meticulous records as to type of growth and number of cell passages were kept throughout the culture period.

It is imperative that sterile techniques be employed at all times when handling amniotic fluids and cultures. The following rules were observed:

i. All sterile procedures were performed in a tissue culture hood with laminar flow system and high efficiency particulate air (HEPA) filtration.

ii. Ultraviolet lights were left on in the hood and tissue culture room, except when people were in the room.

iii. The work surface was disinfected with 70% alcohol both before and after working with cultures.

iv. Hands were meticulously washed both before and after working with cultures.

v. The lips of glassware were well-flamed, and plastic
materials were briefly flamed (proper flaming is good practice, but over heating must be avoided - do not flame a pipette with cells or medium in it and do not use any flamed implement to handle tissues or cells until it has sufficiently cooled down. Overheating plastic material melts the plastic and causes leakage through incorrect fit of flask neck and cap.)

'Planting':

Day 1

i. Contents of the 2 flasks containing the fluid were divided into 2 sterile centrifuge tubes, and centrifuged at 1000 rpm for 10 minutes.

ii. The supernatant was carefully decanted without disturbing the pellet (2ml supernatant was reserved for amniotic alphafoetoprotein estimation and the remaining fluid was deep frozen, should additional biochemical tests be required).

iii. 1ml PCS was added to each tube and the cell pellets were gently resuspended using a disposable pipette and rubber bulb.

iv. Resuspended cells were transferred from each tube into each of the original culture flasks in which the fluid was received, and two other flasks i.e. a total of 4 culture flasks were set up from the 2 cell pellets.

v. The cells were allowed to settle, by leaving the flasks in the laminar flow box for +30 minutes.

vi. 2.5ml whole medium was added to each flask (whole medium = Hams F10 + 20% PCS solution), after gently inverting the flask.

vii. The flasks were gently gassed with 5% CO₂ in air for approximately 10 seconds - the gas flow was directed away from the culture surface so as not to disturb the cells, which should have begun to settle at this stage. To prevent contamination the gas was passed through autoclaved tubing via a disposable microfilter prior to coming into contact with the culture. (This precaution was taken because of a past experience where cultures became contaminated through gassing)

viii. The flasks were incubated at 37°C after ensuring that they were tightly sealed.
Day 3

i. The cultures were examined under an inverted microscope, to note whether cells had adhered to the flask surface.

ii. The original medium was retained and a further 2.5ml whole medium were gently added to each flask.

2.2.3.3 Feeding

Day 7

i. The cultures were examined under an inverted microscope and the extent and type of growth were noted (eg. epithelial/fibroblast).

ii. The spent medium was gently tipped off, retaining ± 0.5ml in each flask.

iii. 5ml whole medium was slowly added, directing the flow away from the culture surface.

iv. The cultures were gassed with 5% CO₂ in air, if spent medium was an incorrect colour, thus indicating that the pH was too acidic (see section 2.2.4.2.1).

Further feeding

Cultures were fed twice weekly until they were ready to be subcultured or terminated.

2.2.3.4 Subculturing

Cultures are ready for subculture (or termination) when ± 5 colonies of 1 to 2 cm diameter are visible on the culture surface.

i. When sufficient growth had occurred, ± 4ml spent medium was 'tipped' off, retaining ± 1ml.

ii. Using a sterile silicon scraper, the previously marked areas of growth were gently scraped off the culture surface, and thereafter, the flasks were shaken lightly.

iii. 5ml newly-suspended cells were carefully transferred by directly pouring from the original flask into a subculture flask.
iv. 5ml whole medium was added to the remaining cells in the original flask.

v. The subculture flask was gently gassed for \( \pm 10 \) seconds.

vi. Original and subcultured flasks were incubated at \( 37^\circ C \).

vii. The flasks were processed as previously described (see section 2.2.3.3) and growth was carefully monitored until ready for subculture or termination.

2.2.3.5 Metaphase arrest

i. Cultures were examined under an inverted microscope for the presence of rounded cells, indicating active cell division.

ii. If sufficient rounded cells were present, colchicine (0.5\( \mu \)g/ml culture) was added.

iii. The culture was reincubated for a further 5 hours at \( 37^\circ C \).

2.2.3.6 Harvesting

From this stage onwards, sterility need no longer be maintained.

i. After 5 hours colchicine treatment, the spent medium was decanted into a centrifuge tube.

ii. The flask was washed with 2ml Ca\(^++\) - and Mg\(^++\) - free phosphate buffered saline (PBS-pH 7.3) and the washings were added to the same centrifuge tube as in (i).

iii. To each flask, 3ml prewarmed (37\( ^\circ \)C) Trypsin - EDTA mixture (see Appendix C) was added.

iv. The flasks were monitored under an inverted microscope for cell lifting (this step usually took \( \pm 2 \) minutes) - each flask was then tapped gently, but sharply, several times to loosen the cells.

v. The trypsinized lifted cells were poured into the same tube as in steps (i) and (ii).

vi. The culture flask was washed with 2ml KCl (0.05M), prewarmed to \( 37^\circ C \) and washings were added to the same centrifuge tube.

vii. After ensuring that the centrifuge tubes were balanced, they were centrifuged at 2500 rpm for 5 minutes.
viii. The supernatant was carefully decanted without disturbing the cell pellet.

2.2.3.6.1 Hypotonic pretreatment

i. 8ml 0.05M KCl (prewarmed to 37°C) plus 1ml FCS was added to each centrifuge tube and the cells were gently resuspended with a Pasteur pipette and rubber bulb.

ii. The cells in hypotonic KCl were incubated at 37°C for ± 35 minutes.

iii. The cultures were centrifuged at 2500 rpm for 5 minutes.

iv. The supernatant was carefully removed, taking care not to disturb the cell pellet.

2.2.3.6.2 Fixation

i. 5ml of 6:1 methanol:glacial acetic acid fixative (freshly prepared and refrigerated) was added by first resuspending the cells in ± 0.5ml fixative, and thereafter, adding the remaining 4.5ml fixative and mixing with the resuspended cell pellet.

ii. The fixed cells were recentrifuged at 2500 rpm for 5 minutes and the supernatant was carefully removed.

iii. 5ml cold fresh 3:1 methanol:glacial acetic acid fixative was added and the cells were resuspended.

iv. The cells in 3:1 fixative were centrifuged at 2500 rpm for 5 minutes, after which the supernatant was carefully decanted.

v. 5ml cold, fresh 1:1 methanol:glacial acetic acid fixative was added and the cells resuspended.

vi. The cells in 1:1 fixative were centrifuged at 2500 rpm for 5 minutes and the supernatant was then carefully removed.

vii. ± 0.5ml 1:1 fixative was again added and the cells were resuspended.
2.2.3.7 Slide preparation

Immediately after harvesting, +3 slides were prepared per case. The technique employed was as described in section 2.1.4; 2.4.3, but 1:1 fixative was used instead of 4:1 fixative.

2.2.3.8 Drug treatment

Amniotic fluid cell cultures to be treated with DEB were planted in the same way as the untreated cultures, as described in section 2.2.3.2. When cultures from the 'at-risk' foetuses and from the normal control foetuses reached near-confluency, the cells were subcultured, usually at a 1:3 'split' ratio, to provide sufficient flasks for experimental and untreated control cultures. Twenty-four hours after subculture, non-toxic concentrations (0.4µg/ml and 0.05µg/ml) of DEB freshly diluted in sterile, distilled water, were added to the growth medium of the experimental and control cultures. Cells were allowed to grow in the drug treated medium until termination (usually 72 to 96 hours later). In addition, some cultures were allowed to grow in the DEB-containing medium for 72 hours, and thereafter, the cells were subcultured in carcinogen-free medium for a further 48 hours, when the cells were harvested for cytogenetic studies (Voss et al., 1981). Replicate cultures of each specimen in DEB-free medium served as controls for spontaneous breakages.

2.2.4 Discussion

Second trimester amniocentesis has developed into the single most important procedure for the early detection and prevention of certain genetic disorders (Emery, 1973, Epstein and Golbus, 1978, Epstein et al., 1983). General indications for amniotic prenatal diagnosis in 'at-risk' foetuses are:

a) chromosomal abnormalities;
b) neural tube defects;
c) certain X-linked disorders for sexing the foetus; and
d) diagnosis of certain single gene disorders biochemically or
molecularly by means of gene probes or restriction fragment length polymorphisms (RFLPs).

Auerbach et al. (1979a) were the first to report the prenatal detection of the PA gene by cytogenetic methods. Their method, a modification of which was employed in the present study, was based on the enhancement of spontaneous chromosome breakage in the amniotic cells of an affected homozygote foetus, by the addition of DEB to the 'at-risk' amniotic fluid cell cultures.

2.2.4.1 Cell types

The cell pellet resulting from low speed centrifugation of whole amniotic fluid consists of at least 3 cell types, which differ in cellular morphology, clonal morphology in culture and growth potential (Hoehn et al., 1974).

Although their tissue sources are as yet not reliably established, certain morphologic cell types may be defined:
- a) epithelial (E) type
- b) fibroblast (F) type
- c) amniotic fluid (AF)

The AF type is intermediate in appearance between the E and F types. AF cells are predominant among cells in amniotic fluid and give rise to the greatest number of colonies upon cultivation. The absolute proportion of the various cell types remains to be determined; however, the distribution of clone types grown from amniotic fluid samples is about 5% fibroblastic clones, 34% epitheloid clones and 61% amniotic fluid cell clones (Barker and Lawce, 1980).

In addition to the above-mentioned 3 major cell types, various types of maternal and foetal blood cells may be present as a result of vascular leakage into the amniotic fluid at the time of amniocentesis. Certain amniotic fluid samples may also contain rapidly adhering (RA) cells which could indicate the presence of neural tube defects (Gosden and Brock, 1977).
2.2.4.2 Technique

2.2.4.2.1 Culturing

Prevention of infection
When culturing amniotic fluid cells it is essential that sterility be meticulously maintained throughout the planting and subculturing processes. Furthermore, it is imperative to monitor the cultures for signs of contamination. Bacterial and fungal contamination may be apparent to the naked eye (a culture may sometimes be rescued by repeated rinsing and appropriate use of antibiotics, but this procedure is not often successful). Mycoplasma contamination is less obvious, but can cause significant problems, such as poor cell growth and chromosome breakage. Mycoplasma infection must thus be avoided at all costs, especially when a positive prenatal diagnosis hinges on chromosome breakage results, as is the case with FA.

Correct pH
The pH of the medium must be maintained within a suitable range (pH 7.0-7.4) during culture. The medium used, Ham's F10, had phenol red, a pH indicator, added to it. After the addition of phenol red to the medium, a yellowish colour is indicative of too acidic a mixture, and a reddish-purple colour indicates that the medium is too alkaline. In most cases, a change of medium, subculturing, or adjustment of CO₂ tension (using sterile 5% CO₂ in air) solves the problem of pH fluctuation.

Serum
It is still poorly understood what factors serum provides cells, but it is nevertheless clear that certain types are better than others and that certain 'batches' are more conducive to growth than others. Foetal calf serum (PCS), as opposed to pooled human AB serum, while relatively expensive, seems to be best suited to amniotic fluid specimens. Because of its animal origin PCS is the most likely of the tissue culture components to contain contaminants, and should therefore be subjected to stringent quality control measures.
Subculture

Cells from amniotic fluid grow as monolayers. These attached cells will continue growing until they cover the entire surface of the flask, a state called 'confluency'. If the cells are not transformed or malignant, they will exhibit 'contact inhibition', and will cease dividing. It is therefore very important to either terminate the culture or subculture prior to confluency being reached.

2.2.4.2.2 Harvesting

As is the case with short-term peripheral blood cultures, the use of a mitotic arresting agent, e.g. colchicine, followed by hypotonic pre-treatment and fixation is essential in securing morphologically distinct, well-spread amniotic cell chromosomes (see section 2.1.5). However, a 0.05M concentration of KCl does appear to be more effective in swelling amniotic cells than the 0.075M KCl solution used for blood cultures. Furthermore, as opposed to the 4:1 fixative used throughout harvesting and slide preparation for blood cultures, the use of successive changes of 6:1, 3:1 and 1:1 fixative appears to improve amniotic chromosome definition.

2.2.4.2.3 Drug-treated cultures

In the writer's experience, it was not possible to subculture all cultures (i.e. 'at-risk' and controls) by a 1:3 split and after a further 3 days, to subculture 1:3 again followed by harvesting 48 hours later, as advocated by Voas et al. (1981), due to non-uniform growth rates in different cultures. It is however, essential that the control and 'at-risk' cultures being investigated be identically processed at the same time, so as to avoid bias. Voas et al. (1981) and Auerbach et al. (1981) recommend that the medium should be decanted and replaced with fresh medium containing DEB 24 hours after subculturing. It was, however, found that this may result in the loss of potentially mitotic cells, because after 24 hours, not all the cells had adhered to the culture surface.
DEB has a half-life of 4 days in aqueous solution (see Auerbach and Wolman, 1976). Therefore, if a chronic exposure time of more than 4 days is to be employed, the DEB containing medium must be decanted 4 days after the addition of DEB, followed by the addition of fresh DEB and medium.

2.3 LONG-TERM SKIN FIBROBLAST CULTURES

Culturing of solid tissue is one of the oldest cytogenetic laboratory techniques. The beginning of successful tissue culture is accepted to be Harrison's experiments in 1907 whereby he managed to culture frog axons (Harrison, 1907). Several reports describing tissue culture appeared prior to 1907, but most of the early attempts to culture tissues and cells were hampered by inadequate media formulations and infection (Paul, 1975).

When cells are cultured from a tissue biopsy (or amniotic fluid), the first outgrowth of cells is referred to as a primary culture. After one subculture, the term serial or long-term culture is used (Priest, 1977). Advantages of using long-term cultures for chromosome analysis include:

i. cells may be stored in liquid nitrogen and retrieved when needed (see section 2.4); and

ii. multiple subcultures are available without returning to the patient.

2.3.1 Individuals studied

Fibroblasts from a diagnosed male FA homozygote were studied; he was the affected proband sibling of the foetus-at-risk. (See section 2.2.1) A skin biopsy of the proband's father grew poorly in culture and failed to yield metaphases. Skin biopsies were indicated in these 2 cases to serve as FA positive and FA heterozygote controls for the amniocentesis to be performed on the pregnant mother of the proband. (See section 2.2.1) A skin biopsy was subsequently taken from the aborted foetus which had been diagnosed as an affected FA
homozygote, in order to verify the amniotic diagnosis.

2.3.2 Skin biopsy procedure

Skin biopsies were aseptically obtained from the inner aspect of the patients' forearms. The 'pinch skin' biopsy technique was used whereby the cleansed skin is pinched between sterile forceps and a small piece is removed using a sharp curved sterile scissors or scalpel (Priest, 1977). The dermis must be included in the specimen, as indicated by bleeding at the incision site. The biopsy was then immediately placed in sterile medium with antibiotics for transfer to the laboratory.

2.3.3 Culture procedure

2.3.3.1 Materials used in the culture process

As for amniotic fluid cell cultures - see section 2.2.3.1.

2.3.3.2 Initiation of fibroblast culture

Sterile techniques must be employed - see section 2.2.3.2.

'Planting':

1. The specimen was placed in a few drops of medium (Hams F10) in a sterile glass petri dish and was constantly kept moist

2. The specimen was cut into 1 mm² pieces using a sharp scalpel, with minimal handling to avoid tearing of tissue (Attachment to the growth surface is crucial to growth: larger pieces tend to float off the growth surface, whilst pieces which are too small, take longer to establish themselves.)

3. Individual explants were picked up with a sterile disposable pipette plus rubber bulb and 12-16 pieces (in 3 or 4 rows) were placed in culture flasks of a minimum size of 25 cm³.
iv. All excess medium was removed from the culture flasks.
v. Each explant was gently surrounded with a drop of FCS.
vi. The explants bathed in serum were incubated at 37°C for 24 hours.
vii. 5ml whole medium (Hams F10 + 20% FCS) was gently added to each flask - the liquid flow was directed to the top of the flask so as to avoid dislodging the explants
viii. The flasks were gassed with 5% CO2 in air, again directing the gas flow towards the top of the flasks.
ix. The cultures were left undisturbed for a further 7 days before they were fed i.e. spent medium was discarded and then replenished with fresh whole medium.

2.3.3.3 Feeding

As for amniotic fluid cell cultures - see section 2.2.3.3.

2.3.3.4 Subculturing

As for amniotic fluid cell cultures - see section 2.2.3.4.

2.3.3.5 Metaphase arrest

As for amniotic fluid cell cultures - see section 2.2.3.5.

2.3.3.6 Harvesting

As for amniotic fluid cell cultures - see section 2.2.3.6.

2.2.3.7 Slide preparation

As for peripheral blood lymphocytes and amniotic fluid cells, but 1:1 fixative was used as opposed to 4:1 fixative - see section
2.1.4.2.4.3.

2.3.3.8 Drug treatment

As for amniotic fluid cell cultures - see section 2.2.3.8.

2.3.4 Discussion

Skin biopsy protocols differ from laboratory to laboratory. The method used in this study is a modification of a number of different techniques (see Paul, 1975). General principles are however the same and involve:

i. inclusion of dermis so that fibroblast cells may grow out from the explant to form a primary culture; by subculturing, long-term (aerial) cultures are obtained (it must however be remembered that normal diploid cells have a finite life-span)

ii. a suitable method to anchor the primary explants in the culture vessel

iii. appropriate growth conditions for cell culture; and

iv. a 3-6 week interval before chromosome studies can be undertaken.

Skin biopsy may cause discomfort to the patient and by comparison to short-term peripheral blood cultures, the techniques involved are laborious and time consuming. Thus, long-term fibroblast cultures are only undertaken when indicated. It was thus decided that the present study did not warrant fibroblast cultures on all the FA homozygotes and heterozygotes studied.

The tissue cultures on the father and proband were established in the same laboratory where the FA 'at-risk' amniocentesis was performed and initiated. The method was very similar if not identical to that described here. Subcultures were subsequently transported by hand to our laboratory. The foetal skin was conveyed directly to our laboratory following delivery of the foetus.
2.4 LONG-TERM STORAGE OF CELL CULTURES

Abnormal or experimental amniotic and fibroblast cell-lines can be preserved for future usage in a frozen state for many years without significant loss of viability. Freezing cells without causing cell death is possible due to the discoveries that glycerol and dimethyl sulphoxide (DMSO) protect cells from ice crystallization during freezing (Lawoe and Hack, 1980).

2.4.1 Cell-lines stored

During the course of the present study 2 FA homozygous and a number of control cell-lines (normal for the FA gene) were frozen.

2.4.2 Materials used in the storage process

1. Whole medium – antibiotic (penicillin and streptomycin) supplemented Hams P10 medium + 20% PCS

2. Dimethyl sulphoxide (DMSO) – autoclaved

3. Sterile 2ml glass ampoules with flame-sealable necks

4. Gas torch sealer

5. 4°C refrigerator

6. -65°C mechanical freezer

7. -196°C liquid nitrogen freezer

2.4.3 Preparation of cells for freezing

The method used is a modification of that of Shannon and Macy (1973). Sterile techniques were employed throughout (see section 2.2.3.2).

i. Cultures which were not 'overly' confluent and would normally be ready for termination or subculturing, were chosen for preservation.

ii. A sterile silicon scraper was used to gently scrape cells off the flask surface in previously marked growth areas and the flask was gently shaken.
iii. The suspended cells were carefully transferred from the original flask into a sterile centrifuge tube.

iv. The cell suspension was then centrifuged at 1000 rpm for 5 minutes.

v. The supernatant was decanted.

vi. The cell pellet was gently resuspended in approximately 1ml freezing mixture consisting of 90% complete medium + 10% sterile DMSO (the amount of freezing mixture is directly proportional to the size of the cell pellet).

vii. + 1 ml cell suspension was added to each glass ampoule by means of a sterile Pasteur pipette plus rubber bulb. (The ampoules were pre-labelled with the date, culture number and name of the patient.)

viii. Immediately thereafter, the ampoule was flame-sealed (prompt sealing of ampoules prevents pH change).

2.4.4 Freezing cells

i. Sealed ampoules were kept at 4°C for several hours.

ii. The ampoules were then kept at -65°C for a further 8-10 hours.

iii. The ampoules were thereafter directly transferred to a liquid nitrogen freezer, where they can be stored indefinitely.

2.4.5 Thawing

i. It is important to know exactly which ampoule is required and its location in the liquid nitrogen freezer.

ii. The ampoule is removed from the freezer and immediately placed in a 37°C water bath: thawing occurs rapidly - within 1 minute.

iii. The thawed cells are aseptically transferred to a culture flask containing at least 5 times the amount of complete medium as the freezing mixture.

iv. After 24 hours, the spent medium is gently poured off and replaced with 5ml whole medium.

v. The culture is gently gassed with 5% CO₂ in air.
vi. Subsequent procedures i.e. feeding/subculturing/harvesting are as for amniotic fluid or fibroblast cell cultures. (Frozen amniotic and fibroblast cells from the PA homozygote have not as yet been thawed, but other frozen cells have been successfully thawed and cultures have been established, using the above-described method.)

2.4.6 Discussion

With the advent of an increasing array of new and exciting developments, particularly in the fields of molecular and biochemical genetics, it is essential that all unusual cell-lines should be preserved by freezing. These stored cell-lines may then be used either for further investigations or as control cultures when other similarly 'at-risk' individuals or amniotic fluids from 'at-risk' foetuses are investigated.

Caution must be excercised in ensuring that all ampoules containing cells to be frozen, are completely sealed - if a small leak is present, liquid nitrogen can enter the frozen ampoule, causing it to explode when thawed, due to the rapid expansion of the liquid nitrogen.

2.5 TRYPsin - GIPMSA BANDING

2.5.1 Method

The method employed was a modification of that developed by Priest et al. (1975):

1. 1-2 day old slides were gently agitated in 50ml Phosphate Buffered Saline (PBS - pH7.3) plus 0.05% Trypsin. The PBS + trypsin solution was initially prewarmed to 37°C. The trypsinization time depended on the age of the slide. (Cold PBS + trypsin was used for amniotic cell and fibroblast chromosomes).
ii. After trypsin treatment, the slides were rinsed in a solution made up of 47.5ml PBS + 2.5ml foetal calf serum (at room T°) for approximately 5 seconds.

iii. The slides were then rinsed in 50ml PBS (at room T°) for approximately 5 seconds.

iv. The rinsed slides were immediately stained in 4% Giemsa in phosphate buffer (pH 6.8) for approximately 4 minutes.

v. Following staining, the slides were washed gently in tap water and carefully blotted dry with facial tissues.

2.5.2 Discussion

Ageing of slides prior to banding, either by standing the freshly made slides at room temperature for about 48 hours or by placing the slides in a 37°C incubator overnight, was found to decrease 'fuzzy' banding and to enhance the contrast of the bands. Fresh slides should thus be avoided (the reason why ageing improves banding quality is unknown).

In order to ascertain the presence/absence of stable structural and/or numerical chromosomal abnormalities (either constitutional or acquired by evolution of a stable abnormal clone from an initially unstable cell), one slide from each case was routinely trypsin-Giemsa banded. Thereafter, 10 metaphases per patient were analyzed, 5 metaphases photographed and 2 of the latter metaphases were karyotyped. (Photographic techniques were the same as described in section 2.6.3 for non-banded preparations).

2.6 QUANTITATION OF CHROMOSOME DAMAGE

2.6.1 Chromosome staining

1. Slides (freshly made or aged) were placed in a Coplin jar containing 10% Giemsa in Phosphate buffer, pH 6.8 for 5 minutes.
2.6.2 Scoring technique

The cells selected in good preparations tend to be of better quality than those in interior preparations. To avoid this type of bias, a standard for cell selection must be set and adhered to, whether the slide quality is good or not.

Consecutive metaphases judged to be complete under high-power magnification, with well-defined chromosome morphology, were selected for study. Chromosome breakage was scored 'blindly' from coded slides so as to avoid further 'scorer' bias (Schwartz et al., 1983).

Breakage analysis was initially performed, where possible, on 50 Giemsa stained metaphases per individual, for every drug concentration studied. After seeking statistical advice from the Institute for Biostatistics (Transvaal Branch) as to the sample size required for statistically valid conclusions to be drawn, it was decided to screen 30 metaphases for each experimental concentration and medium in a particular case - due to the often poor mitotic index in FA homozygotes, 30 metaphases per case, is more uniformly obtainable in most cases. Each metaphase with a complete appearance, under high power magnification (i.e. 1000 times) was scored for number and types of structural abnormalities (see Appendix B for scoring schedule).

The nomenclature and criteria applied for scoring breakage was based on the 'International system for human cytogenetic nomenclature' (ISCN-1978). More specifically, the following definitions were applied:

I. CHROMATID ABERRATIONS - involve only 1 chromatid of a chromosome at a given locus.
   i. Chromatid gap - a distinct achromatic lesion, less than the width of the chromatid in which it appears, in a single
chromatid, with minimal malalignment of the chromatid. (Fig. 2.1-a)

ii. Chromatid break - a distinct achromatic lesion, greater than the width of the chromatid in which it appears, in a single chromatid, either with or without the distal fragment of the chromatid being displaced from the axis of the proximal arm. (Fig. 2.1-b)

iii. Chromatid deletion - a loss of the terminal portion of one chromatid. (Fig. 2.1-c)

iv. Chromatid exchange - the result of two or more chromatid breaks and the subsequent rearrangement of the chromatin material. Exchanges may be between chromosomes of different chromosomes (interchanges) or between chromatids of the same chromosome (intrachanges). Interchanges may be classified as:
   a) triradials - interchanges with 3 arms (Fig. 2.1-d)
   b) quadriradials - interchanges with 4 arms (Fig. 2.1-e)
   c) complex - interchanges with more than 4 arms.

II. ISOCHROMATID (CHROMOSOME) ABERRATIONS - involve both chromatids of a single chromosome at the same locus.

i. Isochromatid gap - defined identically to chromatid gap, but involving an achromatic lesion less than a chromatid in width at the same locus in both chromatids of a single chromosome. (The term 'isochromatid gap' is synonymous with 'chromosome gap' and 'isolocus gap'). (Fig. 2.2-a)

ii. Isochromatid break - identically defined to 'chromatid break', except that both chromatids are broken at the same locus of a single chromosome. (The terms 'chromosome break' and 'isolocus break' are synonymous with 'isochromatid break') (Fig. 2.2-b)

iii. Acentric fragment - paired chromatids that lie parallel to one another, but contain no centromere (Fig. 2.2-c) and are not in close association with an abnormal monocentric chromosome. (Fig. 2.2-d)

iv. Minute - an acentric fragment smaller than the width of a single chromatid.

v. Chromosome exchange - the result of two or more isochochromatid lesions and the subsequent rejoining of both chromatids in a
CHROMATID ABERRATIONS

(a) (b) (c) (d) (e)

GAP BREAK DELETION TRIRADIAL QUADRIRADIAL

Figure 2.1 Chromatid aberrations - diagramatic representation of (a) chromatid gap; (b) chromatid break; (c) chromatid deletion; (d) triradial figure; and (e) quadriradial figure.
Figure 2.2  Isochromatid aberrations - diagramatic representation of
(a) isochromatid gap; (b) isochromatid break; (c) acentric fragment; (d) isochromatid deletion; (e) ring chromosome; (f) reciprocal translocation; (g) dicentric chromosome.
Figure 2.3 Endoreduplication - (A) 10% Giemsa stained, and (B) trypsin-Giemsa banded.
new configuration on the same chromosome, producing for example a ring chromosome, (Fig. 2.2-e) or reciprocal translocation, (Fig. 2.2-f) or a dicentric chromosome, (Fig. 2.2-g)

vi. Pulverization - multiple chromatid and/or isochromatid gaps and/or breaks in such a high frequency that they cannot be accurately enumerated.

vii. Multibreak cells (partial pulverization) - cells containing more than 30 breaks and/or gaps.

viii. Endoreduplication - chromosome doubling occurs without complete separation of the chromatids of the paired bivalents, so that at metaphase, the cell consists of 46, four-stranded structures, (Fig. 2.3)

In this study, structural rearrangements, including chromatid exchange configurations, dicentrics, rings and obvious translocations were considered as 2 break events. Chromatid and isochromatid gaps, breaks and deletions were scored as single breaks. Pulverized or partially pulverized cells and endoreduplications were recorded as unstable cells, but were not included in the final breakage rates. The aberrations were totalled and recorded as:

i. breaks/cell - including chromatid and isochromatid gaps;

ii. breaks/cell - excluding chromatid and isochromatid gaps.

2.6.3 Photography

2.6.3.1 Methods

All spontaneous breaks and unusual drug-induced aberrations were photographed with an automatic 35mm camera attached to an SM-LUX Leitz microscope. The metaphases were photographed under 1000 x magnification (100 x oil immersion objective plus a 10 x photo-eyepiece in the attached adaptor tube). The following materials and settings were used:

i. Kodak Technical-Pan 135 black and white film

ii. ASA 100 and Cal 2 camera settings
iii. a green photographic filter (510nm)

Following photography, the photographic negatives were developed in 'Promicol' ultra-fine grain developer (Maybaker) for 8-12 minutes and then fixed in Ilford Hypam rapid fixer for 5-10 minutes. The negatives were thereafter washed in tap water for 5-10 minutes and air-dried. These negatives were enlarged and printed on Ilford bromide paper (16.5 x 21.6cm) and developed in Ilford PQ universal developer for 1-3 minutes. All prints were fixed in Ilford Hypam rapid fixer for 5-10 minutes and then washed in running tap water for 30-45 minutes. Finally, the photographic prints were heat-dried.

2.6.3.2 Comments

The usual goal of photomicrography is to resolve the details of chromosomes. It is therefore necessary to employ great care in all aspects of microscopy and photography so as to enable the production of 16.5 x 21.6cm enlargements of metaphases suitable for karyotyping. However, the resolution obtained in photographic enlargements is never as good as that seen under direct vision. Furthermore, depending on the photographic development and printing, photographs may mask or enhance certain chromosomal aberrations such as gaps versus breaks and dicentrics which photographically, may appear as twisted chromosomes. For these reasons, all chromosome breakage analyses were done under direct microscopic vision so as to avoid possible bias created by photography.

2.6.4 Discussion

It is generally accepted that isochromatid aberrations usually originate from damage during the pre-DNA synthetic period i.e. the G1 and early S stages of the cell cycle, and conversely, chromatid aberrations are the result of damage in the post-DNA synthetic period i.e. damage during late S, G2 or M (Cohen et al., 1967; Buckton and Evans, 1973). Thus, isochromatid breaks in first division metaphases will appear as chromatid breaks in the following second division
metaphases, whereas chromatid breaks in first division metaphases will result in isochromatid breaks in second division metaphases. Since the cultures studied did not consist of synchronous populations of cells, both chromatid and isochromatid aberrations may be combined for a valid breakage analysis.

The morphology of some chromosome-type aberrations may result in scoring discrepancies. Dicentrics, for example, are often difficult to identify in unbanded chromosomes, as isochromatid constrictions can morphologically imitate centromeric constrictions. Furthermore, an unbanded chromosome with a 'twist' in it may also resemble a dicentric chromosome. Small ring chromosomes are almost impossible to distinguish from small acentric fragments. In addition, the scoring of chromatid/isochromatid gaps/breaks is dependent on relative criteria such as the size of the achromatic lesions involved. Thus, since all analyses are subjective to a certain extent, direct comparisons of published data are unacceptable. It is therefore imperative to maintain uniformity of scoring within a single study by the same scorer analyzing all metaphases of all individuals.

Buckton and Pike (1964) showed that unstable chromosomal abnormalities are more frequent in 48-hour cultures of PHA-stimulated lymphocytes from irradiated adult than in 72-hour cultures, presumably due to some loss of this type of aberration after the first in vitro cell division. Since the proportion of second division metaphases increases after 48 hours, the latter 48-hour period is generally the optimal culture time recommended for studying chromosome breakage in PHA-stimulated cultures, so as to obviate the decline of unstable aberrations and derived changes with subsequent division. However, Hatcher and Hook (1976) noted some second division metaphases in 48-hour cord blood lymphocyte cultures. Mutchinick et al. (1981) confirmed previous reports by others that more than 20% second division metaphases are usually found in standard 48-hour PHA-stimulated lymphocyte cultures, thus introducing considerable bias in the study of so-called first division metaphases.
In the case of FA, there is some debate as to the justification for using 72-hour PHA-stimulated cultures to study chromosome aberrations. Theoretically, if a deficient DNA repair mechanism is involved in the production of the spontaneous chromosome instability seen in FA, (see section 1.8), one would expect a similar frequency of chromosome aberrations in first and/or second division mitoses. However, Mutchinick et al. (1981) found in 3 sibs with FA that the number of structural aberrations, the number of cells with aberrations and the rate of aberrations per cell was markedly higher in first division metaphases as opposed to second division metaphases. Furthermore, a homogeneous distribution of aberrations in different tissues would be expected, which certainly does not appear to be the case in in vitro studies (see section 1.6).

Although these findings tend to indicate that 48-hour cultures may be more informative in breakage studies, Sasaki (1975) has noted that FA cells pass more slowly than normal cells through the S and G2 phases of the cell cycle. Consequently, the majority of FA cells collected at 70 to 74 hours were found still to be in first mitosis.

From a practical viewpoint, 66-hour PHA-stimulated cultures were studied in order to facilitate some measure of comparison with published data by Auerbach et al. (1981) and data obtained by Marx et al. (1983). The present data are also compared to the findings of Cohen et al. (1982) who employed a 96-hour culture period – refer to Tables 3.11 and 3.13). Furthermore, FA lymphocytes often have a low mitotic index (see section 1.7.1) resulting in too few metaphases after 48 hours. Although this problem can be overcome to a certain extent by increasing the colchicine exposure time, longer exposure tends to result in greater contraction of the chromosomes which may then mask the more subtle abnormalities such as chromatid gaps.

With respect to chromosome staining, non-banded chromosomes are usually studied as banding may also mask the appearance of certain aberrations, such as gaps and breaks, particularly in negatively stained G- or R-bands.

One constant source of confusion in comparative chromosome
instability studies is the inclusion/exclusion of chromatid and imochromatid gaps in the reported results. Revell (1959) appears to have been the first to score gaps and breaks separately because he suspected that gaps 'were not true discontinuities but short unstained regions in the chromatids'. For several years, many cytogeneticists have considered gaps to be insignificant artefacts, and although noting them, have not included them in their final aberration scores.

Brogger (1982) has drawn attention to the fact that two morphologically indistinguishable types of gaps occur: the 'clastogenic' (DNA damage) type and the 'turbogenic' (no DNA damage) type. In addition, many genotoxins have been found to induce dose-dependent gaps (Ibid). In preparations sequentially studied by light and electron microscopy, aberrations classified as gaps have appeared as either clean breaks, or attenuations with chromatin of varying thickness extending across the region (Brinkley and Hittleman, 1975). Gaps and breaks thus cannot be unambiguously distinguished using conventional Giemsa staining and light microscopy. Brogger (1982), like Hsu (1979a), therefore has recommended that gaps and breaks should be scored in the same class of aberrations. This recommendation was followed in the present study, but since most previous studies do not include gaps, the results were also tabulated and statistically analyzed excluding gaps.

2.7 STATISTICAL METHODS

2.7.1 Statistical analysis of peripheral blood lymphocyte chromosome breakage results

The writer wishes to acknowledge that all statistical analyses were kindly performed by the staff of the Institute for Biostatistics (Transvaal Branch) of the South African Medical Research Council.

Three stress variables were considered to have sufficiently large
sample sizes for valid statistical conclusions to be drawn. (Refer to Table 3.4.) The 3 categories included in the statistical analysis were:  

i. F10 medium: Spontaneous breakage (no. of cases = 113; no. of cells = 4231)

ii. F10 medium + DEB [0.1 μg/ml] (no. of cases = 53; no. of cells = 2338)

iii. F10 medium + MMC [0.01 μg/ml] (no. of cases = 36; no. of cells = 1055)

The criteria on which all statistical analyses were based was (a) the number of chromosome breaks per cell and (b) the number of unstable cells. Prior to the application of relevant statistical tests, every individual breakage value for all 3 variables was corrected by subtraction of its matching normal control value, so as to exclude the effects of external (i.e. technical/laboratory) factors. Thus, the remaining difference may be considered to be due primarily to the effect of the specific stress agent, or may reflect inherent chromosomal fragility. The correction was not, however, applied when control breakage results were compared to other phenotypic/genotypic results. Alternatively, levels of spontaneous breakage could also have been corrected for, when assessing induced breakage levels - the spontaneous breakage rate would be subtracted out, thereby giving only the damage induced by the specific treatment involved. This approach was not employed since absolute rather than relative breakage values were of interest in the present study.

Non-parametric tests were used for comparison purposes since the samples were reasonably small and the assumption of normality could not be made on the values obtained (Siegel, 1956; Conover, 1971).

'Within classes' analysis of breakage data

For every specific class eg. homozygotes, the Wilcoxon signed ranks test was applied to assess whether induced breakage rates differed significantly from spontaneous breakage rates. This test was not employed for a comparison between DEB and MMC stressing since these clastogens were not used in the same patients. (The MMC studies were commenced at the time that DEB toxicity was being experienced - see section 4.6).
An analysis was performed using breakage rates including gaps (specified in all Tables by '+') and a separate similar analysis was applied to breakage rates excluding gaps (specified by '-'). These separate tests give an indication as to whether the inclusion of gaps in calculating breakage rates has a significant effect on overall results.

'Between classes' analysis of breakage data

For the 3 culture variables under consideration (i.e. spontaneous, DEB- and MMC-induced breakage), all genotypic/phenotypic classes were compared in a pairwise manner by means of a non-parametric test viz Mann-Whitney U-Test.

Once again, as for the Wilcoxon test, before the application of these statistical tests, the 'matched' (i.e. simultaneously and identically cultured) control breakage rates were deducted from those of the subjects under study in an attempt to exclude the possible clastogenic effect of external factors. However, since the homozygote, heterozygote, sib and features classes also had to be compared to the controls, a separate analysis was performed in which the control breakage rates were not deducted from the 'experimental' rates.

Throughout the study, all parents were regarded as 'obligatory heterozygotes'. As the possibility occasionally exists that the so-called father is in fact not the biological father, 2 separate statistical analyses were done - one including both the fathers and the mothers and one including mothers only. (Symbolized in Tables 3.22 to 3.24 by HETM).

Once again, all statistical comparisons were done twice - including gaps and excluding gaps in the calculation of breakage rates (symbolised by '+' and '-' respectively in Tables 3.22 to 3.24).

According to Bonferroni (Neter and Wasserman, 1974), to correct for multiple pair-wise comparisons, the test level of each individual test has to be adapted so that the overall level remains $\alpha$ (in this
case 0.05). Therefore, the significance levels among genotype pairs was taken as $0.05/k$, where $k$ equals the number of comparisons. The level for comparisons between the homozygote, heterozygote, sib and features groups is thus $0.05/6 = 0.0083$ (6 comparisons viz. (1) homozygotes vs heterozygotes; (2) homozygotes vs sibs; (3) homozygotes vs features; (4) heterozygotes vs sibs; (5) heterozygotes vs features and (6) sibs vs features). For significance at the 1% level (i.e. 'highly significant') the equation $0.01/k$ is applied. When the various classes are compared to the control group, the test level for each individual comparison becomes $0.05/4 = 0.0125$ to be significant (4 comparisons viz (1) controls vs homozygotes; (2) controls vs heterozygotes; (3) controls vs sibs and (4) controls vs features). It should be noted that a separate value is required for the latter-mentioned comparisons because slightly different criteria were applied -- as previously discussed, control values were not deducted from 'experimental' values for these comparisons.

2.7.2 Statistical analysis of peripheral blood cellular instability rates

As with the chromosome breakage results (see section 3.4.2), the cellular instability data (see section 3.4.3) were also subjected to statistical analysis. All genotypic/phenotypic classes were compared in a pairwise manner by means of the U-test of Mann-Whitney for the 3 culture variables primarily under consideration (i.e. spontaneous, DEB [0.1 µg/ml]- and MMC [0.01 µg/ml]-induced instability).

Any cell with one or more chromosomal aberrations (i.e. both chromatid - and chromosome-type aberrations) was considered an 'unstable cell'. Gaps were included in the calculations of cellular instability rates since statistical analyses of aberration rates showed no differences when gaps were excluded from the calculation of breakage rates. (See section 4.4.2.4) Similarly the heterozygote class included both mothers and fathers as previous statistical analyses revealed no differences when the fathers were excluded from the heterozygote group in the calculation of breakage rates. (See
For the purposes of the present study, 'breakage-type' aberrations were regarded as chromatic and isochromatid gaps, breaks, fragments and deletions. Rings, dicentrics, triradials, quadriradials and complex exchanges constituted the so-called 'reunion-type' aberrations. Since the cumulative results (i.e. breakage- and reunion-type aberrations) did not, apart from the homozygotes, show clear-cut differences between the different genotypic/phenotypic classes (see section 4.4.2.4), it was envisaged that the separation of the different types of aberrations may ultimately prove useful in the delineation of, for example, putative heterozygotes and normal individuals. Preliminary observations on these 'qualitative' results are discussed in section 4.3.3. The data (divided into 'breakage' and 'reunion' aberrations) were subjected to the U-test of Mann-Whitney.

Unfortunately, the 'reunion figure' results could not be accurately statistically assessed because of the extremely small numbers involved.
3. RESULTS

The data that follow were collected over a period of approximately 2 years. Prior to evaluating these results, the reader should refer to section 2.1.1 to ascertain the criteria employed in genotypically/phenotypically classifying the subjects under study. These individuals were grouped into 5 classes, viz.:

i. FA homozygotes (FA+/FA+)
ii. FA heterozygotes (FA+/FA-)
iii. Normal controls (FA-/FA-)
iv. Sibs
v. Features of FA

3.1 PERIPHERAL BLOOD LYMPHOCYTE STUDIES

3.1.1 Sample size

A total of 115 individuals were studied. This figure was made up of:
24 homozygotes
28 heterozygotes
42 controls
6 sibs
15 features of FA

3.1.2 Ethnic group

The 115 individuals studied were members of 4 ethnic groups viz. Asian Indian, Black, 'Coloured' and White. ['Coloured' is the term used in South Africa to refer to an individual of mixed parentage, with the genetic constitution probably representing Khoisan (Bushman and Hottentot), Negroid and Caucasoid strains.]
TABLE 3.1 Ethnic groups of individuals studied

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3.1.3 Prevalence of homozygous and heterozygous Fanconi's anemia

Of South Africa's 4.5 million Whites, about 57% are Afrikaans-speaking descendants of seventeenth century immigrants from Holland, France and Germany; 39% are English-speaking and are predominantly of British descent, and the remaining 4% consist of a variety of peoples such as Portuguese, Italians and Greeks. A total of 33 White FA homozygotes (excluding patients F.P. and M.G. who may not be FA homozygotes - see sections 4.3.2.1.1.2 and 4.3.2.1.1.3) have over the past approximately 15 years been investigated by the writer's laboratory. A striking feature of these patients was that the great majority appear to be of Afrikaner origin viz. 27 out of 33 (82%). Three patients were referred from the northern Cape Province and one from South West Africa (Namibia). All other patients lived within about 200 km of Johannesburg, most of them in the southern half of the Transvaal Province (Fig. 3.1). The total White population in this area in 1980 was 1 514 340, of whom 684 330 were 24 years of age or younger (South African Statistics, 1982). Approximately 390 068 (57%) of this group would be Afrikaans-speaking (Ibid). In 1980, 15 FA homozygotes (from 12 families) were alive, under the age of 24 years and Afrikaans-speaking. Therefore, the point prevalence of homozygotes in this Afrikaner population was 0.000038 or 1 in 26 005. This figure should be regarded as being a conservative estimate, since all living homozygotes in this Afrikaner population were not systematically ascertained, and it is probable that several other
Figure 3.1  Distribution of 12 White, Afrikaans-speaking PA families with homozygous offspring in the Transvaal Province of South Africa.
Patients may have been diagnosed elsewhere in the area.

Knowing the approximate prevalence of homozygotes in this at-risk group, the prevalence of heterozygotes may be estimated from the Hardy-Weinberg equation (Emery, 1976). If the frequency of the FA allele is q, its frequency is given as \( q = \sqrt{p} \) square root of the homozygote frequency - i.e. the square root of 0.000030 = 0.0062. The frequency of the normal allele, p, is 1 - q = 0.9938. The heterozygote or carrier frequency is given by 2pq, which is 12 in 1000 or 1 in 83.

Similarly, a minimal estimate of the frequency of the FA gene may be derived from the number of FA cases born in the target area from 1955 to 1980. Taking only the patients known to the writer, 23 definite cases have been born in this area, among a total of 890 677 live-births. It may be estimated that 507 686 (57%) of these live-births were to Afrikaner couples. Therefore, the minimal prevalence in this group is 23 in 507 686 or 1 in 22 073. Once again, applying the Hardy-Weinberg law, the expected heterozygote frequency in this at-risk group is 13 in 1000 or 1 in 77.

3.1.4 Sex

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</table>
3.1.5 Age

Of the 115 individuals included in the present study, the ages of 99 of these were on record - it was not possible to ascertain the ages of 9 adult heterozygotes and 7 controls.

<table>
<thead>
<tr>
<th>CLASS</th>
<th>n</th>
<th>MEAN AGE</th>
<th>MIN</th>
<th>MAX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygotes</td>
<td>24</td>
<td>7.43 years</td>
<td>2 months</td>
<td>20 years</td>
</tr>
<tr>
<td>Heterozygotes</td>
<td>26</td>
<td>34.84 years</td>
<td>25 years</td>
<td>51 years</td>
</tr>
<tr>
<td>Controls</td>
<td>42</td>
<td>21.16 years</td>
<td>6 months</td>
<td>65 years</td>
</tr>
<tr>
<td>Sibs</td>
<td>6</td>
<td>12.33 years</td>
<td>2 years</td>
<td>20 years</td>
</tr>
<tr>
<td>Features</td>
<td>15</td>
<td>9.06 years</td>
<td>10 days</td>
<td>20 years</td>
</tr>
</tbody>
</table>

3.2 PERIPHERAL BLOOD LYMPHOCYTE CHROMOSOME ANALYSIS

At least 15 trypsin-Giemsa banded metaphases per case were screened for the presence of spontaneous stable, clonal chromosomal abnormalities. No such clones were noted in any of the cases included in the present study.

3.3 PERIPHERAL BLOOD LYMPHOCYTE CHROMOSOME BREAKAGE STUDIES

3.3.1 Number of cells screened for chromosome breakage under different culture conditions

A summary of the number of cases and the number of cells analyzed under varying culture conditions is shown in Table 3.4. The data in this table are tabulated in more detail according to the phenotypic/genotypic classes (as defined in section 2.1.1) in Table 3.5.
### TABLE 3.4 Summary of number of cases and number of cells analyzed under different culture conditions.

<table>
<thead>
<tr>
<th>CULTURE CONDITION</th>
<th>NO. OF CELLS AND NO. OF CELLS/CASE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>CELLS</td>
</tr>
</tbody>
</table>

|                   | 66  | 43  | 44  | 0    | 113   | 4231 |
| F10; spontaneous  | 4   | 0   | 0   | 0    | 4     | 120  |
| F10+DEB [0.01 μg/ml] | 13  | 38  | 1b  | 1c   | 53    | 2338 |
| F10+DEB [0.10 μg/ml] | 0   | 8   | 0   | 0    | 8     | 400  |
| F10+DEB [0.20 μg/ml] | 5   | 4   | 0   | 0    | 9     | 350  |
| F10+DEB [1.00 μg/ml] | 5   | 0   | 0   | 0    | 5     | 150  |
| F10+MMC [0.01 μg/ml] | 31  | 2   | 3d  | 0    | 36    | 1055 |
| F10+MMC [0.05 μg/ml] | 6   | 0   | 2e  | 0    | 8     | 218  |
| M150; Spontaneous  | 16  | 0   | 0   | 0    | 16    | 480  |
| M150+MMC [0.01 μg/ml] | 5   | 0   | 3f  | 0    | 8     | 204  |

**TOTAL** 9546

- a = 4 cases: 29, 29, 27 and 16 cells/case
- b = 1 case: 12 cells
- c = 1 case: 36 cells
- d = 3 cases: 18, 15 and 2 cells/case
- e = 2 cases: 29 and 9 cells/case
- f = 3 cases: 26, 22 and 6 cells/case

*The total number of cases included in this study was 115; all except 2 with culture failure were screened for spontaneous chromosome breakage and many of these were simultaneously analyzed under the stipulated culture conditions.*
TABLE 3.5 Number of cases (genotypically/phenotypically classified) and number of cells analyzed under different culture conditions

<table>
<thead>
<tr>
<th>CULTURE CONDITION AND CLASS</th>
<th>NO. OF CASES AND NO. OF CELLS/CASE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>CELLS</td>
</tr>
<tr>
<td><strong>F10: Spontaneous</strong></td>
<td></td>
</tr>
<tr>
<td>HOM.</td>
<td>12</td>
</tr>
<tr>
<td>HET.</td>
<td>18</td>
</tr>
<tr>
<td>CON.</td>
<td>25</td>
</tr>
<tr>
<td>SIB.</td>
<td>4</td>
</tr>
<tr>
<td>FEAT.</td>
<td>7</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>66</td>
</tr>
<tr>
<td><strong>F10+DEB [0.01 μg/ml]</strong></td>
<td></td>
</tr>
<tr>
<td>HOM.</td>
<td>0</td>
</tr>
<tr>
<td>HET.</td>
<td>3</td>
</tr>
<tr>
<td>CON.</td>
<td>1</td>
</tr>
<tr>
<td>SIB.</td>
<td>0</td>
</tr>
<tr>
<td>FEAT.</td>
<td>0</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>4</td>
</tr>
<tr>
<td><strong>F10+DEB [0.1 μg/ml]</strong></td>
<td></td>
</tr>
<tr>
<td>HOM.</td>
<td>4</td>
</tr>
<tr>
<td>HET.</td>
<td>4</td>
</tr>
<tr>
<td>CON.</td>
<td>4</td>
</tr>
<tr>
<td>SIB.</td>
<td>1</td>
</tr>
<tr>
<td>FEAT.</td>
<td>0</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>13</td>
</tr>
</tbody>
</table>

Continued
<table>
<thead>
<tr>
<th>CULTURE CONDITION</th>
<th>NO. OF CELLS AND NO. OF CELLS/CASE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 50 &lt;30 31-49 TOTAL TOTAL</td>
</tr>
<tr>
<td></td>
<td>CELLS CELLS CELLS CELLS CASES CELLS</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>F10+DEB [0.2 μg/ml]</td>
<td></td>
</tr>
<tr>
<td>HOM.</td>
<td>0 0 0 0 0 0</td>
</tr>
<tr>
<td>HET.</td>
<td>0 6 0 0 6 300</td>
</tr>
<tr>
<td>CON.</td>
<td>0 2 0 2 100</td>
</tr>
<tr>
<td>SIB.</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>FEAT.</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>0 8 0 0 8 400</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>F10+DEB [0.4 μg/ml]</td>
<td></td>
</tr>
<tr>
<td>HOM.</td>
<td>0 0 0 0 0 0</td>
</tr>
<tr>
<td>HET.</td>
<td>3 2 0 5 190</td>
</tr>
<tr>
<td>CON.</td>
<td>2 2 0 4 160</td>
</tr>
<tr>
<td>SIB.</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>FEAT.</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>5 4 0 9 350</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>F10+DEB [1.0 μg/ml]</td>
<td></td>
</tr>
<tr>
<td>HOM.</td>
<td>0 0 0 0 0 0</td>
</tr>
<tr>
<td>HET.</td>
<td>3 0 0 3 90</td>
</tr>
<tr>
<td>CON.</td>
<td>2 0 2 60</td>
</tr>
<tr>
<td>SIB.</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>FEAT.</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>5 0 0 5 150</td>
</tr>
</tbody>
</table>

Continued
<table>
<thead>
<tr>
<th>CULTURE CONDITION AND CLASS</th>
<th>NO. OF CELLS AND NO. OF CELLS/CASE</th>
<th>30</th>
<th>50</th>
<th>&lt;30</th>
<th>31-49</th>
<th>TOTAL</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CELLS</td>
<td>CELLS</td>
<td>CELLS</td>
<td>CELLS</td>
<td>CASES</td>
<td>CELLS</td>
<td></td>
</tr>
<tr>
<td><strong>F10+MMC [0.01 µg/ml]</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOM.</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>7</td>
<td>157</td>
<td></td>
</tr>
<tr>
<td>HET.</td>
<td>8</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>9</td>
<td>258</td>
<td></td>
</tr>
<tr>
<td>CON.</td>
<td>13</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>440</td>
<td></td>
</tr>
<tr>
<td>SIB.</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>FEAT.</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>31</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>36</td>
<td>1055</td>
<td></td>
</tr>
<tr>
<td><strong>F10+MMC [0.05 µg/ml]</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOM.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>HET.</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>CON.</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>SIB.</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>FEAT.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>6</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>8</td>
<td>218</td>
<td></td>
</tr>
<tr>
<td><strong>M150: Spontaneous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOM.</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>HET.</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>CON.</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>SIB.</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>FEAT.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>480</td>
<td></td>
</tr>
</tbody>
</table>

Continued
The tables that follow depict the mean breakage rates (breaks/cell) followed by the number of unstable cells, the standard deviations about these means and the associated breakage ranges i.e. minimum and maximum breakage values. The numbers of cases and cells screened under differing conditions are also specified as per Table 3.5. These data will first be listed according to the 5 genotypic/phenotypic classes (Tables 3.6 to 3.10) and then compared with each other and with 3 selected similar studies (Tables 3.11 and 3.12). The statistical analysis of these results will follow in section 3.4.

It should be borne in mind that any score (value) by itself is meaningless and can only be interpreted when compared with other scores. Furthermore, a measure of a central tendency, such as the mean, provides only a limited amount of information. To describe a distribution more fully, it is clear that additional information concerning the dispersion of scores about the mean is required i.e. the mean should be seen in conjunction with its standard deviation. Therefore Tables 3.6 to 3.10, include the mean values together with their standard deviations and breakage ranges.
### 3.3.2.1 Chromosome breakage results

#### 3.3.2.1.1 Homozygotes

**TABLE 3.6** Chromosome breakage results (breaks/cell) in homozygotes

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>NO. CASES</th>
<th>NO. CELLS</th>
<th>MIN.</th>
<th>MAX.</th>
<th>MEAN</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>*F10; Spontaneous  -</td>
<td>24</td>
<td>926</td>
<td>0.03</td>
<td>0.87</td>
<td>0.23</td>
<td>0.17</td>
</tr>
<tr>
<td>*F10; Spontaneous  +</td>
<td>24</td>
<td>926</td>
<td>0.08</td>
<td>1.17</td>
<td>0.30</td>
<td>0.21</td>
</tr>
<tr>
<td>M150; Spontaneous  -</td>
<td>3</td>
<td>90</td>
<td>0.17</td>
<td>0.20</td>
<td>0.18</td>
<td>0.02</td>
</tr>
<tr>
<td>M150; Spontaneous  +</td>
<td>3</td>
<td>90</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.00</td>
</tr>
<tr>
<td>*F10+DEB [0.10] -</td>
<td>13</td>
<td>518</td>
<td>0.04</td>
<td>6.73</td>
<td>2.58</td>
<td>1.85</td>
</tr>
<tr>
<td>*F10+DEB [0.10] +</td>
<td>13</td>
<td>518</td>
<td>0.06</td>
<td>8.13</td>
<td>3.41</td>
<td>2.30</td>
</tr>
<tr>
<td>*F10+MMC [0.01] -</td>
<td>7</td>
<td>157</td>
<td>0.07</td>
<td>4.63</td>
<td>1.85</td>
<td>1.85</td>
</tr>
<tr>
<td>*F10+MMC [0.01] +</td>
<td>7</td>
<td>157</td>
<td>0.17</td>
<td>4.67</td>
<td>2.06</td>
<td>1.97</td>
</tr>
<tr>
<td>M150+MMC [0.01] -</td>
<td>3</td>
<td>86</td>
<td>0.20</td>
<td>6.19</td>
<td>2.23</td>
<td>2.80</td>
</tr>
<tr>
<td>M150+MMC [0.01] +</td>
<td>3</td>
<td>86</td>
<td>0.27</td>
<td>6.38</td>
<td>2.36</td>
<td>2.85</td>
</tr>
</tbody>
</table>

Explanatory note on the terms used in Tables 3.6 to 3.10.

'F10' and 'M150' are the different culture media that were used. 'Spontaneous' denotes non-stressed cultures. The figures in parenthesis indicate the DEB and MMC concentrations in \( \mu g/ml \).

The plus and minus signs are indicative of the breakage data including gaps (+) and excluding gaps (-) respectively, but including all other aberrations (as listed in section 2.5.2). ** refers to the data that have undergone extensive statistical analysis, the results of which are available in section 3.4.

MIN. = Minimum
MAX. = Maximum
S.D. = Standard deviation.
In an attempt to unequivocally delineate heterozygotes from homozygotes and normal controls, response to a wide range of culture conditions was investigated. The results obtained using different concentrations of both DEB and MMC together with Hams F10 or M150 culture medium are presented in Table 3.7 and will be discussed in section 4.3.2.1.2.

### Table 3.7 Chromosome breakage results (breaks/cell) in heterozygotes

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>NO. CASES</th>
<th>NO. CELLS</th>
<th>MIN.</th>
<th>MAX.</th>
<th>MEAN</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>F10: Spontaneous -</td>
<td>27</td>
<td>946</td>
<td>0.00</td>
<td>0.08</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>F10: Spontaneous +</td>
<td>27</td>
<td>946</td>
<td>0.00</td>
<td>0.12</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>M150: Spontaneous -</td>
<td>6</td>
<td>180</td>
<td>0.00</td>
<td>0.07</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>M150: Spontaneous +</td>
<td>6</td>
<td>180</td>
<td>0.03</td>
<td>0.10</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td>F10+DEB [0.01] -</td>
<td>3</td>
<td>90</td>
<td>0.03</td>
<td>0.30</td>
<td>0.12</td>
<td>0.13</td>
</tr>
<tr>
<td>F10+DEB [0.01] +</td>
<td>3</td>
<td>90</td>
<td>0.03</td>
<td>0.30</td>
<td>0.12</td>
<td>0.13</td>
</tr>
<tr>
<td>F10+DEB [0.10] -</td>
<td>12</td>
<td>520</td>
<td>0.00</td>
<td>0.23</td>
<td>0.08</td>
<td>0.06</td>
</tr>
<tr>
<td>F10+DEB [0.10] +</td>
<td>12</td>
<td>520</td>
<td>0.04</td>
<td>0.23</td>
<td>0.12</td>
<td>0.06</td>
</tr>
<tr>
<td>F10+DEB [0.20] -</td>
<td>6</td>
<td>300</td>
<td>0.00</td>
<td>0.26</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>F10+DEB [0.20] +</td>
<td>6</td>
<td>300</td>
<td>0.00</td>
<td>0.36</td>
<td>0.15</td>
<td>0.12</td>
</tr>
<tr>
<td>F10+DEB [0.40] -</td>
<td>5</td>
<td>100</td>
<td>0.00</td>
<td>0.06</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>F10+DEB [0.40] +</td>
<td>5</td>
<td>100</td>
<td>0.00</td>
<td>0.12</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>F10+DEB [1.00] -</td>
<td>3</td>
<td>90</td>
<td>0.00</td>
<td>0.10</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>F10+DEB [1.00] +</td>
<td>3</td>
<td>90</td>
<td>0.00</td>
<td>0.10</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>F10+MMC [0.01] -</td>
<td>9</td>
<td>258</td>
<td>0.00</td>
<td>0.17</td>
<td>0.10</td>
<td>0.07</td>
</tr>
<tr>
<td>F10+MMC [0.01] +</td>
<td>9</td>
<td>258</td>
<td>0.00</td>
<td>0.28</td>
<td>0.13</td>
<td>0.10</td>
</tr>
<tr>
<td>M150+MMC [0.01] -</td>
<td>2</td>
<td>52</td>
<td>0.13</td>
<td>0.50</td>
<td>0.32</td>
<td>0.18</td>
</tr>
<tr>
<td>M150+MMC [0.01] +</td>
<td>2</td>
<td>52</td>
<td>0.23</td>
<td>0.64</td>
<td>0.44</td>
<td>0.20</td>
</tr>
<tr>
<td>F10+MMC [0.05] -</td>
<td>4</td>
<td>99</td>
<td>0.03</td>
<td>0.23</td>
<td>0.21</td>
<td>0.15</td>
</tr>
<tr>
<td>F10+MMC [0.05] +</td>
<td>4</td>
<td>99</td>
<td>0.03</td>
<td>0.27</td>
<td>0.23</td>
<td>0.15</td>
</tr>
</tbody>
</table>

(NB: Refer to explanatory note attached to Table 3.6.)
### 3.3.2.1.3 Controls

#### TABLE 3.8 Chromosome breakage results (breaks/cell) in normal controls

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>NO. CASES</th>
<th>NO. CELLS</th>
<th>MIN.</th>
<th>MAX.</th>
<th>MEAN</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>*F10: Spontaneous -</td>
<td>41</td>
<td>1550</td>
<td>0.00</td>
<td>0.07</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>*F10: Spontaneous +</td>
<td>41</td>
<td>1550</td>
<td>0.00</td>
<td>0.07</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>M150: Spontaneous -</td>
<td>5</td>
<td>150</td>
<td>0.00</td>
<td>0.10</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>M150: Spontaneous +</td>
<td>5</td>
<td>150</td>
<td>0.00</td>
<td>0.10</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>F10+DEB [0.01] -</td>
<td>1</td>
<td>30</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>F10+DEB [0.01] +</td>
<td>1</td>
<td>30</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>*F10+DEB [0.10] -</td>
<td>20</td>
<td>920</td>
<td>0.00</td>
<td>0.06</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>*F10+DEB [0.10] +</td>
<td>20</td>
<td>920</td>
<td>0.00</td>
<td>0.06</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>F10+DEB [0.20] -</td>
<td>2</td>
<td>100</td>
<td>0.00</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>F10+DEB [0.20] +</td>
<td>2</td>
<td>100</td>
<td>0.02</td>
<td>0.04</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>F10+DEB [0.40] -</td>
<td>4</td>
<td>160</td>
<td>0.00</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>F10+DEB [0.40] +</td>
<td>4</td>
<td>160</td>
<td>0.00</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>F10+DEB [1.00] -</td>
<td>2</td>
<td>60</td>
<td>0.00</td>
<td>0.03</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>F10+DEB [1.00] +</td>
<td>2</td>
<td>60</td>
<td>0.00</td>
<td>0.03</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>*F10+MMC [0.01] -</td>
<td>14</td>
<td>440</td>
<td>0.00</td>
<td>0.20</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>*F10+MMC [0.01] +</td>
<td>14</td>
<td>440</td>
<td>0.00</td>
<td>0.20</td>
<td>0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>M150+MMC [0.01] -</td>
<td>3</td>
<td>66</td>
<td>0.10</td>
<td>4.00</td>
<td>0.52</td>
<td>1.81</td>
</tr>
<tr>
<td>M150+MMC [0.01] +</td>
<td>3</td>
<td>66</td>
<td>0.23</td>
<td>4.17</td>
<td>0.59</td>
<td>1.85</td>
</tr>
<tr>
<td>F10+MMC [0.05] -</td>
<td>2</td>
<td>60</td>
<td>0.17</td>
<td>0.57</td>
<td>0.37</td>
<td>0.20</td>
</tr>
<tr>
<td>F10+MMC [0.05] +</td>
<td>2</td>
<td>60</td>
<td>0.17</td>
<td>0.57</td>
<td>0.37</td>
<td>0.20</td>
</tr>
</tbody>
</table>

(NB: Refer to explanatory note attached to Table 3.6.)
3.3.2.1.4 Sibs

As previously stated, sibs, since they constitute a somewhat 'unknown quantity', were specifically not requested for the purposes of the present study. Occasionally however, bloods were received and these were then processed.

TABLE 3.9 Chromosome breakage results (breaks/cell) in sibs

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>NO. CASES</th>
<th>NO. CELLS</th>
<th>MIN.</th>
<th>MAX.</th>
<th>MEAN</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>*F10: Spontaneous -</td>
<td>6</td>
<td>220</td>
<td>0.00</td>
<td>0.04</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>*F10: Spontaneous +</td>
<td>6</td>
<td>220</td>
<td>0.00</td>
<td>0.04</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>M150: Spontaneous -</td>
<td>2</td>
<td>60</td>
<td>0.03</td>
<td>0.07</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>M150: Spontaneous +</td>
<td>2</td>
<td>50</td>
<td>0.03</td>
<td>0.07</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>*F10+DEB [0.1] -</td>
<td>3</td>
<td>130</td>
<td>0.00</td>
<td>0.06</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>*F10+DEB [0.1] +</td>
<td>3</td>
<td>130</td>
<td>0.00</td>
<td>0.10</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>*F10+MMC [0.01] -</td>
<td>1</td>
<td>30</td>
<td>0.23</td>
<td>0.23</td>
<td>0.23</td>
<td>0.00</td>
</tr>
<tr>
<td>*F10+MMC [0.01] +</td>
<td>1</td>
<td>30</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.00</td>
</tr>
<tr>
<td>F10+MMC [0.05] -</td>
<td>2</td>
<td>59</td>
<td>0.10</td>
<td>0.83</td>
<td>0.46</td>
<td>0.36</td>
</tr>
<tr>
<td>F10+MMC [0.05] +</td>
<td>2</td>
<td>59</td>
<td>0.17</td>
<td>0.86</td>
<td>0.51</td>
<td>0.35</td>
</tr>
</tbody>
</table>

(NB: Refer to explanatory note attached to Table 3.6.)
3.3.2.1.5 Features

TABLE 3.10 Chromosome breakage results (breaks/cell) in individuals with features of Fanconi's anaemia

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>NO. CASES</th>
<th>NO. CELLS</th>
<th>MIN.</th>
<th>MAX.</th>
<th>MEAN</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>*F10: Spontaneous -</td>
<td>15</td>
<td>589</td>
<td>0.00</td>
<td>0.06</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>*F10: Spontaneous +</td>
<td>15</td>
<td>589</td>
<td>0.00</td>
<td>0.06</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>*F10+DEB [0.1] -</td>
<td>5</td>
<td>250</td>
<td>0.00</td>
<td>0.06</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>*F10+DEB [0.1] +</td>
<td>5</td>
<td>250</td>
<td>0.00</td>
<td>0.06</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>*F10+MMC [0.01] -</td>
<td>5</td>
<td>170</td>
<td>0.00</td>
<td>0.07</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>*F10+MMC [0.01] +</td>
<td>5</td>
<td>170</td>
<td>0.00</td>
<td>0.07</td>
<td>0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

(NB: Refer to explanatory note attached to Table 3.6)

3.3.2.2 Comparison of spontaneous and DEB-induced breakage results with three selected published studies.

In Tables 3.11 and 3.12, the results of the present study are compared with 3 similar, previously published studies, namely those of Auerbach et al. (1981), Cohen et al. (1962a) and Marx et al. (1983).
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>mean (range)</td>
<td>n</td>
<td>mean (range)</td>
</tr>
<tr>
<td>Hms.</td>
<td>24</td>
<td>0.23 (0.03-0.67)</td>
<td>5</td>
<td>0.25 (0.09-0.46)</td>
</tr>
<tr>
<td>Het.</td>
<td>27</td>
<td>0.03 (0.00-0.06)</td>
<td>8</td>
<td>0.02 (0.09-0.06)</td>
</tr>
<tr>
<td>Con.</td>
<td>41</td>
<td>0.01 (0.00-0.07)</td>
<td>15</td>
<td>0.02 (0.00-0.05)</td>
</tr>
<tr>
<td>Sib.</td>
<td>6</td>
<td>0.01 (0.00-0.02)</td>
<td>3</td>
<td>0.01 (0.00-0.02)</td>
</tr>
<tr>
<td>Pestr.</td>
<td>15</td>
<td>0.02 (0.00-0.08)</td>
<td>9</td>
<td>0.03 (0.00-0.07)</td>
</tr>
<tr>
<td>Total</td>
<td>113</td>
<td>40</td>
<td>44</td>
<td>39</td>
</tr>
</tbody>
</table>

** Only results excluding ‘gape’ are tabulated and all cultures were established in Hams F10 medium.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (mean (range))</td>
<td>n (mean (range))</td>
<td>n (mean (range))</td>
<td>n (mean (range))</td>
</tr>
<tr>
<td>Hem.</td>
<td>13 2.58 (0.04-6.73)</td>
<td>5 8.66 (2.10-19.70)</td>
<td>5 1.62 (0.03-6.76)</td>
<td>18 2.58 (2.19-2.90)</td>
</tr>
<tr>
<td>Het.</td>
<td>12 0.08 (0.00-0.23)</td>
<td>8 0.13 (0.06-0.24)</td>
<td>10 0.09 (0.00-0.24)</td>
<td>14 0.88 (0.73-1.05)</td>
</tr>
<tr>
<td>Con.</td>
<td>20 0.02 (0.00-0.06)</td>
<td>15 0.04 (0.00-0.10)</td>
<td>25 0.08 (0.03-0.14)</td>
<td>7 0.06 (0.05-0.07)</td>
</tr>
<tr>
<td>Sib.</td>
<td>3 0.04 (0.00-0.06)</td>
<td>3 0.21 (0.12-0.34)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Feat.</td>
<td>5 0.0  (0.00-0.04)</td>
<td>9 0.05 (0.00-0.12)</td>
<td>4 3.06 (0.13-5.44)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>53*</td>
<td>40</td>
<td>44</td>
<td>39</td>
</tr>
</tbody>
</table>

** Only results excluding 'gaps' are tabulated and all cultures were established in Ham's F10 medium.

* A further 40 cases were successfully studied with MNC.
3.3.2.3 Cellular instability

Only the three culture variables primarily studied with Hams F10 medium (see Table 3.4), will be considered in terms of cellular instability.

**TABLE 3.13 Number of cells screened and percentage unstable cells (%Cu)**

<table>
<thead>
<tr>
<th>Class</th>
<th>F10: Spontaneous</th>
<th>F10+DEB [0.1 μg/ml]</th>
<th>F10+MMC [0.01 μg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean (range)</td>
<td>mean (range)</td>
<td>mean (range)</td>
</tr>
<tr>
<td></td>
<td>n (range)</td>
<td>n (range)</td>
<td>n (range)</td>
</tr>
<tr>
<td>Hom.</td>
<td>926 22 (8-47)</td>
<td>218 79 (8-100)</td>
<td>157 61 (17-100)</td>
</tr>
<tr>
<td>Het.</td>
<td>946 4 (0-13)</td>
<td>520 10 (4-14)</td>
<td>258 11 (0-22)</td>
</tr>
<tr>
<td>Con.</td>
<td>1 550 2 (0-7)</td>
<td>920 2 (0-10)</td>
<td>440 7 (0-20)</td>
</tr>
<tr>
<td>Sib.</td>
<td>220 2 (0-4)</td>
<td>130 5 (0-8)</td>
<td>30 13 (13-13)</td>
</tr>
<tr>
<td>Peat.</td>
<td>589 3 (0-8)</td>
<td>250 3 (0-6)</td>
<td>170 3 (0-7)</td>
</tr>
</tbody>
</table>

Note: n = number of cells screened

'Gaps' included in all calculations of cellular instability rates.

3.3.3 Qualitative analysis of spontaneous and clastogen-induced chromosomal aberrations

In the preceding section (3.3.2), results of chromosomal analysis were quantitively assessed. As discussed in sections 4.4.2 and 4.4.3, spontaneous and/or clastogen-induced chromosome/cellular instability do not suffice in the unequivocal establishment of FA heterozygosity in individual cases. It was therefore hoped that a qualitative analysis of the types of chromosome aberrations found may be helpful in the delineation of FA carriers from normal individuals.
### 3.3.3.1 Spontaneous chromosomal aberrations

<table>
<thead>
<tr>
<th>Type and number of spontaneous chromosomal aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TYPE OF ABERRATION</strong></td>
</tr>
<tr>
<td>-------------------------</td>
</tr>
<tr>
<td>No. cells screened</td>
</tr>
<tr>
<td>Chromatid gap</td>
</tr>
<tr>
<td>Isochromatid gap</td>
</tr>
<tr>
<td>Centromeric gap</td>
</tr>
<tr>
<td>Chromatid break</td>
</tr>
<tr>
<td>Isochromatid break</td>
</tr>
<tr>
<td>Centromeric break</td>
</tr>
<tr>
<td>Acentric fragment</td>
</tr>
<tr>
<td>Chromatid deletion</td>
</tr>
<tr>
<td>Isochromatid deletion</td>
</tr>
<tr>
<td>Total 'Breaks'</td>
</tr>
<tr>
<td>'Breaks'/cell</td>
</tr>
<tr>
<td>Ring</td>
</tr>
<tr>
<td>Dicentric</td>
</tr>
<tr>
<td>Triradial</td>
</tr>
<tr>
<td>Quadriradial</td>
</tr>
<tr>
<td>Complex exchange</td>
</tr>
<tr>
<td>Total 'Reunion figures'</td>
</tr>
<tr>
<td>'Reunion figures'/cell</td>
</tr>
<tr>
<td>Endoreduplication</td>
</tr>
</tbody>
</table>

'Breaks' = gaps + breaks + fragments + deletions
'Reunion figures' = rings + dicentrics + triradials + quadriradials + complex exchanges

Culture medium = Hams F10
### TABLE 3.15 Spontaneous 'breakage'- and 'reunion-type' aberrations

<table>
<thead>
<tr>
<th>NO. CASES</th>
<th>NO. CELLS</th>
<th>MEAN S.D.</th>
<th>MIN.</th>
<th>MAX.</th>
</tr>
</thead>
</table>

**'BREAKS'/CELL**

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Cases</th>
<th>Cells</th>
<th>Mean</th>
<th>S.D.</th>
<th>Min.</th>
<th>Max.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygotes</td>
<td>24</td>
<td>926</td>
<td>0.25</td>
<td>0.16</td>
<td>0.08</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>Heterozygotes</td>
<td>27</td>
<td>946</td>
<td>0.04</td>
<td>0.04</td>
<td>0.00</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>41</td>
<td>1550</td>
<td>0.02</td>
<td>0.02</td>
<td>0.00</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Sibs</td>
<td>6</td>
<td>220</td>
<td>0.02</td>
<td>0.02</td>
<td>0.00</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Features</td>
<td>15</td>
<td>589</td>
<td>0.03</td>
<td>0.03</td>
<td>0.00</td>
<td>0.08</td>
<td></td>
</tr>
</tbody>
</table>

**'REUNION FIGURES'/CELL**

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Cases</th>
<th>Cells</th>
<th>Mean</th>
<th>S.D.</th>
<th>Min.</th>
<th>Max.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygotes</td>
<td>24</td>
<td>926</td>
<td>0.02</td>
<td>0.04</td>
<td>0.00</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>Heterozygotes</td>
<td>27</td>
<td>946</td>
<td>0.001</td>
<td>0.004</td>
<td>0.00</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>41</td>
<td>1550</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Sibs</td>
<td>6</td>
<td>220</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Features</td>
<td>15</td>
<td>589</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

'Breaks' = gaps + breaks + fragments + deletions
'Reunion figures' = rings + dicentrics + triradians + quadriradians + complex exchanges

Culture medium = Hams F10

Three partial metaphases (10% Giemsa-stained) displaying spontaneous chromosomal aberrations are shown in Fig 3.2.
Figure 3.2 Spontaneous chromosomal aberrations found in homozygotes: 
(a) chromatid break, (b) chromatid gap, (c) quadriradial figure, and (d) triradial figure.
3.3.3.2 DEB [0.1 μg/ml] - induced chromosomal aberrations

**TABLE 3.16 Type and number of DEB [0.1 μg/ml] - induced chromosomal aberrations using Hams F10 medium**

<table>
<thead>
<tr>
<th>TYPE OF ABERRATION</th>
<th>HOM.</th>
<th>HET.</th>
<th>CON.</th>
<th>SIB.</th>
<th>FEAT.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. cells screened</td>
<td>518</td>
<td>520</td>
<td>920</td>
<td>130</td>
<td>250</td>
</tr>
<tr>
<td>Chromatid gap</td>
<td>338</td>
<td>13</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Isochromatid gap</td>
<td>79</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Centromeric gap</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chromatid break</td>
<td>825</td>
<td>19</td>
<td>7</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Isochromatid break</td>
<td>63</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Centromeric break</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acentric fragment</td>
<td>76</td>
<td>10</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Chromatid deletion</td>
<td>74</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Isochromatid deletion</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total 'Breaks'</td>
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<td>9</td>
<td>8</td>
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<tr>
<td>'Breaks'/cell</td>
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<td>0.02</td>
<td>0.07</td>
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<tr>
<td>Ring</td>
<td>14</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dicentric</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Triradial</td>
<td>116</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Quadriradial</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Complex exchange</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total 'Reunion figures'</td>
<td>136</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>'Reunion figures'/cell</td>
<td>0.26</td>
<td>0.002</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Endoreduplication</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

'BREAKs' = gaps + breaks + fragments + deletions

'Reunion figures' = rings + dicentrics + triradials + quadriradials + complex exchanges

Culture medium = Hams F10
**TABLE 3.17** DEB [0.1 µg/ml]-induced 'breakage'- and 'reunion-type' aberrations

<table>
<thead>
<tr>
<th></th>
<th>NO.</th>
<th>NO.</th>
<th>CASES</th>
<th>CELLS</th>
<th>MEAN</th>
<th>S.D.</th>
<th>MIN.</th>
<th>MAX.</th>
</tr>
</thead>
<tbody>
<tr>
<td>'BREAKS'/CELL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygotes</td>
<td>13</td>
<td>518</td>
<td>2.99</td>
<td>1.95</td>
<td>0.08</td>
<td>6.93</td>
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<tr>
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<td>520</td>
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<td>0.06</td>
<td>0.04</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>20</td>
<td>920</td>
<td>0.02</td>
<td>0.02</td>
<td>0.00</td>
<td>0.08</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>130</td>
<td>0.06</td>
<td>0.05</td>
<td>0.00</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Features</td>
<td>5</td>
<td>250</td>
<td>0.03</td>
<td>0.03</td>
<td>0.00</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>'REUNION FIGURES'/CELL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygotes</td>
<td>13</td>
<td>518</td>
<td>0.25</td>
<td>0.19</td>
<td>0.00</td>
<td>0.60</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>520</td>
<td>0.002</td>
<td>0.006</td>
<td>0.00</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
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<td>920</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sibs</td>
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<td>130</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
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<tr>
<td>Features</td>
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<td>250</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

'Breaks' = gaps + breaks + fragments + deletions
'Reunion figures' = rings + dicentrics + triradials + quadriradials + complex exchanges
Culture medium = Ham's F10

In Fig. 3.3, examples of some chromosomal aberrations noted after DEB [0.1µg/ml]-stressing are shown in 2 partial metaphases (10% Giemsa-stained).
Figure 3.3 DEB [0.1 μg/ml]-induced chromosomal aberrations found in homozygotes: (a) chromatid break, (b) isochromatid break, (c) acentric fragment, (d) dicentric chromosome, and (e) complex ring rearrangement.
### 3.3.3.3 MMC [0.01 μg/ml]-induced chromosomal aberrations

**TABLE 3.18** Type and number of MMC [0.01 μg/ml]-induced chromosomal aberrations using Hams F10 medium

<table>
<thead>
<tr>
<th>TYPE OF ABERRATION</th>
<th>HOM.</th>
<th>HET.</th>
<th>CON.</th>
<th>SIB.</th>
<th>FEAT.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. cells screened</td>
<td>157</td>
<td>258</td>
<td>440</td>
<td>30</td>
<td>170</td>
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<tr>
<td>Chromatid gap</td>
<td>10</td>
<td>6</td>
<td>5</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Isochromatid gap</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Chromatid break</td>
<td>142</td>
<td>18</td>
<td>20</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Isochromatid break</td>
<td>8</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Centromeric break</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acentric fragment</td>
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<td>2</td>
<td>1</td>
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<td>0</td>
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<tr>
<td>Chromatid deletion</td>
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<td>0</td>
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<td>0</td>
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<tr>
<td>Isochromatid deletion</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>Total 'breaks'</td>
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<td>3</td>
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<td>0.07</td>
<td>0.20</td>
<td>0.02</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dicentric</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Triradial</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Quadriradial</td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
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<td>Complex exchange</td>
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<td>0</td>
<td>0</td>
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<td>Total 'Reunion figures'</td>
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<td>1</td>
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<td>0.00</td>
<td>0.03</td>
<td>0.006</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

'Breaks' = gaps + breaks + fragments + deletions
'Reunion figures' = rings + dicentrics + triradials + quadriradials + complex exchanges

Cultures medium = Hams F10
<table>
<thead>
<tr>
<th>NO.</th>
<th>NO.</th>
<th>CASES</th>
<th>MEAN</th>
<th>S.D.</th>
<th>MIN.</th>
<th>MAX.</th>
</tr>
</thead>
<tbody>
<tr>
<td>'BREAKS'/CELL</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Homozygotes</td>
<td>7</td>
<td>157</td>
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<td>1.62</td>
<td>0.17</td>
<td>4.00</td>
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<tr>
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<td>9</td>
<td>258</td>
<td>0.13</td>
<td>0.10</td>
<td>0.00</td>
<td>0.28</td>
</tr>
<tr>
<td>Controls</td>
<td>14</td>
<td>440</td>
<td>0.07</td>
<td>0.06</td>
<td>0.00</td>
<td>0.20</td>
</tr>
<tr>
<td>Sibs</td>
<td>1</td>
<td>30</td>
<td>0.20</td>
<td>0.00</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Features</td>
<td>5</td>
<td>170</td>
<td>0.02</td>
<td>0.02</td>
<td>0.00</td>
<td>0.03</td>
</tr>
<tr>
<td>'REUNION FIGURES'/CELL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygotes</td>
<td>7</td>
<td>157</td>
<td>0.19</td>
<td>0.28</td>
<td>0.00</td>
<td>0.60</td>
</tr>
<tr>
<td>Heterozygotes</td>
<td>9</td>
<td>258</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Controls</td>
<td>14</td>
<td>440</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<td>30</td>
<td>0.30</td>
<td>0.00</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Features</td>
<td>5</td>
<td>170</td>
<td>0.007</td>
<td>0.015</td>
<td>0.00</td>
<td>0.03</td>
</tr>
</tbody>
</table>

'Breaks' = gaps + breaks + fragments + deletions
'Reunion figures' = rings + dicentrics + triradials + quadiradials + complex exchanges
Culture medium = Hams F10

Examples of MMC [0.01 μg/ml]-induced chromosomal aberrations are shown in 2 partial metaphases (10% Giemsa-stained) in Fig. 3.4.
Figure 3.4 MMC [0.01 μg/ml]-induced chromosomal aberrations found in homozygotes: (a) chromatid break, (b) acentric fragment, (c) triradial figure, and (d) complex exchange.
3.3.4 The effect of ethnic origin on chromosome breakage results

As noted in section 3.1.2, the homozygote group consisted of 16 White patients and 8 Black patients. Prior to the submission of the chromosome breakage data to in-depth statistical analyses (see section 3.4), the mean spontaneous chromosome breakage rates (both including and excluding gaps) from these 2 groups of patients were compared by the U-test of Mann-Whitney. (This test was kindly performed by Doctor J Kuyl of the Department of Biochemistry, South African Institute for Medical Research, Johannesburg).

<table>
<thead>
<tr>
<th>TABLE 3.20</th>
<th>Comparison of spontaneous chromosome breakage rates in Black and White patients.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Including Gaps</td>
</tr>
<tr>
<td></td>
<td>White</td>
</tr>
<tr>
<td>No. patients</td>
<td>16</td>
</tr>
<tr>
<td>Mean</td>
<td>0.30</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.25</td>
</tr>
<tr>
<td>Range (breaks/cell)</td>
<td>0.08-1.17</td>
</tr>
</tbody>
</table>

Results of U-test of Mann-Whitney
White vs. Black (+gaps) : \( T = 0.153864 \) (\( p = 0.56 \))
White vs. Black (-gaps) : \( T = 1.0449 \) (\( p = 0.15 \))

Therefore, the White and Black patients did not exhibit statistically significant differences in spontaneous breakage rates. It was thus decided to pool all breakage data for future statistical analyses. (See discussion section 4.3.4)
3.4 **STATISTICAL ANALYSIS OF PERIPHERAL BLOOD LYMPHOCYTE CHROMOSOME BREAKAGE RESULTS, CELLULAR INSTABILITY RATES AND NUMBER OF 'BREAKAGE-TYPE' ABERRATIONS**

3.4.1 'Within classes' analysis of breakage data

**TABLE 3.21** Statistical comparisons within genotypic/phenotypic classes (Test used: Wilcoxon's signed-rank test)

<table>
<thead>
<tr>
<th>Class</th>
<th>Comparison</th>
<th>n</th>
<th>Means</th>
<th>Wilcoxon Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOM</td>
<td>SPONT+ vs DEB+</td>
<td>10</td>
<td>0.26</td>
<td>3.22 1.0 0.0069**</td>
</tr>
<tr>
<td>HOM</td>
<td>SPONT- vs DEB-</td>
<td>10</td>
<td>0.21</td>
<td>2.51 1.0 0.0069**</td>
</tr>
<tr>
<td>HOM</td>
<td>SPONT+ vs MMC+</td>
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<td>0.21</td>
<td>1.98 0.0 0.0180*</td>
</tr>
<tr>
<td>HOM</td>
<td>SPONT- vs MMC-</td>
<td>7</td>
<td>0.18</td>
<td>1.78 1.0 0.0280*</td>
</tr>
<tr>
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<td>SPONT+ vs DEB+</td>
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<td>0.07 11.5 0.3627</td>
</tr>
<tr>
<td>HET</td>
<td>SPONT- vs DEB-</td>
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<td>0.03</td>
<td>0.03 18.0 1.0000</td>
</tr>
<tr>
<td>HET</td>
<td>SPONT+ vs MMC+</td>
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<td>SPONT- vs MMC-</td>
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<td>0.01</td>
<td>0.04 1.5 0.2012</td>
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<tr>
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<td>SPONT+ vs DEB+</td>
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<td>0.02 44.5 0.9443</td>
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<tr>
<td>CON</td>
<td>SPONT- vs DEB-</td>
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<td>0.01</td>
<td>0.02 24.5 0.4498</td>
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<tr>
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<td>SPONT+ vs MMC+</td>
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<td>0.01</td>
<td>0.07 0.0 0.0051**</td>
</tr>
<tr>
<td>CON</td>
<td>SPONT- vs MMC-</td>
<td>8</td>
<td>0.01</td>
<td>0.06 0.0 0.0117*</td>
</tr>
</tbody>
</table>

Continued
<table>
<thead>
<tr>
<th>Class</th>
<th>Comparison</th>
<th>Means</th>
<th>Wilcoxon</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1)</td>
<td>(2)</td>
<td>n</td>
<td>(1)</td>
</tr>
<tr>
<td>SIB</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOTE**

+/- = including/excluding gaps respectively

* = significant at 5% level (i.e. p<0.05)

** = significant at 1% level (i.e. p<0.01)

n = number of non-zero differences (i.e. difference between spontaneous and induced breakage not equal to zero)

means = homozygote, heterozygote, sib and features mean values calculated after subtraction of control values; hence occasional negative values; expressed as 'breaks/cell'.

---

FEAT SPONT+ vs DEB+  5  -0.01  0.02  1.5  0.1056
FEAT SPONT- vs DEB-  4  -0.00  0.01  1.5  0.2012
FEAT SPONT+ vs MMC+  3  0.02  -0.02  0.0  0.1088
FEAT SPONT- vs MMC-  4  0.01  -0.03  0.0  0.0679
3.4.2 'Between classes' analysis of breakage data

3.4.2.1 Spontaneous chromosome breakage

Table 3.22 Pair-wise comparisons of spontaneous breakage values
(Test used: Mann-Whitney)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Comparison</th>
<th>Sample Size</th>
<th>Means</th>
<th>Mann-Whitney Value</th>
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</tr>
</thead>
<tbody>
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<td>(1)</td>
<td>(2)</td>
<td>(1)</td>
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**NOTE:**

* = significant at 5% level (i.e. p<0.0125 for comparisons with controls, and p<0.0083 for all other comparisons — refer to section 2.7.1)

** = significant at 1% level (i.e. p<0.0025 for all comparisons with controls and p<0.0017 for all other comparisons — refer to section 2.7.1)

+/- = including/excluding gaps respectively

HETM = heterozygotes — mothers only (i.e. excluding fathers)

means = mean values after subtraction of control values (not applicable to comparisons with controls); expressed as 'breaks/cell'.
Table 3.23  Pair-wise comparisons of DEB [0.1 μg/ml]-induced breakage values (Test used: Mann-Whitney)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Comparison</th>
<th>Sample Size</th>
<th>Means</th>
<th>Mann-Whitney Value</th>
<th>p</th>
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<td>7</td>
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<th>Mann–Whitney Value</th>
<th>p</th>
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<td>16.5</td>
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<td>0.03</td>
<td>39.0</td>
</tr>
<tr>
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<td>CON vs FEAT</td>
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<td>0.02</td>
<td>0.02</td>
<td>43.0</td>
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**NOTE:**

* = significant at 5% level (i.e. p<0.0125 for comparisons with controls, and p<0.0083 for all other comparisons - refer to section 2.7.1)

** = significant at 1% level (i.e. p<0.0025 for all comparisons with controls and p<0.0017 for all other comparisons - refer to section 2.7.1)

+/− = including/excluding gaps respectively

HETM = heterozygotes – mothers only (i.e. excluding fathers)

means = mean values after subtraction of control values (not applicable to comparisons with controls); expressed as ‘breaks/cell’.
### 3.4.2.3 MMC [0.01 µg/ml] - induced chromosome breakage

**TABLE 3.24** Pair-wise comparisons of MMC [0.01 µg/ml] - induced breakage values (Test used: Mann Whitney)

<table>
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<tr>
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<th>p</th>
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<th>Mann-Whitney Value</th>
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**NOTE:**

* = significant at 5% level (i.e. p<0.0125 for comparisons with controls, and p<0.0083 for all other comparisons - refer to section 2.7.1)

** = significant at 1% level (i.e. p<0.0025 for all comparisons with controls and p<0.0017 for all other comparisons - refer to section 2.7.1)

+/- = including/excluding gaps respectively

HETM = heterozygotes - mothers only (i.e. excluding fathers)

Means = mean values after subtraction of control values (not applicable to comparisons with controls): expressed as 'breaks/cell'.
3.4.3 Between classes analysis of cellular instability rates

3.4.3.1 Spontaneous cellular instability

**TABLE 3.25** Pair-wise comparisons of spontaneous cellular instability rates (Test used: Mann-Whitney)

<table>
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<th>Sample Size</th>
<th>Means</th>
<th>Mann-Whitney Value</th>
<th>p</th>
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<td>(1) (2)</td>
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</tr>
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<td>641.0</td>
<td>&lt;0.0001**</td>
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<td>21.87  2.11</td>
<td>144.0</td>
<td>0.0002**</td>
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<tr>
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<td>21.87  2.71</td>
<td>359.0</td>
<td>&lt;0.0001**</td>
</tr>
<tr>
<td>HOM vs CON</td>
<td>24 41</td>
<td>21.87  1.71</td>
<td>0.0</td>
<td>&lt;0.0001**</td>
</tr>
<tr>
<td>HET vs SIB</td>
<td>27 6</td>
<td>4.16   2.11</td>
<td>98.5</td>
<td>0.3973</td>
</tr>
<tr>
<td>HET vs PEAT</td>
<td>27 15</td>
<td>4.16   2.71</td>
<td>230.5</td>
<td>0.4452</td>
</tr>
<tr>
<td>HET vs CON</td>
<td>27 41</td>
<td>4.16   1.71</td>
<td>388.0</td>
<td>0.0269</td>
</tr>
<tr>
<td>SIB vs PEAT</td>
<td>6 15</td>
<td>2.11   2.71</td>
<td>46.5</td>
<td>0.9035</td>
</tr>
<tr>
<td>SIB vs CON</td>
<td>6 41</td>
<td>2.11   1.71</td>
<td>104.0</td>
<td>0.5125</td>
</tr>
<tr>
<td>PLENT vs CON</td>
<td>15 41</td>
<td>2.71   1.71</td>
<td>253.0</td>
<td>0.275*</td>
</tr>
</tbody>
</table>

* = significant at 5% level i.e. p<0.005 (or 0.05/10)
** = significant at 1% level i.e. p<0.001 (or 0.01/10)
* and ** - for explanations refer to section 2.7.1
means - expressed as % unstable cells
### 3.4.3.2 DEB [0.1 µg/ml] - induced cellular instability

**TABLE 3.26** Pair-wise comparisons of DEB [0.1 µg/ml] - induced cellular instability rates (Test used: Mann-Whitney)

<table>
<thead>
<tr>
<th>Comparison (1) vs (2)</th>
<th>Sample Size</th>
<th>Means (1)</th>
<th>Means (2)</th>
<th>Mann-Whitney Value</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOM vs HET</td>
<td>13</td>
<td>78.62</td>
<td>9.56</td>
<td>148.5</td>
<td>0.0001**</td>
</tr>
<tr>
<td>HOM vs SIB</td>
<td>13</td>
<td>78.62</td>
<td>5.33</td>
<td>38.0</td>
<td>0.0118</td>
</tr>
<tr>
<td>HOM vs FEAT</td>
<td>13</td>
<td>78.62</td>
<td>2.80</td>
<td>65.0</td>
<td>0.0013*</td>
</tr>
<tr>
<td>HOM vs CON</td>
<td>13</td>
<td>78.62</td>
<td>2.23</td>
<td>1.0</td>
<td>&lt;0.0001**</td>
</tr>
<tr>
<td>HET vs SIB</td>
<td>12</td>
<td>9.56</td>
<td>5.33</td>
<td>27.0</td>
<td>0.1899</td>
</tr>
<tr>
<td>HET vs FEAT</td>
<td>12</td>
<td>9.56</td>
<td>2.80</td>
<td>57.5</td>
<td>0.0035</td>
</tr>
<tr>
<td>HET vs CON</td>
<td>12</td>
<td>9.56</td>
<td>2.23</td>
<td>9.0</td>
<td>&lt;0.0001**</td>
</tr>
<tr>
<td>SIB vs FEAT</td>
<td>3</td>
<td>5.33</td>
<td>2.80</td>
<td>4.0</td>
<td>0.2789</td>
</tr>
<tr>
<td>SIB vs CON</td>
<td>3</td>
<td>5.33</td>
<td>2.23</td>
<td>18.5</td>
<td>0.2762</td>
</tr>
<tr>
<td>FEAT vs CON</td>
<td>5</td>
<td>2.80</td>
<td>2.23</td>
<td>40.5</td>
<td>0.5028</td>
</tr>
</tbody>
</table>

* = significant at 5% level i.e. p<0.005 (or 0.05/10)
** = significant at 1% level i.e. p<0.001 (or 0.01/10)

* and ** - for explanations refer to section 2.7.1

Means - expressed as % unstable cells
### 3.4.3.3 MMC [0.01 μg/ml] - Increased cellular instability

**Table 3.27 Pair-wise comparisons of MMC [0.01 μg/ml] - induced cellular instability rates (Test used: Mann-Whitney)**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Sample Size</th>
<th>Means</th>
<th>Mann-Whitney Value</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) (2)</td>
<td>(1) (2)</td>
<td>(1)</td>
<td>(2)</td>
<td></td>
</tr>
<tr>
<td>HOM vs HET</td>
<td>7 9</td>
<td>60.95</td>
<td>11.36</td>
<td>60.0</td>
</tr>
<tr>
<td>HOM vs SIB</td>
<td>7 1</td>
<td>60.95</td>
<td>13.33</td>
<td>7.0</td>
</tr>
<tr>
<td>HOM vs FEAT</td>
<td>7 5</td>
<td>60.95</td>
<td>2.67</td>
<td>35.0</td>
</tr>
<tr>
<td>HOM vs CON</td>
<td>7 14</td>
<td>60.95</td>
<td>6.48</td>
<td>1.0</td>
</tr>
<tr>
<td>HET vs SIB</td>
<td>9 1</td>
<td>11.36</td>
<td>13.33</td>
<td>3.5</td>
</tr>
<tr>
<td>HET vs FEAT</td>
<td>9 5</td>
<td>11.36</td>
<td>2.67</td>
<td>36.5</td>
</tr>
<tr>
<td>HET vs CON</td>
<td>9 14</td>
<td>11.36</td>
<td>6.48</td>
<td>39.5</td>
</tr>
<tr>
<td>SIB vs FEAT</td>
<td>1 5</td>
<td>13.33</td>
<td>2.67</td>
<td>0.0</td>
</tr>
<tr>
<td>SIB vs CON</td>
<td>1 14</td>
<td>13.33</td>
<td>6.48</td>
<td>1.0</td>
</tr>
<tr>
<td>FEAT vs CON</td>
<td>5 14</td>
<td>2.67</td>
<td>6.48</td>
<td>51.5</td>
</tr>
</tbody>
</table>

* = significant at 5% level i.e. p<0.005 (or 0.05/10)

** = significant at 1% level i.e. p<0.001 (or 0.01/10)

* and ** - for explanations refer to section 2.7.1

Means - expressed as % unstable cells
3.4.4 'Between classes' analysis of the number of 'breakage-type' aberrations

3.4.4.1 Spontaneous 'breakage-type' aberrations

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Sample Size (1)</th>
<th>Sample Size (2)</th>
<th>Means (1)</th>
<th>Means (2)</th>
<th>Mann-Whitney Value</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOM vs HET</td>
<td>24</td>
<td>27</td>
<td>0.25</td>
<td>0.04</td>
<td>642.0</td>
<td>&lt;0.0001**</td>
</tr>
<tr>
<td>HOM vs SIB</td>
<td>24</td>
<td>6</td>
<td>0.25</td>
<td>0.02</td>
<td>144.0</td>
<td>0.0002**</td>
</tr>
<tr>
<td>HOM vs FEAT</td>
<td>24</td>
<td>15</td>
<td>0.25</td>
<td>0.03</td>
<td>359.0</td>
<td>0.0001**</td>
</tr>
<tr>
<td>HOM vs CON</td>
<td>24</td>
<td>41</td>
<td>0.25</td>
<td>0.02</td>
<td>0.0</td>
<td>0.0001**</td>
</tr>
<tr>
<td>HET vs SIB</td>
<td>27</td>
<td>6</td>
<td>0.04</td>
<td>0.02</td>
<td>98.5</td>
<td>0.3975</td>
</tr>
<tr>
<td>HET vs FEAT</td>
<td>27</td>
<td>15</td>
<td>0.04</td>
<td>0.03</td>
<td>229.5</td>
<td>0.4620</td>
</tr>
<tr>
<td>HET vs CON</td>
<td>27</td>
<td>41</td>
<td>0.04</td>
<td>0.02</td>
<td>37.7</td>
<td>0.0189</td>
</tr>
<tr>
<td>SIB vs FEAT</td>
<td>6</td>
<td>15</td>
<td>0.02</td>
<td>0.03</td>
<td>48.5</td>
<td>0.7780</td>
</tr>
<tr>
<td>SIB vs CON</td>
<td>6</td>
<td>41</td>
<td>0.02</td>
<td>0.02</td>
<td>102.5</td>
<td>0.4754</td>
</tr>
<tr>
<td>FEAT vs CON</td>
<td>15</td>
<td>41</td>
<td>0.03</td>
<td>0.02</td>
<td>239.0</td>
<td>0.1702</td>
</tr>
</tbody>
</table>

* = significant at 5% level i.e. p<0.005 (or 0.05/10)
** = significant at 1% level i.e. p<0.001 (or 0.01/10)

* and ** - for explanations refer to section 2.7.1

means - expressed as 'breaks'/cell
3.4.4.2 DEB [0.1 μg/ml] - induced 'breakage-type' aberrations

**TABLE 3.29** Pair-wise comparisons of DEB [0.1 μg/ml] - induced 'breakage-type' aberration rates (Test used: Mann-Whitney)

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Sample Size</th>
<th>Means</th>
<th>Mann-Whitney Value</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1) (2)</td>
<td>(1)</td>
<td>(2)</td>
<td></td>
</tr>
<tr>
<td>HOM vs HET</td>
<td>13 12</td>
<td>2.99</td>
<td>0.12</td>
<td>148.0</td>
</tr>
<tr>
<td>HOM vs SIB</td>
<td>13 3</td>
<td>2.99</td>
<td>0.06</td>
<td>37.5</td>
</tr>
<tr>
<td>HOM vs FL</td>
<td>13 5</td>
<td>2.99</td>
<td>0.03</td>
<td>65.0</td>
</tr>
<tr>
<td>HOM vs CON</td>
<td>13 20</td>
<td>2.99</td>
<td>0.02</td>
<td>0.5</td>
</tr>
<tr>
<td>HET vs SIB</td>
<td>12 3</td>
<td>0.12</td>
<td>0.06</td>
<td>27.0</td>
</tr>
<tr>
<td>HET vs FEAT</td>
<td>12 5</td>
<td>0.12</td>
<td>0.03</td>
<td>55.5</td>
</tr>
<tr>
<td>HET vs CON</td>
<td>12 20</td>
<td>0.12</td>
<td>0.02</td>
<td>7.0</td>
</tr>
<tr>
<td>SIB vs FEAT</td>
<td>3 5</td>
<td>0.06</td>
<td>0.03</td>
<td>4.0</td>
</tr>
<tr>
<td>SIB vs CON</td>
<td>3 20</td>
<td>0.06</td>
<td>0.02</td>
<td>17.0</td>
</tr>
<tr>
<td>FEAT vs CON</td>
<td>5 20</td>
<td>0.03</td>
<td>0.02</td>
<td>39.0</td>
</tr>
</tbody>
</table>

* = significant at 5% level i.e. p<0.005 (or 0.05/10)
** = significant at 1% level i.e. p<0.001 (or 0.01/10)
* and ** - for explanations refer to section 2.7.1
means - expressed as 'breaks'/cell
### TABLE 3.30  Pair-wise comparisons of MMC [0.01 µg/ml] - induced 'breakage-type' aberration rates (Test used: Mann-Whitney)

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Sample Size</th>
<th>Means</th>
<th>Mann-Whitney Value</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) (2)</td>
<td>(1) (2) (1) (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOM vs HET</td>
<td>7 9</td>
<td>1.69</td>
<td>0.13</td>
<td>59.0</td>
</tr>
<tr>
<td>HOM vs SIB</td>
<td>7 1</td>
<td>1.69</td>
<td>0.20</td>
<td>6.0</td>
</tr>
<tr>
<td>HOM vs FEAT</td>
<td>7 5</td>
<td>1.69</td>
<td>0.02</td>
<td>35.0</td>
</tr>
<tr>
<td>HOM vs CON</td>
<td>7 14</td>
<td>1.69</td>
<td>0.07</td>
<td>1.0</td>
</tr>
<tr>
<td>HET vs SIB</td>
<td>9 1</td>
<td>0.13</td>
<td>0.20</td>
<td>2.5</td>
</tr>
<tr>
<td>HET vs FEAT</td>
<td>9 5</td>
<td>0.13</td>
<td>0.02</td>
<td>37.0</td>
</tr>
<tr>
<td>HET vs CON</td>
<td>9 14</td>
<td>0.13</td>
<td>0.07</td>
<td>41.0</td>
</tr>
<tr>
<td>SIB vs FEAT</td>
<td>1 5</td>
<td>0.20</td>
<td>0.02</td>
<td>0.0</td>
</tr>
<tr>
<td>SIB vs CON</td>
<td>1 14</td>
<td>0.20</td>
<td>0.07</td>
<td>0.5</td>
</tr>
<tr>
<td>FEAT vs CON</td>
<td>5 14</td>
<td>0.02</td>
<td>0.07</td>
<td>55.0</td>
</tr>
</tbody>
</table>

* = significant at 5% level i.e. p<0.005 (or 0.05/10)
** = significant at 1% level i.e. p<0.001 (or 0.01/10)
* and ** - for explanations refer to section 2.7.1
means - expressed as 'breaks'/cell
3.5 AMNIOTIC FLUID CELL AND FIBROBLAST STUDIES

The amniotic fluid cell and fibroblast studies are to be considered together since the fibroblast cultures from an affected homozygote, together with normal amniotic fluid cell cultures, served as controls for the 'at-risk' amniotic cell culture that was investigated in the course of the present study.

Only one amniotic fluid from a pregnancy at-risk for FA was cytogenetically analyzed with a view to prenatal diagnosis of FA. An amniotic fluid specimen being investigated because of the mother's age, was simultaneously cultured and served as a 'normal' control. A fibroblast culture established from a skin biopsy of a FA homozygote (the brother of the foetus being prenatally investigated) was also simultaneously studied. Following termination of the 'at-risk' pregnancy, foetal skin fibroblasts were cultured and screened for the presence of increased chromosomal fragility.

3.5.1 Results of amniotic fluid cell and fibroblast studies

The karyotype of the cultured 'at-risk' amniotic cells was 46,XX. Results of spontaneous and DEB-induced [0.1 μg/ml and 0.5 μg/ml] chromosome breakage studies in cultured amniotic fluid cells, FA homozygote skin fibroblasts, foetal skin fibroblasts (obtained after termination of pregnancy) and normal control amniotic cells are summarized in Tables 3.31 and 3.32.
TABLE 3.31 Chromosome breakage and cellular instability in amniotic cell cultures

<table>
<thead>
<tr>
<th>Source of cells</th>
<th>Culture Condition</th>
<th>Number Cells</th>
<th>Number Endoreduplications</th>
<th>Number 'Breaks'</th>
<th>Number Reunion Figs.</th>
<th>'Breaks' /Cell</th>
<th>Unstable Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AMNIOTIC CELLS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i) Foetus at Risk</td>
<td>Spontaneous</td>
<td>30</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>0.23</td>
<td>13.33</td>
</tr>
<tr>
<td></td>
<td>0.1 μgDEB/ml</td>
<td>30</td>
<td>0</td>
<td>12</td>
<td>2</td>
<td>0.53</td>
<td>30.00</td>
</tr>
<tr>
<td></td>
<td>0.1 μgDEB/ml*</td>
<td>30</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0.27</td>
<td>23.33</td>
</tr>
<tr>
<td></td>
<td>0.5 μgDEB/ml</td>
<td>27</td>
<td>1</td>
<td>12</td>
<td>3</td>
<td>0.67</td>
<td>51.85</td>
</tr>
<tr>
<td></td>
<td>0.5 μgDEB/ml*</td>
<td>30</td>
<td>1</td>
<td>14</td>
<td>1</td>
<td>0.53</td>
<td>33.33</td>
</tr>
<tr>
<td>ii) Control</td>
<td>Spontaneous</td>
<td>30</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0.03</td>
<td>3.33</td>
</tr>
<tr>
<td></td>
<td>0.1 μgDEB/ml</td>
<td>30</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>0.13</td>
<td>16.67</td>
</tr>
<tr>
<td></td>
<td>0.1 μgDEB/ml*</td>
<td>30</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0.03</td>
<td>3.33</td>
</tr>
<tr>
<td></td>
<td>0.5 μgDEB/ml</td>
<td>30</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0.03</td>
<td>3.33</td>
</tr>
<tr>
<td></td>
<td>0.5 μgDEB/ml*</td>
<td>30</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0.03</td>
<td>3.33</td>
</tr>
</tbody>
</table>

'BREAKS' = all gaps, breaks, fragments and deletions

* = DEB removed 48 hours prior to harvesting (refer to section 2.2.3.8)
<table>
<thead>
<tr>
<th>Source of cells</th>
<th>Culture Condition</th>
<th>Number Cells</th>
<th>Number Endoreduplication</th>
<th>Number 'Breaks' Figs.</th>
<th>Number Reunion/Cell</th>
<th>'Breaks' Unstable Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKIN FIBROBLASTS</td>
<td>i) FA homozygote</td>
<td>Spontaneous 30</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1 μgDEB/ml 11</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1 μgDEB/ml* 30</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 μgDEB/ml 14</td>
<td>0</td>
<td>12</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 μgDEB/ml* 19</td>
<td>1</td>
<td>11</td>
<td>1</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>ii) Aborted Fetus</td>
<td>Spontaneous 30</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1 μgDEB/ml 30</td>
<td>0</td>
<td>16</td>
<td>0</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1 μgDEB/ml* 30</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 μgDEB/ml 23</td>
<td>0</td>
<td>25</td>
<td>1</td>
<td>1.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 μgDEB/ml* 30</td>
<td>0</td>
<td>18</td>
<td>1</td>
<td>0.67</td>
</tr>
</tbody>
</table>

'BREAKS' = all gaps, breaks, fragments and deletions
* = DEB removed 48 hours prior to harvesting (refer to section 2.2.3.8)
4. DISCUSSION OF RESULTS

4.1 PERIPHERAL BLOOD LYMPHOCYTE STUDIES

4.1.1 Sample size

The 'sins' class is noticeably small (n=6) since brothers and sisters of affected FA patients were specifically not requested for the purposes of this study as siblings constitute a somewhat 'unknown quantity'. At present, it is impossible to ascertain whether these individuals are heterozygous for the FA gene or normal, or alternatively, may in the future develop pancytopenia.

In view of the 24 homozygotes studied, clearly more than 28 heterozygotes (i.e. parents of FA homozygotes) could have been studied. Unfortunately, although bloods were requested on all obligatory heterozygotes, private clinicians were often unable to comply with this request. Furthermore, most children are usually accompanied by only one parent (usually the mother) on visits to the participating haematology clinics. With respect to the Black patients, both parents usually work resulting in the children being accompanied by another member of the family e.g. the grandmother.

It should be noted that the present study is larger than any other previously published comparable study on enhancement of chromosomal breakage in FA. (See Tables 3.11 and 3.12)

4.1.2 Ethnic group

The homozygote group and consequently by definition, the heterozygote group, were composed entirely of either Whites or Blacks. The majority of White homozygotes (14 out of a total of 16) stem from predominantly Afrikaans-speaking families. (It should be noted that Afrikaans is the home language of approximately 57% of South African Whites - see section 3.13). This situation is probably attributable
to a founder effect in a once small Afrikaans community. (See section 4.1.3) Of the 8 Black patients included in the present study, 2 were Zulu, 4 were Sotho, 1 was a Tswana and 1 individual was from Zimbabwe. Since the Coloured people trace part of their ancestry back to White Afrikaners and Blacks, some affected Coloureds were expected. Rather surprisingly, no Coloured patients were referred for investigation.

The high number of Blacks with 'features of FA' compared to Whites in this group could be due to a postulated variant sub-class of FA in the Blacks, which manifests with incomplete expression of the FA gene. Alternatively, the small group of referring doctors at Baragwanath hospital, the source of all Black bloods studied, may simply have been more interested in the syndrome and would consequently have referred a greater proportion of their patients to our cytogenetics unit, including those which did not fit the 'classic' FA pattern.

4.1.3 Prevalence of homozygous and heterozygous Fanconi's anaemia

The estimated prevalences of FA homozygotes and heterozygotes in the Afrikaner population under study are much higher than those elsewhere. For example, Swift (1976) estimated that heterozygotes in New York State occur at a minimum frequency of 1 in 300, while according to Arlett and Lehmann (1978), Wonder arrived at a frequency of 1 in 125 in a 'mid-European' population. By comparison, the present study has revealed that Afrikaans-speaking heterozygotes in the Johannesburg area (see Fig. 3.1) occur with a minimum prevalence of approximately 1 in 80 (1 in 77 based on live-births or 1 in 83 based on point prevalence data).

It is believed then that the gene for FA is unusually common among the Afrikaans community for the same reason that a variety of genetic disorders including lipoid proteinosis (Heyl, 1970), porphyria variegata (Dean, 1971), Oudtshoorn skin (Findlay et al., 1977), sclerosteosis (Heighton et al., 1977) and familial
hypercholesterolaemia (Jenkins et al., 1980) are common in this community - namely, random genetic drift in the form of a founder effect.

It has been pointed out by Dean (1973) that the Afrikaans population, during a period of about 300 years, underwent an increase in size of approximately 12 500-fold, while Britain's population, for example, increased by about 6-fold during that time. The Afrikaans community started with a small number of 'founders' when the Dutch East India Company established a refreshment station at the Cape in 1652. By 1663 there were 13 immigrant families at the Cape and some 15 years later the White population totalled 359. A very significant addition to this mainly Dutch and German population was made in 1688 when 200 Huguenots (mainly stable family groups) arrived in the Cape. By 1701 the White population of the Cape totalled 1265 and by the end of the 18th century it had reached 15 000 (Ross, 1975).

If one or two of the original 'founders' had happened to possess the FA allele, it is quite conceivable that the gene could be present in the high frequency postulated from the present study. Dean (1973) convincingly showed that such a founder effect accounted for the extremely high prevalence (about 1 in 250) of porphyria variegata among Afrikaners in the 1960's. It should be noted that although the present study was confined to the Johannesburg area, a relatively large number of FA patients is known in the Bloemfontein area (Marx et al., 1983).

It is unlikely that 19th century immigration from European countries, chiefly Britain, would have significantly reduced the frequency of the FA gene by a dilution effect. Firstly, the numbers were small and secondly there was minimal integration. For example, of the 36 000 emigrants who arrived from the U.K. before 1869, only half remained in South Africa. During the 19th century South Africa attracted only 0.7% of British emigrants, while Australia took 14% (Christopher, 1976).

Unfortunately, the present social structure in South Africa prevents any reliable estimate of the prevalence of the FA gene in the Black
community being made. FA cases occurring in the rural areas may well go unrecorded and the relatively poor health care services in these areas will often result in diagnosed cases being lost to follow-up. In addition, there is some doubt about the accuracy of population census figures, especially as they relate to the rural areas. It should, however, be pointed out that at least 25 unequivocal Black FA homozygotes have been treated at Baragwanath Hospital, a large referral hospital on the outskirts of Johannesburg, over recent years (L. McDougall, personal communication, 1985). (Based on the 1980 Population Census, it may be estimated that the Black community age 24 years or less residing in the Transvaal numbers approximately 3,461,369). A number of Black patients have also been reported in the Bloemfontein area (Marx et al., 1983). Clearly then, these findings are in contrast with those of Meme et al. (1975) who reported FA as being rare in individuals of 'Bantu' extraction.

4.1.4 Sex

It is of interest that the homozygote group consisted of an equal number of males and females. This distribution is in conflict with previous reports which indicate a shift in the sex ratio towards males. (See section 1.3) However, a sample size of 24 is probably grossly inadequate in attempting to clarify whether affected males do in fact outnumber homozygous females. As is to be expected, a greater number of heterozygous females than males were investigated. The explanation for this is that it is far more common for a child to be accompanied to the clinician by its mother rather than by the father. It thus follows that female heterozygotes were more accessible for study. In section 2.1.1, it was pointed out that the control group consisted of 'normal' bloods randomly selected from those being investigated in the laboratory plus laboratory personnel. Among our laboratory staff, males are outnumbered by about 15:1, explaining the 1.6:1::female:male ratio in the control class.
It is clear that all classes consisted of subjects differing widely in age. Correlation of age with chromosomal breakage would consequently be difficult to study and would not be expected to contribute greatly to our understanding of the basis of chromosomal breakage. Furthermore, most previous reports have indicated no direct relationship between age and the extent of chromosomal fragility. (See section 1.6.1.8) With respect to the homozygote class, it should be noted that the mean age of these patients in no way reflects the mean age of onset of pancytopenia. The mean age is simply the average age of the patients at the time of cytogenetic analysis.

4.2 PERIPHERAL BLOOD LYMPHOCYTE CHROMOSOME ANALYSIS

As recorded in section 3.2, no stable structural/numerical chromosomal abnormalities were noted in any of the subjects included in the present study. It is however realized that the analysis of only 15 metaphases per case is not sufficient to detect a minor acquired clonal abnormality.

4.3 PERIPHERAL BLOOD LYMPHOCYTE CHROMOSOME BREAKAGE STUDIES

4.3.1 Number of cells screened for chromosome breakage under different culture conditions

4.3.1.1 Number of cells screened

As discussed in section 2.6.2, initially, where possible, 50 metaphases per case per mutagenic parameter were screened for breakage. On the advice of the consulting statisticians, this criterion for studying chromosomal fragility was later changed to 30 metaphases. The rationale behind this lowering of the number of
cells screened was basically that 'normality' can be assumed if 30 metaphases per case in a total of at least 30 cases are studied. Unfortunately, as to be expected from any biological study, it was not always possible to analyze the required preset number of metaphases due to poor growth of some of the cultures. Furthermore, the number of cases studied per culture condition varied greatly and thus for comparative statistical purposes, the statisticians subsequently deemed it necessary to apply non-parametric tests. (At this point, it is perhaps appropriate to mention that no difference in growth was noted between untreated FA homozygote, FA heterozygote and normal control cultures.)

4.3.1.2 Rationale for using varying concentrations of Diepoxybutane and Mitomycin C

It will be noted in Table 3.5, that not all cases were subjected to all mutagenic treatments. Varying concentrations of DEB and MMC were tested only in heterozygotes and their controls (with the one exception of a single sib tested with 2 dosages of MMC). It was not deemed necessary to test homozygotes with different DEB and MMC concentrations because the initial concentrations of 0.1 µg/ml DEB and 0.01 µg/ml MMC resulted in highly significant increases in chromosome breakage without affecting mitotic index. (See section 4.4.1) However, due to the equivocal results obtained in obligatory heterozygotes using the latter concentrations, it was decided to vary these dosages in an attempt to enhance clastogenesis in carriers (one of the aims of this study).

At the outset of the present study it was decided to investigate the enhancement of in vitro chromosome breakage after the addition of DEB at a concentration of 0.1 µg/ml. This DEB dosage was initially chosen in an attempt to compare data with those of Auerbach et al.'s (1981) impressive classical study. In the latter study, 5 homozygotes, 8 obligatory heterozygotes and 3 sibs were investigated for the effects of the in vitro addition of 0.1 µg/ml DEB to peripheral blood cultures. Although Auerbach et al.'s results appear conclusive, it was nevertheless necessary to test this dosage (0.1
μg/ml) in our study sample under local culture conditions. Subsequent to the commencement of the present study, additional DEB-induced breakage data have been published using concentrations of DEB varying from 0.01 μg/ml to 1 μg/ml. In no study was more than 1 μg/ml DEB used. Some of these findings are recorded in Table 3.12.

MMC was tested at concentrations of 0.01 and 0.05 μg/ml culture. The published studies using MMC to clastogenically stress FA cells primarily made use of 0.01 μg/ml MMC. Once again, in order to facilitate a comparative study, this concentration was preferentially chosen.

4.3.1.3 Choice of culture media

Two different culture media were used viz. Hams F10 and M150. As pointed out in section 2.1.5.2, M150 like the well-known TC199, is a folate deficient medium which enhances the expression of the fragile X in X-linked mental retardation and other folate dependent fragile sites in the normal human genome. (See section 5.3.5.2.2) It was of interest to investigate whether this medium, which is less rich in nutrients than F10, might also enhance the expression of chromosomal fragility in FA. In previous studies similar to the present, Auerbach et al. (1981) used Eagles MEM medium and Cohen et al. (1982a) used chromosome medium 1A. Medium 199 was used by Marx et al. (1983). (The reader may refer to the GIBCO catalogue, for example, for the formulation of the various above-specified culture media.)

The results obtained with the above-described variations in number of cells, concentrations of DEB and MMC and differing culture media are discussed in section 4.3.2. It should be pointed out that the number of individuals tested using M150 medium was small and the influence of this medium as opposed to Hams F10 medium in producing breakage, was far from conclusive. The results of the statistical analysis of these data are considered in section 4.4.
4.3.2 Quantitative analysis of spontaneous and clastogen induced chromosomal aberrations

4.3.2.1 Chromosome breakage

4.3.2.1.1 Homozygotes

4.3.2.1.1.1 Spontaneous chromosome breakage in Fanconi's anaemia homozygotes

a) Hams F10 culture medium

The mean spontaneous breakage rate in the homozygote class was 0.23 breaks/cell. This mean has an associated standard deviation of 0.17 breaks/cell, which is indicative of a broad interpatient range of spontaneous chromosome damage within the 24 FA patients. In fact, the breakage rate varied from a minimum of 0.03 to a maximum of 0.87 breaks/cell. When gaps are taken into consideration, the mean was 0.30 ± 0.21 breaks/cell and an even greater breakage range was recorded viz. 0.06 to 1.17 breaks/cell.

Inter-patient variation is a frequently reported finding, as is intra-patient variation in spontaneous chromosomal instability. (See section 1.6.1) The present study does not include repeated investigations on patients, but the majority of patients who have undergone repeated investigations in our laboratory all exhibited some fluctuation in breakage rates, to a lesser or greater extent.

b) M150 culture medium

Only 3 homozygotes were studied using M150 as the culture medium. Very little interpatient variation was noted - the mean breakage rate was 0.18 breaks/cell with an associated standard deviation of 0.02. The minimum breakage noted was 0.17 breaks/cell and the maximum was 0.20 breaks/cell. When the same 3 bloods were concurrently cultured
in Hams F10 medium, the mean breakage rate was 0.11 breaks/cell (range 0.03 to 0.20 breaks/cell). It is interesting to note that if gaps found in these 3 cases are included in the breakage rates, these values become 0.20 breaks/cell for M150 and 0.18 breaks/cell for F10, indicating the presence of far more gaps in the F10 cultures. Remembering that the mean breakage with F10 in 24 homozygotes was 0.23 breaks/cell, it is obvious that the latter values are skewed by the small sample size. Thus, although higher breakage may be expected using M150 (see 2.1.5.2), no definite conclusions can be drawn at present since a sample size of only 3 is much too small.

4.3.2.1.1.1 Comparison of homozygote spontaneous chromosome breakage data with other studies

Some interesting observations can be made when comparing findings of spontaneous chromosome breakage investigations similar to the present study. (See section 3.3.2.2) It must however be emphasized that direct comparisons for spontaneous (and induced) breakage should always be treated with great reserve because the assessment of chromosome breakage is subjective and individual protocols, although similar, do vary.

The mean spontaneous breakage rate noted by Auerbach et al. (1981) (0.25 breaks/cell) is very similar to the present findings with Hams F10 medium (0.23 breaks/cell), while that published by Cohen et al. (1982) is slightly lower (0.16 breaks/cell) than the breakage rate noted with Hams F10 medium, but very similar to that found in the 3 subjects investigated with M150 medium (0.18 breaks/cell). This small degree of variation is expected since only 5 FA individuals were studied by both these groups.

The 18 FA patients investigated by Marx et al. (1983) exhibited a mean breakage of 0.77 breaks/cell. This value is much higher than almost all previously published findings. (See section 3.3.2.2) Interestingly, the latter group of workers found the mean spontaneous breakage rate in 11 FA none marrow cultures (32-hour culture period) to be 0.72 breaks/cell (range 0.40 to 0.89 breaks/cell), a mean
strikingly close to that obtained in their lymphocyte cultures. As discussed in section 1.6.1.4, published data indicate that only the minority of direct bone marrow cultures exhibit chromosome breakage, and when present, the breakage rates are generally much lower than those found in peripheral blood lymphocyte cultures. This dramatic increase in observed breakage may be attributable to the fact that Marx et al. established their cultures in Medium 199 (Gibco) with 3 times the usual concentration of glutamine (i.e. + 300 mg/l). Although the Hams F10 medium and M150 used in the present study contained only 100 mg glutamine/l, Eagles MEM [used by Auerbach et al. (1981)] also contains glutamine at a concentration of + 300 mg/l. It is therefore surprising that the results of Marx et al. (1983) differ considerably from those of Auerbach et al. (1981). (See Table 3.11)

4.3.2.1.1.2 DEB-induced chromosome breakage in Fanconi's anaemia homozygotes

Peripheral blood lymphocyte cultures from 13 FA homozygotes were established in Hams F10 medium and were clastogenically stressed for 42 hours with DEB at a concentration of 0.10 μg DEB/ml culture. The DEB was added 24 hours after culture initiation. This stressing resulted in an 11-fold increase in the number of chromosomal aberrations compared to the spontaneous breakage rate. Based on the 518 cells screened for breakage, a mean breakage rate of 2.58 breaks/cell was obtained. The associated standard deviation was 1.85 breaks/cell and the breakage range was 0.04 to 6.73 breaks/cell.

At this point some attention should be focused on the lower end of the above-mentioned breakage range. In particular, 3 patients from 2 families will fall under discussion.

Patient F.P. (case no. 77) and his brother W.P. (case no. 78) were initially referred as fulfilling the necessary diagnostic criteria for FA homozygosity. The proband W.P. who was 6 years of age at the time, had a markedly raised foetal haemoglobin (HbF) level of 27% (normal range being 0-2% after the age of 1 year). Three previous
chromosome analyses on W.P. showed high spontaneous breakage, ranging from 0.2 to 0.3 breaks/cell. A few endoreduplications and 2 chromosome rearrangements were also noted. However, his spontaneous breakage rate in the present study was only 0.12 breaks/cell (plus 1 endoreduplication, but zero rearrangements) and exposure to 0.1 µg DEB/ml culture yielded only 0.12 breaks/cell. It is therefore clear that although W.P. clinically appears to be an FA homozygote, both his present spontaneous and DEB-induced breakage rates fall well below the respective means of 0.23 and 2.58 breaks/cell.

W.P.'s younger brother, P.P. was also referred to our laboratory in 1976 when he was 2 years old, because he had had a slightly raised level of foetal haemoglobin viz. 4%. Three repeated spontaneous chromosome breakage studies over the next 4 years revealed slightly raised breakage values ranging from 0.08 to 0.13 breaks/cell (no rearrangements or endoreduplications were observed). The present investigation yielded a spontaneous breakage rate of only 0.06 breaks/cell and a DEB-induced value of only 0.04 breaks/cell. Quite clearly, this patient's mean spontaneous and DEB-induced breakage rates are considerably lower than expected for FA homozygotes.

At a very recent meeting with the consulting clinician, subsequent to the completion of all statistical analyses, some doubt was expressed as to the genotypic classification of P.P. At the age of 9 years, he is healthy and has not as yet developed any evidence of pancytopenia. This, together with the repeated cytogenetic findings are perhaps indicative of patient P.P. being an FA heterozygote rather than homozygote. Alternatively, since both P.P. and W.P. are not remarkably sensitive to the clastogenic effects of DEB, these 2 sibs may represent an unusual variant form of FA. The latter postulate is in concordance with the findings of Cohen et al. (1982a) who noted that 2 of their 5 FA patients did not exhibit the expected increase in chromosome damage after in vitro staining with DEB. Furthermore, as discussed in section 1.6.1, a minority of FA patients do not exhibit increased spontaneous chromosome fragility.

Unfortunately, neither P.P. nor W.P. were available for repeat confirmatory studies with DEB and/or MMC.
The third lowest DEB-induced breakage score was noted in a 20 year-old female patient, K.H. (case 23). K.H.'s spontaneous and DEB-induced breakage rates were 0.37 and 0.5 breaks/cell respectively. Thus, although she had a relatively high rate of spontaneous chromosome aberrations (compared with the mean of 0.23 breaks/cell), this patient does not appear to be unduly sensitive to the clastogenic effects of DEB. (Unfortunately, K.H. was not available for MMC studies) In this respect, it is of interest that K.H. was at least 7 years older than any other FA patient included in this study. K.H. apparently only presented with aplastic anaemia at 10 years of age, indicating a milder perhaps variant form of the disease. Following this patient's induced value of 0.5 breaks/cell, the next breakage rate in this range was 1.8 breaks/cell, a point from which the induced scores increased more or less steadily to 6.73 breaks/cell.

The data presented here, together with those of Cohen et al. (1982a), indicate that there may in fact be 2 groups of FA patients, with one set (the minority) not being hypersensitive to DEB. With respect to spontaneous breakage, Sasaki (1978) postulated the presence of 2 groups of FA patients with mean spontaneous breakage values of 55.7% and 5.9% respectively. This was however, not noted in the present study. As to the existence of similar sub-classes after DEB stressing, the number of DEB-stressed cases constituting the present study is too small to reach any definite conclusion. In this respect, it is of interest that Kwee et al. (1983) have reported on one 22 year-old patient who exhibited clastogen sensitivity in only 40% of his cultured cells, while the other 60% of his cells responded like those from normal healthy controls. It should also be remembered that differences in the genotypic/phenotypic classification of patients may lead to discrepancies - for example, the three patients at the lower end of the present DEB-induced breakage scale may not have been classified as 'true' FA homozygotes by all workers in the field.

As a consequence of the above-described variation in DEB-sensitivity in FA homozygotes, one is forced to conclude that the induction of chromosome aberrations by DEB should not be regarded as an absolute...
criterion in the diagnosis of FA.

4.3.2.1.2.1 Comparison of hemizygote DEB-induced chromosome breakage data with other studies

With reference to Table 3.12 in section 3.3.2.2, showing the results of four studies, Marx et al. (1983), like the present study, also found a mean breakage rate of 2.58 breaks/cell, while that noted by Cohen et al. (1982a) was slightly lower (1.62 breaks/cell). Auerbach et al. (1981) reported a relatively high breakage rate (8.66 breaks/cell). In the latter 2 studies, wide ranges and consequent large standard deviations were recorded. This is in concordance with the present findings. It is however, rather surprising that the present study's mean breakage rate is identical to that reported by Marx et al. (1982) since these workers found a much higher spontaneous breakage rate in their patients.

4.3.2.1.3 MMC-induced chromosome breakage in Fanconi's anaemia homozygotes

a) Hams F10 culture medium

Peripheral blood lymphocyte cultures from 7 FA homozygotes were cultured in Hams F10 medium and stressed with 0.01 μg MMC/ml culture for the final 42 hours of the usual 66-hour culture period. This resulted in an approximately 8-fold increase in breakage over the mean spontaneous breakage rate. Breakage (excluding gaps) ranged from a minimum of 0.07 breaks/cell to a maximum of 4.63 breaks/cell. The mean breakage rate was 1.85 breaks/cell and the associated standard deviation was 1.85. Quite clearly, as for DEB-induced breakage, a vast degree of interpatient variation in MMC-induced breakage was evident.

The lower end of this MMC-induced breakage range warrants discussion. Patient 19 (M.G.), was first referred to our laboratory as a 2
Author  Rosendorff J
Name of thesis  Chromosome breakage studies in Fanconi's Anaemia homozygotes and Heterozygotes  1985

PUBLISHER:
University of the Witwatersrand, Johannesburg
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