month-old infant with dysmorphic features and abnormal thumbs. During a routine chromosome analysis at that time, (not included in this study), a spontaneous breakage rate of 0.2 breaks/cell was noted. The patient's clinicians subsequently diagnosed probable FA homozygosity. At that point it was decided to include this little girl in the present study. A repeat cytogenetic investigation revealed a spontaneous breakage rate of only 0.13 breaks/cell and an MMC-induced rate of only 0.07 breaks/cell. A repeat investigation (not included in the present study) at 15 months of age, again revealed a low spontaneous breakage rate, viz. 0.02 breaks/cell. However, and most importantly, on clinical examination at this age, no somatic stigmata of FA, apart from abnormal thumbs, were noted and a history of severy epilepsy was obtained. M.G. had apparently been on a number of anti-convulsant drugs since the neo-natal period. These drugs could very well have caused the increased spontaneous breakage first seen in this child when she was 2 months-old. The low MMC-induced breakage rate is therefore not suprising since this patient is probably not an FA homozygote. This case thus serves to illustrate the ease with which a patient may be misdiagnosed in the absence of a full clinical history.

Patient 14 (S.F.), a positively diagnosed FA homozygote, showed only 0.30 breaks/cell after MMC stressing. Interestingly, this patient's spontaneous breakage rate was only 0.10 breaks/cell, which also falls at the lower end of the spontaneous breakage scale. It therefore appears that the magnitude of induced chromosome breakage may in fact reflect an individual susceptibility to chromosomal instability. Similar findings have been reported by Cohen et al. (1982a).

b) M150 Culture Medium

Using M150 as the culture medium and stressing 3 FA homozygotes with 0.01 μg MMC/ml culture, resulted in a mean breakage rate of 2.23 breaks/cell (range 0.20 - 6.19 breaks/cell) and an associated standard deviation of 2.80. Once again, vast interpatient variation is evident. Interestingly, patient 14 (S.F.) exhibited only 0.20 breaks/cell, while her simultaneous spontaneous breakage rate with M150 was only 0.17 breaks/cell. This patient also showed

comparatively low spontaneous and MMC-induced breakage when her peripheral blood lymphocytes were cultured in F10 medium (0.10 and 0.30 breaks/cell respectively).

4.3.2.1.1.3.1 Comparison of homozygote MMC-induced chromosome breakage data with other studies

There appear to be relatively few studies with similar protocols on the clastogenic effects of MMC in FA. No comparative table was therefore drawn up.

Sasaki and Tonomura (1973) stressed peripheral blood lymphocytes from 5 FA homozygotes with 0.01 μg MMC/ml culture for the last 24 hours of the usual 72-hour culture period (as opposed to the last 42 hours in 66-hour cultures in the present study). These authors reported a breakage range of 0.28 to 28.64 chromatid aberrations/cell (mean = 13.27). The present results in FA homozygotes, although not as dramatic in fluctuation, thus confirm this wide interpatient variation in susceptibility to the clastogenic effects of MMC. The same authors noted a mean spontaneous breakage rate of 1.73 breaks/cell in 6 FA patients. This value is relatively high, possibly explaining their much higher MMC-induced breakage range compared to the present study. Also, a 24-hour exposure to the mutagen results in most cells only being exposed to MMC for 1 cell cycle. In the present study (of 48-hour exposure periods) a number of cells, especially the more severely damaged, would have been 'lost' (probably blocked in the G2 phase of the cell cycle) prior to the second mitotic cycle.

Cohen et al. (1982b), stressed 3 FA lymphoblastoid cell-lines for 24 hours with 0.01 μg MMC/ml culture. This resulted in an average of 1.89 breaks/cell. This figure, although only based on 3 cell-lines (but 300 cells) is strikingly close to the means obtained in the present study viz. 1.85 breaks/cell with F 10 (n = 7) and 2.23 break/cell with M150 (n = 3).

4.3.2.1.2. Heterozygotes

4.3.2.1.2.1 Spontaneous chromosome breakage in Fanconi's anaemia heterozygotes

a) Hams F10 culture medium

A total of 27 obligatory heterozygotes were screened for the presence of spontaneous chromosomal aberrations after culturing for 66 hours in Hams F10. A standard deviation of 0.03 breaks/cell was found to be associated with a mean breakage rate of 0.03 breaks/cell (range 0.00 - 0.08 breaks/cell). It should be noted that the upper limits of this range overlap with the lower limits of the range found in affected homozygotes. The mean breakage values do however, differ significantly. (See section 4.4.2.1) (Refer to section 4.3.2.1.3 for a comparison with normal control subjects.)

b) M150 culture medium

M150 cultures of 6 heterozygotes yielded almost identical results to simultaneous F10 cultures of the same bloods. (See Appendix A) Using M150, a mean of 0.03 breaks/cell, with an associated standard deviation of 0.02, was noted. Breakage ranged from 0.00 - 0.07 breaks/cell. With F10, an average of 0.035 breaks/cell was recorded and the range was 0.00 - 0.08 breaks/cell in these 6 individuals. In the 3 homozygote M150 cultures, slightly higher breakage rates were noted compared to the F10 cultures from the same 3 patients. (See section 4.3.2.1.1.1) No such difference was evident in the heterozygotes.

4.3.2.1.2.1.1 Comparison of heterozygote spontaneous chromosome breakage data with other studies

The breakage results obtained using both F10 and M150 media, although based on a far larger sample size, correlate extremely well with

previously reported figures. (See Table 3.11) The heterozygote breakage range observed by Cohen et al. (1982a) also overlaps with the lower limits of their homozygote range. Auerbach et al. (1981) and Marx et al. (1983) did not note a similar overlap.

4.3.2.1.2.2 DEB-induced chromosome breakage in Fanconi's anaemia heterozygotes

Peripheral blood lymphocytes from obligatory heterozygotes were stressed with DEB at concentrations ranging from 0.01 to 1.0 μg/ml. All cultures were established in Hams F10 medium.

DEB at a concentration of 0.01 μg/ml

0.01 μg DEB/ml culture was added to 3 FA heterozygote cultures. The mean breakage rate was 0.12 breaks/cell (range 0.03 to 0.30 breaks/cell) and the associated standard deviation was 0.13 (0.03 breaks/cell were noted in 2 of the heterozygotes, while the third exhibited 0.30 breaks/cell; the latter value being skewed by the presence of 1 cell with 7 acentric fragments). Using the same concentration of DEB, Cohen et al. (1982a) found a mean breakage value of 0.04 breaks/cell in 10 heterozygote cases, whereas Marx et al. (1983) recorded a mean of 0.30 breaks/cell in 14 heterozygotes. Quite clearly, it is evident that different laboratories, although employing identical concentrations and ostensibly very similar techniques, report vastly different results.

DEB at a concentration of 0.1 μg/ml

Stressing heterozygote peripheral blood lymphocytes from 12 subjects with 0.1 μg DEB/ml culture resulted in a mean breakage rate of 0.08 breaks/cell. A standard deviation of 0.06 was found to be associated with this mean. The observed breakage rates varied from a minimum of 0.00 to a maximum of 0.23 breaks/cell. Once again, as with spontaneous breakage, this range overlaps with that of the homozygotes, eventhough the means differ significantly. (See section 4.4.2.2) It should be noted that only 1 homozygous patient (F.P.,

patient 77) was found to fall within the heterozygote range. As discussed in section 4.3.2.1.1.2, there has been some debate as to whether F.P. is in fact a heterozygote, rather than a homozygote. If he were excluded from the homozygote group, there would have been no overlap with the heterozygote group. (For comparisons with normal controls, see section 4.3.2.1.3.)

DEB at a concentration of 0.2 μg/ml

A breakage range of 0.00 - 0.26 breaks/cell was recorded in 6 heterozygote cultures stressed with 0.2 μg DEB/ml culture. The mean breakage rate and the associated standard deviation were 0.08 and 0.09 breaks/cell respectively, the latter value giving an indication of how great a variation existed. This mean is identical to that recorded after stressing with half this concentration of DEB (i.e. 0.1 μg/ml) and slightly lower than that noted when 0.01 μg DEB/ml was added. However, no conclusions can at this stage be drawn since only 3 heterozygotes were investigated for the clastogenic effect of 0.01 μg DEB/ml and, as mentioned previously, one of these three heterozygotes showed excessive breakage.

DEB at a concentration of 0.4 μg/ml

The mean breakage rate noted after screening 0.4 μg DEB/ml treated cultures from 5 obligatory heterozygotes was 0.03 breaks/cell. The standard deviation was 0.03, while breakage rates ranged from 0.00 to 0.06 breaks/cell. Thus, the mean breakage rate was in fact lower than that found after stressing the cells with lower concentrations of DEB. This finding is perhaps an indication that although higher concentrations of the mutagen may induce proportionally more chromosomal aberrations, the more severely damaged a cell, the less chance it has of reaching the mitotic stage of the cell cycle. Consequently, only those cells with relatively little breakage will be cytogenetically detectable. It should however be pointed out that the mitotic index was not noticeably lower with higher DEB concentrations, but this aspect was not specifically assessed.

DEB at a concentration of 1.0 μg/ml

Only 3 heterozygotes were stressed with 1.0 μg DEB/ml culture since at this concentration a lower mitotic index was apparent. Using this clastogenic concentration, a mean breakage value of 0.04 ± 0.04 breaks/cell was recorded. Breakage ranged from 0.00 to 0.1 breaks/cell. Once again, it appears that cells with a large number of chromosomal aberrations did not reach mitosis. The lower than expected breakage rates in these 3 cases were not due to individual low susceptibility to chromosomal fragility since they showed higher breakage when simultaneously stressed with 0.1 μg DEB/ml culture (refer to appendix A).

Therefore, it is clear that a direct relationship between mutagenic concentration and clastogenic response was in most instances not apparent. These findings confirm those of Cohen et al. (1982a), who, in some of their heterozygote cases, also did not find the number of chromosomal aberrations to be directly proportional to the mutagenic concentration employed. It therefore appears that cytogenetic evaluation is limited by cytotoxicity in the determination of dose-response curves, since the higher the dose of mutagen the smaller the number of growing cells. Furthermore, selection is involved since only that part of the cell population which is less damaged, or is less susceptible, survives.

The mean breakage recorded in the present study (viz. 0.04 breaks/cell) was extremely low compared to that noted by Cohen et al. (1982a) and Marx et al. (1983). These authors recorded mean breakage values of 1.30 and 1.19 respectively after stressing their heterozygotes with 1.0 μg DEB/ml culture. The reasons for this large discrepancy are uncertain, but may have been related to the excessive DEB toxicity being experienced in the laboratory at the time. (See section 4.6)

4.3.2.1.2.3 MMC-induced chromosome breakage in Fanconi's anaemia heterozygotes

MMC at a concentration of 0.01 μg/ml

a) Hams F10 culture medium

Nine heterozygotes were screened for chromosomal aberrations after being stressed with 0.01 μg MMC/ml culture and cultured in Hams F10 medium. The result[illegible] mean breakage rate was 0.10 breaks/cell and the associated standard deviation was 0.07 breaks/cell. Breakage values ranged from a low of 0.00 to a high of 0.17 breaks/cell. Thus, a ± 3-fold increase was noted over the spontaneous breakage rate. (A 4-fold increase was noted with an equivalent concentration of DEB i.e. 0.01 μg/ml, but fewer subjects were included in that investigation.) Once again, as with DEB, although the mean breakage rates of homozygotes and heterozygotes differ significantly (see section 4.4.3.3), the upper limits of the heterozygote breakage range overlap with the lower limits of the homozygote breakage range. On closer inspection, it may be noted that only 1 homozygote falls within the heterozygote range - patient 19 (M.G.). As discussed in section 4.3.2.1.1.3, this little girl may well have been misclassified and appears not to be an FA homozygote. If this 'patient' were excluded from the homozygote group, there would be no overlap between homozygote and heterozygote responses to treatment with 0.01 μg MMC/ml culture.

b) M150 culture medium

The 2 heterozygotes cultured in M150 and stressed with MMC at a concentration of 0.01 μg/ml culture yielded 0.13 and 0.50 breaks/cell respectively, (i.e. a mean of 0.32 ± 0.18 breaks/cell). Breakage rates of 0.17 and 0.11 breaks/cell respectively, were recorded in simultaneous F10 plus 0.01 μg MMC/ml cultures. Although no definite conclusions can be based on only 2 patients, it does appear that considerable intrapatient variation was evident.

4.3.2.1.2.3 MMC-induced chromosome breakage in Fanconi's anaemia heterozygotes

MMC at a concentration of 0.01 μg/ml

a) Hams F10 culture medium

Nine heterozygotes were screened for chromosomal aberrations after being stressed with 0.01 μg MMC/ml culture and cultured in Hams F10 medium. The resultant mean breakage rate was 0.10 breaks/cell and the associated standard deviation was 0.07 breaks/cell. Breakage values ranged from a low of 0.00 to a high of 0.17 breaks/cell. Thus, a ± 3-fold increase was noted over the spontaneous breakage rate. (A 4-fold increase was noted with an equivalent concentration of DEB i.e. 0.01 μg/ml, but fewer subjects were included in that investigation.) Once again, as with DEB, although the mean breakage rates of homozygotes and heterozygotes differ significantly (see section 4.4.3.3), the upper limits of the heterozygote breakage range overlap with the lower limits of the homozygote breakage range. On closer inspection, it may be noted that only 1 homozygote falls within the heterozygote range - patient 19 (M.G.). As discussed in section 4.3.2.1.1.3, this little girl may well have been misclassified and appears not to be an FA homozygote. If this 'patient' were excluded from the homozygote group, there would be no overlap between homozygote and heterozygote responses to treatment with 0.01 μg MMC/ml culture.

b) M150 culture medium

The 2 heterozygotes cultured in M150 and stressed with MMC at a concentration of 0.01 μg/ml culture yielded 0.13 and 0.50 breaks/cell respectively, (i.e. a mean of 0.32 ± 0.18 breaks/cell). Breakage rates of 0.17 and 0.11 breaks/cell respectively, were recorded in simultaneous F10 plus 0.01 μg MMC/ml cultures. Although no definite conclusions can be based on only 2 patients, it does appear that considerable intrapatient variation was evident.

MMC at a concentration of 0.05 μg/ml

0.05 μg MMC/ml culture induced a mean breakage rate of 0.21 breaks/cell (standard deviation = 0.15) in Hams F10 cultures from 4 obligatory heterozygotes. Breakage values ranged from 0.03 to 0.23 breaks/cell. Although this mean is higher than that noted in F10 plus 0.01 μg MMC/ml cultures, the breakage ranges were very similar, both showing wide fluctuations. Cohen et al. (1982a) reported breakage rates of 0.73 and 0.19 breaks/cell in 2 patients after stressing their cultures with 0.05 μg MMC/ml. The present findings confirm the latter results and highlight the marked interpatient variation in chromosome breakage which may be seen both spontaneously and/or after clastogenic treatment.

4.3.2.1.3 Controls

4.3.2.1.3.1 Spontaneous chromosome breakage in normal controls

a) Hams F10 culture medium

A total of 41 subjects served as controls for non-stressed cultures established in Hams F10 medium. The control breakage values varied from 0.00 to 0.07 breaks/cell. The mean breakage rate was 0.01 breaks/cell and this was associated with a standard deviation of 0.02. It can thus be seen that the average basal level of 'normal' spontaneously occurring (i.e. probably technical in origin) breakage in the writer's laboratory is extremely low. However, the range of breakage values coincided almost exactly with that found in the heterozygote class and overlapped with the lower limits of the homozygote range. Two of the 24 homozygotes investigated fell into the upper normal breakage range - cases 57 (L.M.) and 77 (F.P. - discussed in section 4.3.2.1.1.2).

b) M150 culture medium

Using M150 as the culture medium, an average spontaneous chromosome breakage rate of 0.03 ± 0.04 breaks/cell was detected in 5 controls. Breakage values ranged from 0.00 to 0.10 breaks/cell. Thus, a slightly higher breakage rate was evident, compared to Hams F10 cultures. This M150 control range does however, overlap completely with that found in heterozygotes cultured under similar conditions and the mean spontaneous breakage rate in the latter class is identical to that found in normal controls. However, there was no overlap with homozygotes cultured under similar conditions.

4.3.2.1.3.1.1 Comparison of control spontaneous chromosome breakage data with other studies

The breakage ranges and means noted in normal controls in the present study are similar to those reported in previous studies employing far smaller sample sizes (refer to Table 3.11 which compares Hams F10 results from this study with other similar studies using different culture media).

4.3.2.1.3.2 DEB-induced chromosome breakage in normal controls

Peripheral blood lymphocytes from normal controls were stressed with DEB at concentrations ranging from 0.01 to 1.0 μg/ml. All cultures were established in Hams F10 medium.

DEB at a concentration of 0.01 μg/ml

The one peripheral lymphocyte culture which served as a control for the 3 simultaneously established heterozygote cultures stressed with 0.01 μg DEB/ml culture (see section 4.3.2.1.2.2), exhibited no breakage.

DEB at a concentration of 0.1 μg/ml

Twenty controls were screened for the clastogenic effect of 0.1 μg DEB/ml culture. The mean breakage rate was 0.02 ± 0.02 breaks/cell and breakage values ranged from 0.00 to 0.06 breaks/cell. Although this mean is very slightly higher than that noted in untreated (i.e. spontaneous) cultures (mean = 0.01 breaks/cell), the two ranges are almost identical, indicating that these cultures, from 'normal', healthy individuals, were in no way adversely affected by this dose of DEB. The present mean breakage rate is slightly lower than those found in previous studies, but the comparative ranges do not differ considerably. (See Table 3.12)

It must however, be noted that in this study, there was once again considerable overlap of the control and heterozygous breakage ranges and 1 'homozygote' (patient F.P. - discussed in section 4.3.2.1.1.2), falls within the normal and heterozygous ranges. Both Auerbach et al. (1981) and Cohen et al. (1982a) also noted overlapping between their normal and heterozygous classes following stressing with 0.1 μg DEB/ml culture. Cohen et al. (1982a) found that their whole control range overlapped with their FA homozygote range.

DEB at a concentration of 0.2 μg/ml

The 2 control cultures stressed with 0.2 μg DEB/ml culture exhibited 0.00 and 0.02 breaks/cell respectively, giving a mean breakage rate of 0.01 ± 0.01 breaks/cell. This mean is identical to the spontaneous breakage rate with Hams F10 medium. Two of the heterozygotes simultaneously studied also showed no breakage and a further one had only 0.02 breaks/cell. Therefore, based on only 2 cases, at this concentration of DEB (as with 0.01 and 0.1 μg/ml), no distinction could be made between heterozygotes and controls. (No homozygotes were investigated at this concentration.)

DEB at a concentration of 0.4 μg/ml

A mean breakage rate of 0.01 breaks/cell and an associated standard deviation of 0.01 were recorded in 4 control cultures treated with

0.4 µg DEB/ml culture. Two of these controls exhibited no breakage, whereas 0.02 breaks/cell were present in the other two cultures. Therefore, this dose, similar to the previously discussed concentrations of DEB, did not appear to enhance the number of chromosome aberrations in these control cultures - the values reported here are identical to those found in the much larger non-treated control sample. (See section 4.3.2.1.3.1). Some of the heterozygote cultures stressed with an equal amount of DEB (i.e. 0.4 µg/ml) had breakage rates falling within this stressed control range, but the heterozygote mean was higher (viz. 0.03 breaks/cell).

DEB at a concentration of 1.0 µg/ml

The 2 control cultures treated with 1.0 µg DEB/ml culture had breakage rates of 0.00 and 0.03 breaks/cell, while the 3 heterozygotes stressed with an equal concentration of DEB had 0.00; 0.03 and 0.10 breaks/cell respectively. Although the sample size was small, it nevertheless appears that increasing the concentration of DEB does unfortunately not result in the unequivocal differentiation of most heterozygotes and normal controls.

4.3.2.1.3.3 MMC-induced chromosome breakage in normal controls

MMC at a concentration of 0.01 µg/ml

a) Hams F10 culture medium

The 14 normal controls treated with 0.01 µg MMC/ml culture exhibited, on average, a 6-fold increase in breakage over the mean normal spontaneous breakage rate. The mean breakage rate in stressed cultures was 0.06 breaks/cell and the associated standard deviation was 0.06. Breakage ranged from a low of 0.00 breaks/cell to a high of 0.20 breaks/cell. Thus, unlike DEB, MMC at a concentration of 0.01 µg/ml does appear to have a statistically significant effect on normal controls. (See section 4.4.1) This normal range of induced breakage unfortunately overlaps completely with the MMC-stressed

heterozygote range. One homozygote (M.G.) also falls within this control range, but as discussed in section 4.3.2.1.1.3, this patient appears to have been misclassified and may in fact not be an FA homozygote.

b) M150 culture medium

Extensive interpatient variation in chromosome breakage was recorded following the addition of 0.01 μg MMC/ml culture to 3 control cultures. One control culture exhibited 0.23 breaks/cell, another 0.10 breaks/cell and the third had 4.0 breaks/cell, a value 10 times higher than that found in one simultaneously studied homozygote! With respect to the latter control, the entire slide revealed only 6 metaphases, giving an indication of the extreme toxic effect of MMC in this culture. (It should be pointed out that the same untreated specimen cultured simultaneously with M150 had a normal mitotic index.) This toxicity and accompanying high aberration rate, was not evident in the F10 + MMC culture which was processed at the same time and to which the same MMC solution was added: 0.10 breaks/cell were noted in 30 cells screened.

MMC at a concentration of 0.05 μg/ml

Using a Hams F10 medium, a dose of 0.05 μg MMC/ml culture resulted in 1 control exhibiting 0.17 breaks/cell and the other having 0.57 breaks/cell. The latter value was higher than that found in 2 heterozygotes simultaneously studied with the same concentration of MMC and also established in Hams F10 medium (0.44 and 0.03 breaks/cell respectively), while a healthy 21 year-old sibling also investigated at the same time, exhibited 0.86 breaks/cell. (It should be pointed out that the control in question is a healthy, English-speaking fellow laboratory worker, and is therefore unlikely to be an FA heterozygote.) These cases serve to emphasize the importance of simultaneously studying normal controls - occasionally normal control cultures may be highly sensitive to MMC, especially at higher concentrations, and based on breakage values alone, misdiagnosis is possible.

4.3.2.1.4. Sibs

As previously stated (see section 3.3.2.1.4), siblings were specifically not requested for the purposes of the present study. Occasionally however, bloods were received and these were then processed. Because of the low number of sibs investigated, it is almost impossible to draw any definite conclusions. Nevertheless, for the sake of completeness, the results obtained will be briefly discussed.

4.3.2.1.4.1 Spontaneous chromosome breakage in siblings

a) Hams F10 culture medium

All 6 sibs included in the present study were screened for the presence of spontaneously occurring chro some aberrations using Hams F10 as the culture medium. The mean breakage rate was 0.01 breaks/cell (range 0.00 to 0.04 breaks/cell) and the associated standard deviation was 0.02 breaks/cell. This mean is identical to that found in the normal control group and the breakage range falls within that noted in the heterozygote group. These results are somewhat expected since siblings of affected individuals may be either completely normal or heterozygous for the FA gene.

b) M150 culture medium

Two sibling M150 cultures exhibited 0.03 and 0.07 breaks/cell respectively. A breakage rate of 0.03 breaks/cell was recorded in the simultaneous F10 cultures of both subjects.

4.3.2.1.4.1.1 Comparison of sibling spontaneous chromosome breakage data with other studies

The 3 siblings investigated by Auerbach et al. (1981) exhibited, on average, 0.01 breaks/cell. This value is identical to that noted in

the 6 sibling F10 cultures.

4.3.2.1.4.2 DEB-induced chromosome breakage in siblings

Following stressing of Hams F10 peripheral blood lymphocyte cultures with 0.1 μg DEB/ml culture, a mean induced breakage rate of 0.04 breaks/cell $\pm$ 0.04 was recorded in 3 sibling Hams F10 cultures. Breakage ranged from 0.00 to 0.06 breaks/cell. This range is identical to that found in the DEB stressed control group. In contrast, Auerbach et al. (1981) reported a mean breakage rate of 0.21 breaks/cell (range : 0.12 to 0.34 breaks/cell) in the 3 siblings they studied. This finding is rather unexpected since the same 3 siblings had a mean spontaneous breakage rate equal to that found in the present study with Hams F10 medium, i.e. 0.01 breaks/cell. It is, of course, possible that the 3 siblings investigated by these authors may have been heterozygotes, while those included in the present study may, by chance, all have FA^-/FA^- genotypes.

4.3.2.1.4.3 MMC-induced chromosome breakage in siblings

MMC at a concentration of 0.01 μg/ml

Only one sib Hams F10 culture was stressed with 0.01 μg MMC/ml culture; 0.23 breaks/cell were recorded. This is approximately an 8-fold increase over the spontaneous breakage rate in this sibling. It should be remembered that the control group was also sensitive to the clastogenic effects of MMC and therefore the observation in this sibling is probably of no significance.

MMC at a concentration of 0.05 μg/ml

Two siblings showed 0.10 and 0.83 breaks/cell respectively after stressing Hams F10 cultures with 0.05 μg MMC/ml culture. Although the latter value is high, the normal control investigated at the same time exhibited 0.57 breaks/cell, emphasizing the importance of simultaneously, under identical conditions, studying normal controls.

4.3.2.1.5 Features

4.3.2.1.5.1 Spontaneous chromosome breakage in subjects with features of Fanconi's anaemia

A total of 15 individuals displaying features of FA, but not having sufficient clinical and/or haematological stigmata to be classified as FA homozygotes (refer to section 2.1.1 for criteria), were screened for the presence of spontaneously occurring chromosomal aberrations. A mean breakage rate of 0.02 breaks/cell was recorded in Hams F10 cultures. The associated standard deviation was 0.03 breaks/cell and breakage in this group ranged from 0.00 to 0.08 breaks/cell. This range overlaps to a greater or lesser extent with the ranges noted in all the genotypic/phenotypic classes. The latter finding is quite feasible since individuals belonging to the 'features' class may be normal for the FA gene, heterozygous for the FA gene (since some FA heterozygotes may exhibit some clinical features of FA - see section 1.4.3), or have a variant form of FA with variable expression of the clinical characteristics of the disorder.

4.3.2.1.5.1.1 Comparison of spontaneous chromosome breakage data from the 'features' class with other studies

The 'features' class investigated by Auerbach et al. (1981) also had a mean spontaneous breakage rate of 0.03 breaks/cell (0.00 - 0.07 breaks/cell), while that of Cohen et al. (1982a) exhibited a mean value of 0.29 breaks/cells (0.06 - 0.41 breaks/cell). (See Table 3.11) It is quite possible that the application of more stringent guidelines to the classification of FA homozygotes may have resulted in subjects being included in the latter author's 'features' class which other workers may have included as FA homozygotes. Unfortunately, a stringent clinical and haematological classification before chromosome studies were done, was not always possible in the present study, because of the numerous centres from which patients

were referred for investigation. Furthermore, full clinical details were not always supplied at the time of chromosomal investigation. These factors may have contributed to the misclassification of, for example, some individuals with features of FA.

4.3.2.1.5.2 DEB-induced chromsome breakage in subjects with features of Fanconi's anaemia

DEB at a concentration of 0.1 μg/ml culture induced a mean breakage rate of 0.02 ± 0.02 breaks/cell (range 0.00 - 0.04 breaks/cell) in Hams F10 cultures from 5 patients displaying some stigmata of FA. This mean value is equal to that found in non-stressed cultures, indicating that the cells studied were not unduly susceptible to the clastogenic effects of DEB at this concentration. These figures are very slightly lower than those of Auerbach et al. (1981) and much lower than those of Cohen et al. (1982a). (See Table 3.12) As previously explained (section 4.3.2.1.5.1.1) differences in genotypic classification methodology may have led to the latter discrepancies.

4.3.2.1.5.3 MMC-induced chromosome breakage in subjects with features of Fanconi's anaemia

As with DEB, MMC [0.01 μg/ml] also did not greatly enhance the number of spontaneously occurring aberrations in 5 'features' individuals. Breakage ranged from 0.00 to 0.03 breaks/cell and the mean breakage rate was 0.02 breaks/cell in Hams F10 cultures. This mean was associated with a standard deviation of 0.02 breaks/cell. Interestingly, in contrast with these results, MMC was found to greatly enhance chromosome breakage in normal controls. (See section 4.4.1) This inconsistency highlights the variability in results following MMC treatment.

It therefore follows that, based on induced breakage rates in this group, the patients exhibiting some features of FA are either normal, heterozygous, or have a form of FA not displaying abnormal

chromosomal fragility. Furthermore, the stressing of these cells did not contribute to a definitive diagnosis.

In conclusion, when reviewing the chromosome breakage results obtained spontaneously and following clastogenic stressing (section 4.3.2.1), it is evident from the breakage ranges that considerable interpatient variation was observed. This problem of individual variability not only pertains to overlaps in the observed breakage rates among genotypic classes, but also to the variability observed within each class and the spread among the different genotypic/phenotypic classes. This marked 'within' class variability is reflected in the wide breakage ranges; those individuals at the extremes of the ranges are discussed in the relevant sections. Even though the significance of the overlap in breakage ranges has been alluded to in section 4.3.2.1, it is nevertheless necessary to once again emphasize the problems relating to these marked differences in breakage which may be noted even within the homozygote group, for example (see section 4.3.2.1.1).

Because of the broad phenotypic diversity characteristic of FA, chromosome breakage has often been suggested as a laboratory diagnostic tool to detect affected individuals (see section 1.6). Clearly, the frequency of aberrant cells detected varied widely among FA homozygotes, heterozygotes, normal controls, sibs and individuals with features of FA. Although these differing breakage rates may represent actual individual differences, this postulate does not always hold true, since intrapatient variation is also well documented (see sections 1.6.1.2.1 and 4.3.2.1.1.1).

The above-mentioned considerations are, of course, of prime importance in a chromosome breakage study and focus attention on the fact that breakage results must always be viewed in conjunction with clinical and haematological data.

4.3.2.2 Cellular instability

It should be pointed out that the cellular instability values in no way reflect the breakage rates *per se* i.e. the mean number of cells with breaks as opposed to the mean number of breaks per cell.

4.3.2.2.1. Spontaneous cellular instability

In the present study, the 24 homozygotes displayed a mean spontaneous cellular instability rate of about 22%. (This figure includes cells showing chromatid or isochromatid gaps). (See Table 3.13) This finding correlates extremely well with the classic report of Schroeder *et al.* (1964); they described two brothers with FA who exhibited spontaneous chromosomal aberrations in about 25% of cells examined. Although the mean instability rate in the present study is similar to that of Schroeder *et al.*, it should be noted that a wide range in the percentage affected cells was evident in the 24 patients investigated; 8% to 47% of cells were unstable.

As expected, the heterozygote, control, sib and features classes were all characterised by mean spontaneous instability rates much lower than those seen in the homozygote class (these differences have been statistically analysed in section 4.4.3). Relatively small instability ranges were observed. (Refer to Table 3.13)

4.3.2.2.2 DEB- and MMC-induced cellular instability

The 13 homozygotes stressed with 0.1 μg DEB/ml had a mean cellular instability rate of 79% (range 8% to 100%). (Refer to Table 3.13) Patient F.P., a possible homozygote but probable heterozygote (see section 4.3.2.1.1.2), displayed aberrations in only 8% of his cells, while the next lowest rate was 33%. Eight of the 13 patients had a

cellular instability value of more than 90%. It therefore follows that very little 'clustering' occurred, i.e. the chromosome aberrations were fairly evenly distributed throughout the cellular population that was screened. Similar observations were made following MMC (0.01 μg/ml) treatment - a mean cellular instability rate of 70% was recorded (range 17% to 100%).

DEB and MMC slightly increased the instability rates (and chromosome breakage values - see section 4.4.1) in the heterozygote, sib, features and control groups. (See section 4.4.3 for statistical analysis.) Once again, the observed ranges in the mean percentage unstable cells were generally fairly small after DEB stressing. Wider ranges were noted in the heterozygotes and controls following MMC stressing. It should be remembered that only 1 sib was screened for the presence of MMC-induced chromosomal aberrations and thus no meaningful observations can be made with respect to instability rates in this group.

4.3.3 Qualitative analysis of spontaneous and clastogen-induced chromosomal aberrations

4.3.3.1 Spontaneous chromosomal aberrations

The most common spontaneous chromosomal aberrations in untreated cultures were chromatid breaks followed by chromatid gaps. (See section 3.3.3) Chromosome-type (i.e. isochromatid) aberrations were in turn more frequent than exchange configurations. Similar findings have previously been reported by Schroeder and German, 1974; Polani, 1976 and Schroeder, 1982.

The FA homozygotes collectively had many more spontaneous rearrangements than the other classes - 17 in 926 cells as opposed to 1 in 946 cells in heterozygotes and the complete absence of reunion figures in the sib, feature and control groups.

Only 3 of 926 cells were endoreduplicated in FA homozygotes. The

heterozygotes and controls each had 1 endoreduplication in 946 and 1550 cells respectively. Although an excessive tendency for endoreduplication to occur spontaneously in FA lymphocytes in culture has been reported, the present results do not substantiate those findings. (Schroeder et al. 1964; von Koskull and Aula, 1973).

4.3.3.2 DEB [0.1 μg/ml]- and MMC [0.01 μg/ml]-induced chromosomal aberrations

Qualitatively, the breakage caused by the clastogens DEB and MMC at concentrations of 0.1 and 0.01 μg/ml culture respectively, was similar to that which occurred spontaneously in cells belonging to all 5 genotypic/phenotypic classes - chromatid breaks were the most frequent aberration both before and after stressing. These results are in concordance with the original findings of Auerbach and Wolman (1976). Chemical treatment did however, increase the incidence of all types of aberrations. (See section 3.3.3) The large numbers of induced chromosomal aberrations in the homozygotes were not found to be concentrated in a few cells, but were usually distributed throughout the majority of the cells screened. This is substantiated by the results of statistical analyses employing 'breakage rates' and 'cellular instability rates' - the latter two sets of data yielded identical conclusions. (See section 4.4)

Neither DEB nor MMC increased the number of endoreduplications in FA homozygotes. Endoreduplications were never found in both treated and untreated cells from the same patient.

When treated with 0.1 μg DEB/ml culture, FA homozygote lymphocytes responded with approximately a 14-fold increase in chromosomal rearrangements/reunion figures compared with the number of spontaneously occurring exchange configurations. MMC at a concentration of 0.01 μg/ml culture caused an 8-fold increase in the number of reunion figures. The high number of induced rearrangements noted is probably a function of the greatly increased number of 'open' breaks seen in treated cells, so allowing exchanges to occur. If there are increased 'open' breaks, there should also be an

increase in the number of concurrently occurring acentric fragments - acentrics were indeed increased from 1.6% to 14.7% with DEB and to 8.3% with MMC treatment.

Clearly then, the FA homozygotes certainly show a tendency towards higher numbers of spontaneous and mutagen-induced structural rearrangements, when compared to normal controls, obligatory heterozygotes, sibs and individuals with features of FA. It should be noted that not all homozygotes exhibited reunion-type aberrations, indicating that some overlap does exist. It is however, possible that if a large number of cells were screened, reunion figures might have been observed in all homozygotes. If this were found to be a consistent finding, some workers may in the future prefer to screen for these figures only, should this technique prove to be labour-saving. Since a large number of cells would be required for such a technique, a practical difficulty may be encountered in that FA homozygotes are usually typified by low mitotic indices. (See section 1.7.1)

Although DEB significantly increased overall chromosome breakage in heterozygotes (see section 3.4.1), the number of rearrangements was not increased by DEB stressing - 1 ring in 946 cells was noted spontaneously, while 1 ring in 520 cells was noted following DEB stressing. (These rings did not occur in the same individuals.) No rearrangements were seen in MMC-stressed heterozygote cells. Control, sib and feature individuals exhibited no spontaneous or DEB-induced exchange configurations. The one and only sib stressed with MMC had 1 quadriradial figure in 30 cells screened and 1 dicentric was noted in 170 MMC-stressed cells of patients with features of FA.

Therefore, the mean frequences of DEB- and MMC-induced rearrangements in heterozygotes do not differ greatly from those in controls, sibs and individuals exhibiting some features of FA. It should however be noted that the number of rearrangements observed is extremely small and in future a far larger number of cells should be screened in suspected heterozygotes before truly valid statistical conclusions can be drawn. In accordance with the present findings, Cohen et al.

(1982a) noted no significant difference ($p>0.05$) between the number of DEB-induced rearrangements in heterozygotes and normals. This finding is in contrast to the results obtained by Auerbach et al. (1981). The latter authors noted that in addition to the increased number of chromosome breaks/cell following DEB [0.1 μg/ml] treatment, a significantly increased frequency of induced triradial and quadriradial configurations in the lymphocytes from all non-FA individuals was also recorded. This finding was certainly not confirmed by the present study.

To sum up, chromatid breaks were the most frequent chromosomal aberrations in all 5 genotypic/phenotypic classes both before and after mutagen stressing. Endoreduplication occurred at a lower than anticipated frequency in FA homozygotes and DEB- and MMC-stressed cells did not show an increased tendency towards endoreduplication. The number of spontaneously occurring rearrangements was relatively high in FA homozygotes, and especially so after treatment with DEB and MMC. However, stressing the cells of heterozygotes, normal controls, sibs or patients with features of FA did not obviously increase the number of exchange configurations. It follows that the screening for 'reunion-type' aberrations only, may possibly be of use in the detection of homozygotes, and this technique warrants future accurate investigation.

4.3.4 The effect of ethnic origin on chromosome breakage results

As noted in section 3.3.4, the Black and White homozygote spontaneous breakage rates did not differ significantly. It should also be pointed out that a chromosome breakage study by Pinto (1983) on 23 Black and 63 White South African individuals did not reveal any significant difference between spontaneous breakage rates in these normal controls.

The findings of strikingly similar breakage values in the Black and White patients indicates that the molecular defect could be the same in these 2 groups of patients. However, this presumption may not be valid since, for example, different molecular mutations in β-

thalassaemia (Orkin, 1983) and Duchenne muscular dystrophy (Monaco et al., 1985) are known to result in the same phenotypic expression. Furthermore, the clinical course appears to be similar in Black and White patients (this aspect is still in the process of being accurately assessed - Prof. L. MacDougall, 1985 - personal communication).

Therefore, to sum up, there is at present no reason to suspect that the genetic defect differs in White and Black patients.

4.4 STATISTICAL ANALYSIS OF SPONTANEOUS AND CLASTOGEN- INDUCED PERIPHERAL BLOOD LYMPHOCYTE CHROMOSOME BREAKAGE RESULTS, CELLULAR INSTABILITY RATES AND NUMBER OF 'BREAKAGE-TYPE' ABERRATIONS

4.4.1 'Within classes' analysis of breakage data

DEB at a concentration of 0.1 µg/ml culture induced a highly significant increase in the number of chromosomal aberrations when compared to spontaneous breakage values of patients homozygous for the FA gene. By contrast, DEB did not have a significant effect on chromosome breakage in obligatory heterozygotes, controls and individuals with features of FA. (The reader is referred to section 3.4.1)

In the homozygote group, 0.01 µg MMC/ml culture also significantly enhanced chromosome breakage values. Although no such effect was noted in the heterozygote and features groups, MMC was also noted to have a significant clastogenic effect in the normal control group. The latter observation is difficult to explain since if MMC has a significant clastogenic effect in normal controls, a similar effect would be expected in the heterozygote and features classes.

It is of interest to note the similarity between data including gaps and data excluding these aberrations. In the homozygote group, DEB was found to have a highly significant effect ($p<0.01$) both when gaps were included and excluded in the calculation of breakage rates.

Similar findings were noted when homozygote cultures were stressed with MMC. In the control group, MMC had a highly significant effect ($p<0.01$) when gaps were taken into consideration and a significant effect ($p = 0.0177$) when gaps were not included in the breakage rates. This slight discrepancy is probably attributable to there being a smaller number of 'non-zero differences' in the 'Spont- vs MMC-' comparison than in the 'Spont+ vs MMC+' comparison. Clearly then, the inclusion of gaps in the screening for chromosomal aberrations appears to have no effect on the final results.

4.4.2 'Between classes' analysis of breakage data

The reader is referred to section 3.4.2.

4.4.2.1 Spontaneous chromosome breakage

Collectively, the homozygote group exhibited spontaneous breakage rates significantly greater than those of the heterozygote, sib, features and control groups. This significant difference is evident both when gaps are included and excluded in the calculation of aberration rates. No other genotypic/phenotypic class comparisons revealed significant differences in inherent (i.e. spontaneous) breakage values. Therefore, although homozygotes may, as a group, be distinguished from all other classes on the basis of spontaneous breakage rates, no distinction is possible between FA heterozygotes, sibs, patients with features of FA and normal individuals. It is however imperative to remember that the lower limits of the homozygote spontaneous breakage range overlap with the upper limits of the heterozygote, sib, features and control spontaneous breakage ranges. (See section 3.3.2.1.)

So as to exclude possible cases of 'non-paternity', a separate analysis was performed using breakage values observed in mothers only (HET^M). The exclusion of fathers from the heterozygote group did not have any statistically noticeable effect on the overall results.

4.4.2.2 DEB [0.1 μg/ml]-induced chromosome breakage

DEB-stressed FA homozygote cells had significantly higher breakage rates than similarly stressed heterozygote (both including and excluding the fathers), features and normal control cells. The fact that only 2 sibs were included in this analysis probably accounts for there being no significant difference between the sib and homozygote groups. Although DEB-induced chromosome breakage values were similar in some of the heterozygotes and normal controls, these 2 groups as a whole, responded significantly differently to 0.1 μg DEB/ml culture. (This difference was not noted in non-stressed cultures.)

Thus, even though the heterozygote and control groups are statistically highly distinguishable, DEB stress cannot be considered a definitive discriminator of individual putative carriers. This finding is in concordance with other published results, (Auerbach et al., 1981; Cohen et al., 1982a, b; Cervenka and Hirsch, 1983). No distinction could however, be made between the heterozygote, sib and features classes. Once again, the inclusion of gaps in the breakage rates had no noticeable effect on the overall conclusions. Similarly, the exclusion of fathers from the heterozygote group did not alter the results.

4.4.2.3 MMC [0.01 μg/ml]-induced chromosome breakage

Stressing with MMC enables clear differentiation between FA homozygotes and heterozygotes, individuals with features of FA and normal controls. When the homozygotes were compared to heterozygote mothers, a significant difference in breakage was noted when gaps were included in the calculation of aberration rates but not when they were excluded - the fact that only 4 MMC-treated mothers were included in this analysis probably accounts for this discrepancy. Only 1 sib was stressed with MMC, explaining the non-significant difference between sibs and homozygotes. Unlike DEB, MMC did not have a significant effect on heterozygotes when compared to normal controls. It should however, be remembered that MMC at a concentration of 0.01 μg/ml was found to have a significant

clastogenic effect in normal controls. (See section 4.4.1)

4.4.2.4 Summary and conclusions

'Within classes' comparison

The Wilcoxon signed ranks test showed both MMC and DEB to significantly enhance the number of spontaneously occurring chromosomal aberrations in FA homozygotes. DEB appeared to have no significant effect in normal control subjects, while control cells exhibited increased susceptibility to MMC. Heterozygotes, sibs and individuals with features of FA showed no increased sensitivity to DEB or MMC clastogenicity.

'Between classes' comparison

For the 3 variables primarily considered i.e. F10 - spontaneous breakage; F10 + DEB [0.1 μg/ml] induced breakage and F10 + MMC [0.01 μg/ml] induced breakage, the genotypic/phenotypic classes were compared in a pair-wise fashion by means of the U-test of Mann-Whitney. With respect to spontaneous breakage, only the FA homozygote group could be distinguished from all other groups. However, following DEB stressing, both homozygotes and heterozygotes could be distinguished from normal controls. The homozygote group could also be distinguished from the heterozygote and features classes. (The sib group was too small for statistically valid conclusions to be drawn.) MMC at a concentration of 0.01 μg/ml had a similar effect to DEB in the homozygotes i.e. following MMC stressing, the homozygote breakage values were found to differ significantly from those of all the other classes.

The inclusion of gaps in the calculation of breakage rates was at no time found to alter any conclusion, while the exclusion of fathers from the heterozygote group was also generally found not to have any effect on the overall results.

Therefore, on the basis of these observations, it appears that both

DEB and MMC stressing are reliable and accurate techniques for the definitive cytogenetic diagnosis of FA homozygosity. However, the extent of chromosomal fragility (both spontaneous and in response to clastogenic stress) must always be assessed within the context of the family under study and relevant family data are essential so as to ascertain possible familial levels of sensitivity. Furthermore, it is imperative to simultaneously establish normal control cultures so as to detect any unusual external clastogenic factors which may be operating at the time to produce a higher than expected number of chromosomal aberrations. The latter considerations are of the utmost importance when the prenatal diagnosis of FA, using induced chromosome breakage, is being considered. It is therefore advocated that the DEB-stress test in combination with the MMC-stress test be applied in all problematic cases of aplastic anaemia and in all cases where some stigmata of FA are exhibited. However, the diagnosis of FA must under no circumstances be made purely on the basis of an increased response to clastogenic stress - only in the presence of full clinical and cytogenetic data should a definitive diagnosis be made.

With respect to heterozygotes, the present study provides no evidence that individual putative carriers may be differentiated from normal individuals on the basis of spontaneous, DEB- or MMC-induced chromosome 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 possible carrier can be interpreted. This conclusion was also reached by Auerbach et al. (1981) and Cohen et al. (1982b) using DEB, Cervenka et al. (1981) using MMC and Berger et al. (1980a) using nitrogen mustard. Only Marx et al. (1983) noted a clear, individual distinction between heterozygotes and normal controls.

4.4.3 'Between classes' analysis of cellular instability rates

Both spontaneous and drug-induced cellular instability rates were subjected to statistical analysis.

4.4.3.1 Spontaneous cellular instability

The homozygotes, as a group, had spontaneous cellular instability rates significantly greater than those noted in the heterozygotes, sib features and control groups. No other phenotypic/genotypic class comparisons revealed significant differences in inherent cellular instability. These results are identical to those obtained when the spontaneous breakage data were subjected to statistical analysis. (See section 4.4.2.1) The latter observation is not unexpected since a clustering of aberrations was never noted and very few cells revealed more than 1 break/cell. Consequently, in this case, the cellular instability rate is in fact a direct reflection of the breakage rates within the cells screened.

4.4.3.2 DEB [0.1 μg/ml]-induced cellular instability

Following DEB stressing with 0.1 μg/ml culture, FA homozygote cells showed cellular instability rates significantly higher than those recorded in similarly stressed heterozygote, feature and normal control cells. Although the sib and homozygote classes did not differ significantly, it must be remembered that only 3 siblings were included in this analysis. The heterozygote and control groups differed significantly following DEB stressing. These results are once again identical to those obtained when the DEB-induced breakage data were statistically analyzed. (See section 4.4.2.2)

4.4.3.3 MMC [0.01 μg/ml]-induced cellular instability

MMC [0.01 μg/ml]-stressed homozygote peripheral blood lymphocytes exhibited significantly more cellular instability than similarly treated heterozygote, feature and control cells. Although the statistical results show homozygotes not to be distinguishable from sibs, it must be remembered that only 1 sib was included in this analysis and therefore no valid conclusions can be drawn. It is of importance to note that based on cellular instability rates, MMC does not enable the differentiation of heterozygotes and controls. The

latter finding is in contrast to the DEB-induced findings. (See section 4.4.3.2) These results are identical to those obtained when the MMC-induced chromosome breakage data were subjected to statistical analysis. (See section 4.4.2.3)

4.4.3.4 Summary

Statistical analysis of spontaneous, DEB- and MMC-induced cellular instability rates (% unstable cells) yielded results identical to those obtained when the respective chromosome breakage rates (breaks/cell) were statistically analyzed. (See section 4.4.2.4.) It is therefore apparent that in the present study, the cellular instability rates may be regarded as being a reflection of the chromosome instability rates.

4.4.4 'Between classes' analysis of the number of 'breakage-type' aberrations

The basic data tabulated in Tables 3.14 to 3.19 clearly show that once again the breakage ranges overlap to a greater or lesser extent. This was also noted when all aberrations (i.e. 'breakage-' and 'reunion-type' aberrations) were included in the calculations of breakage rates. (See section 3.3.2.1)

The U-test of Mann-Whitney revealed that the spontaneous homozygote 'breakage-type' aberration rates differed significantly from the heterozygote (i.e. mothers and fathers), sib, feature and control values. Following DEB and MMC stressing, the homozygotes once again had significantly more 'breakage-type' aberrations than the heterozygotes, feature and control groups. In addition, the heterozygotes and controls differed significantly after treatment with DEB (no such difference was noted following MMC treatment). Although no distinction was apparent between the homozygote and sib groups subsequent to DEB and MMC stressing, it must be remembered that very few sibs were subjected to mutagenic treatment, thus detracting from the validity of these statistical conclusions.

The above-mentioned findings are identical to the results obtained when the cumulative breakage values (i.e. all aberrations) were subjected to the U-test of Mann-Whitney. This indicates that the 'reunion-type' aberrations in fact contributed very little to the observed rates. The only class showing a relatively high number of 'reunion-type' aberrations, especially after clastogenic treatment, was the homozygotes, but this group also exhibited high numbers of 'breakage-type' aberrations. (The 'reunion-type' aberration rates unfortunately could not be subjected to statistical analysis since in all classes except the homozygotes, both spontaneously and following DEB- and MMC-stressing, exceptionally few 'reunion-type' aberrations were noted - see section 4.3.3).

4.4.4.1 Summary

On the basis of 'breakage-type' aberrations only, the homozygotes could be distinguished from all other groups. However, it is quite clear that even the separation of 'breakage-' and 'reunion-type' aberrations does not allow for the definitive distinction of FA heterozygotes and normals. Although the FA carriers, as a group, displayed significantly more DEB-induced 'breakage-type' aberrations than the controls, the breakage ranges overlapped considerably. However, the screening for 'reunion-type' aberrations only, may possibly be of use in the detection of homozygotes. (See section 4.3.3)

4.5 AMNIOTIC FLUID CELL AND FIBROBLAST STUDIES

4.5.1 Discussion of results of amniotic fluid cell and fibroblast studies

The mean spontaneous chromosome breakage rate in cultured 'at-risk' amniotic fluid cells was 0.23 breaks/cell. The most frequent type of aberration observed was chromatid breaks. One triradial figure was also noted in the 30 metaphases screened. DEB at a concentration of

0.1 µg/ml culture induced 0.53 breaks/cell. When the DEB [0.1 µg/ml]-containing medium was removed 48 hours prior to harvesting, only 0.27 breaks/cell were noted. A breakage rate of 0.67 breaks/cell was noted following treatment with 0.5 µg DEB/ml culture. Cells exposed to DEB at a concentration of 0.5 µg/ml culture and then grown in fresh medium for 48 hours before harvesting, exhibited 0.53 breaks/cell. (See Table 3.31)

Skin fibroblasts from an FA homozygote had a spontaneous breakage rate of 0.33 breaks/cell. DEB-induced breakage values in this homozygote ranged from 0.47 to 1.27 breaks/cell. (Refer to Table 3.32 for details)

By comparison, 0.03 spontaneous breaks/cell were noted in 'normal' control amniotic cells. Treatment with DEB at a concentration of 0.1 µg/ml culture resulted in 0.13 breaks/cell plus 1 endoreduplication in 30 control cells. All other DEB treatments in this control yielded only 0.03 breaks/cell i.e. the same as the spontaneous breakage rate.

Quite clearly, both the spontaneous and drug-induced chromosome breakage noted in the 'at-risk' amniotic cells are very similar to that seen in the simultaneously cultured FA fibroblasts, and markedly elevated in comparison to the normal control amniotic cells. Amniotic cultures from the same 'at-risk' pregnancy were simultaneously investigated in another laboratory in South Africa. These workers also noted increased spontaneous and DEB-induced breakage, confirming the present findings (Marx et al., 1982). On the basis of the above results, the foetus was diagnosed as being homozygous for the FA gene, and the parents decided to terminate the pregnancy.

Abortion was induced in the 22nd week of pregnancy. The level of spontaneous chromosome breakage in foetal skin fibroblasts was 0.20 breaks/cell and DEB-induced breakage values ranged from 0.47 to 1.17 breaks/cell. This DEB-induced breakage range is strikingly similar to that noted in the FA fibroblasts studied (0.47 to 1.27 breaks/cell).

After termination of the pregnancy, the female foetus was closely examined and the only external abnormality found was a proximally placed malformed thumb. No other congenital malformations were noted externally. An autopsy examination was not performed. The significantly increased chromosome breakage detected in the foetal fibroblasts together with the presence of an abnormal thumb were considered to be highly suggestive confirmatory evidence that the prenatal diagnosis of FA homozygosity was correct.

With reference to Tables 3.31 and 3.32, it is clear that 'breakage-type' aberrations were far in excess of 'reunion-type' aberrations. Similar to the peripheral blood lymphocyte results (see section 3.3.3), remarkably few rearrangements were noted, even after DEB-treatment. However in the control amniotic cells, not one reunion figure was seen. A very low number of endoreduplicated cells was noted in the affected foetus and FA fibroblasts. One endoreduplication was seen in control cells. The latter findings confirm the peripheral blood lymphocyte results which indicated that FA homozygotes and heterozygotes are not characterized by greatly increased numbers of endoreduplicated cells. (See section 3.3.3) More breakage was generally recorded after stressing the cells with 0.5 μg DEB/ml culture compared with a dose of 0.1 μg DEB/ml culture. Furthermore, removal of DEB 48 hours prior to harvesting usually resulted in less breakage, but a higher mitotic index. DEB at a concentration of 0.1 μg/ml induced far less breakage in the affected amniotic cells and FA fibroblasts when compared with the effects of this optimal 'stressing' concentration of DEB on FA lymphocytes. It is therefore advocated that future 'at-risk' amniotic cell cultures be treated with at least 2 concentrations of DEB and that the cells be exposed to DEB for as long as possible without adversely affecting the mitotic index.

4.5.1.1 General comments

FA was first recognised as being potentially prenatally diagnosable in 1979, when Auerbach et al. (1979a) cultured cells from foetal membranes obtained following an elective abortion in a pregnancy

'at-risk' for FA. These cells exhibited spontaneous and DEB-induced chromosomal breakage consistent with that seen in FA heterozygotes previously investigated by these workers (Auerbach and Wolman, 1978). These encouraging results led to attempted diagnoses of FA in cultured amniotic cells using DEB as a clastogen.

To date, at least 30 pregnancies 'at-risk' for FA have been monitored for the presence of increased spontaneous and DEB-induced chromosome breakage (Auerbach et al., 1985). Seven of these foetuses were prenatally diagnosed as affected by FA; 2 were carried to term and resulted in the birth of clinically affected children. The other 5 pregnancies were terminated. One newborn and 2 abortuses had congenital malformations, including abnormalities of the thumb and radius. The other affected liveborn (age 5½ years at the time of writing), has severe growth retardation and pancytopenia. The prenatal diagnosis of normal foetuses in the other 23 pregnancies 'at-risk' for FA were confirmed after birth by the absence of congenital abnormalities and by the normal response of peripheral blood lymphocytes to DEB in all cases in which tissue was available for study.

Amniocentesis is generally regarded as being of low risk to the mother and foetus, but cannot be performed prior to 15 - 16 weeks of gestation. The time required for culturing the amniotic cells and for analysis of cytogenetic and biochemical or molecular data, is such that a definitive diagnosis is often only available at 20 to 22 weeks of gestation. It would therefore be advantageous if the diagnosis of an abnormal foetus could be made in the first trimester of pregnancy, as this would offer a greater benefit in terms of a reduced waiting period for results and a safer method of abortion should an abnormality be identified. To this end, a number of investigators have recently made much progress in developing a safe and reliable technique for obtaining [illegible]nic villus biopsies in the first trimester of pregnancy, and in [illegible], have established the practicality of these methods. (Simoni et al., 1983; Ward et al., 1983; Smidt-Jensen and Hahnemann, 1984.)

Recently, Auerbach et al. (1985) reported on an investigation to

determine the feasibility of performing prenatal diagnosis in 'at-risk' pregnancies for FA, using chorionic villus cells cultured in the presence of DEB. Four of their series of 30 prenatal diagnoses were diagnosed as normal on the basis of chromosomal breakage studies in cells obtained by chorionic villus biopsy. Subsequent amniocentesis performed at 16 weeks of gestation also revealed no increased chromosomal breakage in amniotic cells from these 4 cases. The technique of chorionic villus biopsy thus holds great promise for the future diagnosis of affected foetuses.

Shipley et al. (1984) reported the first case in which foetal blood was investigated for the presence of increased chromosome breakage. Following 2 amniotic cell culture failures at 16 and 18 weeks of gestation, their patient was offered, and agreed to, foetal blood sampling, which was performed as described by Rodeck (1980). The 'at-risk' foetus was shown to be affected by FA on the basis of excessive spontaneous and MMC-induced breakage in 72-hour cultured peripheral blood lymphocytes. Shipley et al. (1984) found no evidence that foetal blood responds differently from children's or adult's blood to MMC and they were able to detect marked hypersensitivity to this clastogen in the foetus 'at-risk' for FA. Thus, no differences in drug metabolism between foetal and non-foetal blood cultures were evident. These authors therefore advocate that even though the risk of foetal loss (2% to 3%) following foetal blood sampling is higher than that associated with amniocentesis (0.5% to 1%), the former procedure should be considered as an alternative technique in the diagnosis of chromosome breakage syndromes.

The technical merits associated with foetal blood sampling include a shorter time in which results can be obtained (4 days as opposed to ± 3 weeks following amniocentesis); the greater ease in setting up parallel control cultures for obligatory heterozygotes and the index patient; and the higher mitotic index generally obtained in blood cultures relative to fibroblast or amniotic cell cultures. However, these advantages must be considered in the light of the slightly higher risk of foetoscopy with regard to foetal loss and possible prematurity and perinatal morbidity.

4.5.1.2 Summary and conclusions

The results of the present study on amniotic cell and fibroblast cultures are in accordance with previously published findings and conclusively show that cytogenetic analysis of amniotic fluid cells can provide a reliable and relatively safe method for the prenatal diagnosis of FA. The fact that FA heterozygotes are not clearly distinguishable from normal controls on the basis of chromosome breakage is of no consequence in the prenatal diagnosis of FA, since heterozygotes are generally 'silent' carriers of the FA gene. (See section 1.4.3)

It is advocated that ultrasonography accompany amniocentesis, in an attempt to identify congenital malformations such as radial defects. However, it is imperative that the ultrasound findings be considered in conjunction with amniotic cell chromosome breakage studies since the inability to demonstrate abnormalities on sonar scan, or the absence of physical abnormalities does not rule out a diagnosis of FA. Furthermore, some FA heterozygotes may exhibit some of the somatic features typical of FA homozygosity. (See section 1.4.3) Finally, it must also be remembered that some FA homozygotes are not characterized by increased chromosomal fragility. (See section 1.6) Therefore, in order to contemplate prenatal diagnosis of FA in any one particular family, the affected member/s of the family must exhibit spontaneous and/or induced chromosome breakage clearly distinguishable from that seen in heterozygotes from the same family and normal unrelated controls. Given these conditions, amniotic cells, foetal blood and chorionic villi can be used for the prenatal diagnosis of FA.

4.6 'TROUBLE-SHOOTING'

Mention must be made of a crippling problem which was encountered some 9 months after the commencement of the present study - the addition of DEB to the peripheral blood lymphocyte cultures apparently resulted in the cell cycle being 'blocked' at a stage prior to mitosis. All DEB-treated cultures, irrespective of the DEB

concentration or the FA genotype, displayed PHA-transformed, 'healthy-looking' cells, but no metaphases.

Problem solving strategy

Initially it was believed that the DEB stock solution had undergone a chemical alteration leading to a greater toxicity potential, perhaps the result of fluctuations in refrigeration temperature due to mechanical failure. No evidence was found to support this latter theory. At least 3 new bottles of DEB (Merck) were purchased but, again, all peripheral blood lymphocyte and fibroblast cultures stressed with DEB failed to yield any metaphases. It therefore seemed clear that it was not the DEB *per se* causing the problem, but rather an interaction between the DEB and another component, either biological (eg. an ingredient of the culture medium) or physical (eg. laboratory materials). Consequently, a number of experimental strategies were adopted in an attempt to identify the causative mechanism behind this problem.

Firstly, all physical materials used in the culturing process were in turn substituted, one at a time, for other brands or components. For example, sterile glass McCartney bottles were employed instead of Falcon centrifuge tubes; different brands of hypodermic needles and syringes were used, while some cultures were established without the use of needles. In a further set of experiments, M150 medium and foetal calf serum were used as substitutes for Hams F10 medium and pooled human AB serum. DEB was diluted in sterile saline or nutrient medium instead of the usual sterile distilled water. In addition to routine 66-hour cultures, 48-hour and 92-hour cultures were also established. Furthermore, DEB was added at initiation of culture and after 24, 36 and 48 hours of culture. The DEB concentrations used ranged from 0.01 μg/ml to 1.0 μg/ml. All experiments proved fruitless - in all cases the mitotic index was zero, whereas simultaneous cultures established on the *same* blood samples without DEB yielded satisfactory numbers of metaphases.

Because the cells were healthy and transformed in appearance, it was decided to investigate whether DNA synthesis was still occurring. A

number of different cultures were simultaneously established using the methods described in section 2.1.4. Some 42 hours after initiation of culture (i.e. 24 hours prior to harvesting), 3 x 1ml samples of each culture were transferred to separate microculture wells, allowing each sample to be investigated in triplicate. To each of these wells, 0.1 μCi [6-^{3}H] thymidine/ml culture was added for a further 24 hours at 37^{0}C. This culture 'mix' was then spotted onto filter paper discs, each of which was subsequently dissolved in 5ml scintillation 'cocktail' ('Estagel'). These samples were subsequently 'counted' in a scintillation counter. In the first such investigation, 4 individuals (1 control, 2 heterozygotes and 1 subject with features of FA) were studied under 12 different culture conditions. These results are tabulated in Table 4.1.

TABLE 4.1 Mean DPM* (disintegrations/minute) following uptake of [6-^{3}H] thymidine

CULTURE CONDITION	CON.	HET.	HET.	FEAT.
F10 SPONTANEOUS	44 645	11 965	28 498	57 258
F10; 0.1 μg DEB/ml	7 849	2 188	5 545	8 205
F10; 0.5 μg DEB/ml	9 205	3 061	6 320	8 569
F10; 1.0 μg DEB/ml	4 052	1 751	2 261	2 702
F10; 0.01 μg MMC/ml	47 018	14 806	27 260	52 783
F10; 0.1 μg MMC/ml	15 915	4 803	10 917	20 312
F10; 0.5 μg MMC/ml	7 747	1 480	8 029	9 020
M150; SPONTANEOUS	87 020	21 636	47 637	119 601
M150; 0.1 μg DEB/ml	14 768	3 420	7 297	9 824
M150; 0.5 μg DEB/ml	10 728	1 938	8 821	11 206
M150; 0.01 μg MMC/ml	63 546	18 138	13 711	81 044
M150; 0.1 μg MMC/ml	20 189	7 501	15 469	27 215

4 x BLANK samples : mean DPM = 17 (= background)

* Mean DPM based in each case on 3 samples.

The results in Table 4.1 indicate that thymidine was being incorporated i.e. DNA synthesis was occurring in all the cultures. The rate of synthesis was however lower in the spontaneous heterozygote culture than in the control and 'features' cultures. (Unfortunately, no homozygote peripheral bloods were available for study.) The cultures which did not have DEB or MMC added to them showed, not suprisingly, the highest uptake of [6-^{3}H] thymidine. It is of interest that the M150 cultures simultaneously processed from each of 4 cases (20 samples) nearly all showed a greater incorporation of thymidine than the F10 cultures - Hams F10 medium is generally regarded as being far richer in nutrients than M150. Due to the obvious increase in toxicity with increasing mutagen concentration, an inverse correlation between DEB/MMC concentration and thymidine uptake was generally noted. Strangely, there was a very small difference between those cultures stressed with 0.1 μg DEB/ml and those stressed with 0.5 μg DEB/ml - a slightly lower incorporation of thymidine was seen in the former cultures.

At the same time as setting up the above-mentioned cultures, duplicate cultures were established and subjected to identical conditions; [6-^{3}H] thymidine was however, not added to these duplicate cultures. These cultures were harvested after the usual 66 hours and screened for the presence of metaphases. Although metaphases were always noted in the F10 SPONTANEOUS, M150 SPONTANEOUS and plus MMC cultures, the mitotic index in those cultures to which DEB was added was always zero, eventhough the MMC and DEB synthesis results were fairly similar. (See Table 4.1)

It should be noted that the above test was repeated using 5 different bloods and 7 different culture conditions. Very similar results to those here described were once again obtained.

The results of these experiments measuring the uptake of thymidine therefore indicated that the DEB-stressed cells were probably passing through the S-phase of the cell cycle and thereafter being blocked in the G2 phase, prior to mitosis. Interestingly, flow cytometric studies of FA fibroblasts have revealed an accumulation of MMC-treated [0.05 or 0.1 μg/ml] cells in the G2 phase of the cell

cycle. (Kaiser et al., 1980, 1982; Latt et al., 1982). The reason for this accumulation of FA cells prior to mitosis remains to be determined. Latt et al. (1982) pointed out that damaged cells are often blocked in or near the G2 phase of the cell cycle and that this phenomenon is not unique for FA cells or to cells treated with MMC.

Quite plausibly then, in the present study, an unknown factor may have enhanced the toxicity of the DEB, resulting in damage so severe that the cells were blocked in G2.

At the time of experiencing the above-described culture problems, a similar study on FA was in progress at the University of the Orange Free State (U.O.F.S.), Bloemfontein. (Fig. 3.1) (See Marx et al., 1983). The DEB stock solution which was successfully being employed in the latter study was forwarded to the writer and duly added to a few control peripheral blood lymphocyte cultures - once again, the mitotic index was zero. This served as an indication that perhaps factors other than the mutagen itself, were causing this apparent block in the cell cycle.

The next step in pursuit of a solution was to travel to U.O.F.S. and establish cultures in that laboratory using their materials as well as materials identical to those being used in the writer's laboratory. It should be stressed that all materials - DEB, medium, serum, sterile water, fixative, syringes, needles, culture flasks, blood samples etc. were taken to Bloemfontein by the writer, but obviously this did not include centrifuges and incubators. However, the latter two pieces of equipment were checked and no problems had been encountered by the routine laboratory staff using the same equipment. A large series of different cultures were then established, basically comprising different combinations of 'Bloemfontein materials' and 'Johannesburg materials'. All these cultures were established in duplicate - one batch was set up by the writer and the other by an experienced laboratory worker. All cultures yielded metaphases. The self-same materials were then transported back to Johannesburg and DEB-treated cultures were established in the writer's laboratory and simultaneously in another laboratory in Johannesburg - the resultant mitotic index was in all cases zero!

A logical and rational explanation as to the extreme toxic effect that the DEB was at the time producing in Johannesbury was unfortunately never uncovered.

Section 5

5. GENERAL DISCUSSION AND CONCLUSIONS

Fanconi's anaemia (FA) is usually characterized by a variable natural history and age of onset, together with a wide range of clinical and pathological malformations which may be present either singly or in combination. (See section 1.4.2) Untreated children with FA have a poor prognosis - the average duration of life after the onset of pancytopenia is approximately 2 years, although much variation does exist (Gmyrek and Sylla-Rapoport, 1964). Death is commonly caused by haemorrhage or infection. Evidence is accumulating that those patients who survive their bone marrow aplasia are likely to develop a malignancy, either as a result of treatment, or more probably, due to the inherent nature of the FA disease process itself. (See section 5.3.5)

The present study has once again confirmed the extreme susceptibility of the chromosome complement of FA homozygotes (and to a limited extent, FA heterozygotes), to spontaneous and mutagen-induced chromosome breakage. This chapter is aimed at evaluating the significance of chromosome instability in the pathogenesis of this disorder, specifically with respect to the broader aetiological and pathogenetic aspects of oncogenesis.

5.1 CELLULAR IMPLICATIONS OF CHROMOSOME BREAKAGE IN FANCONI'S ANAEMIA

The consequences of chromosome breakage to the individual cell become evident at anaphase and telophase: acentric fragments will not be attached to the spindle and will therefore be lost from the cell; dicentric chromosomes would form bridges which would eventually break; chromatin masses which have not migrated properly to either pole could be enveloped by nuclear membranes, which would then manifest as micronuclei. All these events would lead to an imbalance of the genetic material in the daughter cells resulting in a reduction in their lifespan. There are however, important exceptions to this generalization, as some cells with gross changes in their genetic constitution may prove stable and survive to continue their

path through the cell cycle. (In this respect it should be noted that chromosomally abnormal clones have been observed in FA patients - see section 5.3.3).

It is probable that cells bearing chromosome abnormalities will not be able to divide. These cells are more likely to be eliminated *in vivo* than *in vitro*. This is evident in the finding of far more aberrations in cultured lymphocytes than in direct bone marrow preparations. (See section 1.6.1.4) Of greater significance are those cells bearing microscopically invisible structural anomalies such as point mutations, gene deletions and duplications, which may result from breakage-reunion events. Divisions of such cells would usually be unrestrained. Such cells may be regarded as the seeds of malignancy and together with cell loss, could initiate teratogenesis.

5.2 CONGENITAL MANIFESTATIONS OF CHROMOSOME BREAKAGE IN FANCONI'S ANAEMIA

It is possible that the chromosome aberrations seen in FA cells are simply epiphenomena which are secondary to a more basic metabolic disturbance. Alternatively, they may be a direct expression of the basic genetic defect and could be causally related to the haematologic findings and perhaps the congenital abnormalities seen in afflicted individuals (Bloom *et al.*, 1966). The feasibility of the latter hypothesis demands discussion.

Prior to implicating chromosome breakage in the pathogenesis of FA, it is essential to first establish the relationship between the *in vitro* findings and the actual *in vivo* events. This association may be clarified to some extent through the analysis of direct chromosome preparations from the bone marrow and cytological inspection of bone marrow smears from FA homozygotes. As discussed in some detail in section 1.6.1.4, chromosome breakage in direct FA bone marrow preparations is observed distinctly less frequently than in those cells subjected to *in vitro* culture conditions. In those cases where chromosomal instability is increased, the observed breakage rates are low. Reports of anaphase bridges and micronuclei in bone marrow

smears and the existence of chromosomally abnormal clones in freshly aspirated FA bone marrow, also point to the presence of *in vivo* chromosomal fragility. (See section 1.6.1.4)

Although direct bone marrow preparations are generally not characterized by unusually high chromosome breakage, it must be realized that *in vitro* results cannot be numerically applied to *in vivo* events. It should be borne in mind that a pre-existing clastogenic mechanism could be enhanced and/or provoked in culture. or alternately, a cellular deficiency which is evident *in vivo* could be corrected by the enriched culture medium (Schroeder and Kurth, 1971). Furthermore, as pointed out by Swift and Hirschhorn (1966), the mitogenic activity of PHA (phytohaemagglutinin) on slowly dividing lymphocytes has the effect of causing the simultaneous display of aberrations which have accumulated over a period of time, since many of those cells transformed by PHA may very well never have divided *in vivo*.

Overall then, there does appear to be evidence that chromosome breakage exists *in vivo* and could therefore be correlated to the clinical and haematologic stigmata which characterize FA.

In the majority of patients with FA, associated congenital abnormalities (if present) are usually recognized at birth or in early infancy. By comparison, the average age of onset of marrow aplasia in male FA patients is 6.6 years and 8.8 years in female FA patients (Gmyrek and Sylm-Rapoport, 1964). Therefore, any theory implicating chromosome breakage in the onset of marrow aplasia must take into account the fact that there exists an asymptomatic period in affected individuals who may have various congenital abnormalities before anaemia becomes manifest.

It should also be remembered that in individuals with acquired aplastic anaemia, chromatid and chromosome aberrations are an infrequent finding and leukaemia has seldom been recorded in such cases (Bloom *et al.*, 1966). Patients with Bloom's syndrome or ataxia telangiectasia for example, also exhibit greatly increased spontaneous chromosome breakage. (See section 1.6.5) These

patients however, do not develop pancytopenia. It would therefore appear that the postulated DNA repair defect in FA (see section 1.8) is selectively responsible for aplastic anaemia. However, paradoxically, the latter hypothesis appears unlikely, especially in view of the fact that increased chromosome breakage is observed even following steroid treatment. All these data point to the conclusion that the profound haematologic disturbances characterizing FA are not being directly and entirely caused by chromosome breakage.

It is, of course, possible that the genetic defect in FA is manifold and that progressive stem cell failure could result from the increased sensitivity of FA cells to cytotoxic effects and from their reduced growth rates. (See section 1.7.1) In this respect, it is interesting that De Vroede et al. (1982) described 2 siblings with FA in whom a simultaneous onset of pancytopenia was noted after 'possible exposure to common external agents'. Environmental, as well as genetic factors may therefore play an important aetiological role in the pathogenesis of this disorder. Alternatively, the marrow aplasia may not be evident for several years until the increasing haematologic demands of the growing child eventually overwhelm a system which may be defective in producing adequate enzyme(s) for normal erythropoiesis (Meisner et al., 1978).

To sum up, in FA it is a most tempting assumption to incriminate the fragility of the chromosomes in the haematological findings. However, on the basis of presently available evidence, it is extremely difficult to prove any association between chromosome aberrations and progressive pancytopenia and any causative explanation must be considered as being entirely speculative.

By contrast, some of the clinical stigmata characterizing FA can plausibly be linked to chromosome breakage. For example, cell death commencing in utero could, quite conceivably, cause overall growth retardation. In this respect it should be remembered that Bloom's syndrome is also characterized by short stature, but to a more marked extent than that seen in FA or AT. (See section 1.4.4) Similarly, the mental deficiency noted in some FA patients may be a reflection of a reduction in the overall number of brain cells. In tissues

with relatively slow rates of cell replacement, a low aberration frequency of 10% for example, would be functionally insignificant. However, during embryogenesis, areas of rapid cell multiplication such as the radial aspect of the forelimb buds, would be vulnerable to mitotic irregularities. This could explain the skeletal defects of the hands and forearms which are present in about 66% of FA patients (Alter et al., 1981).

Pigmentary anomalies, usually in the form of cafe-au-lait spots, have been described in approximately 77% of FA homozygotes (Ibid) and are common in Bloom's syndrome patients. (See section 1.4.4) Pigmentary changes are also present occasionally in XP and AT patients. (See section 1.4.4) Polani (1976) has suggested that this patchy pigmentation could reflect division difficulties during embryogenesis, occurring especially at particular developmental stages. Alternatively, the division error, resulting from widespread unrepaired genetic damage, could involve cells of the neural crest that ultimately contribute to the formation and function of pigment cells. It has also been proposed by German (1964; 1973a) that cafe-au-lait spots and depigmented spots could conceivably be the equivalent of twin spots in maize and Drosophila with the darker coloured spots representing a clone of cells homozygous for genes for darker pigmentation and the areas of hypopigmentation being representative of a clone homozygous for genes determining lighter pigmentation. This clonal homozygosity could be the result of somatic crossing-over or an increased mutation rate which is usually associated with increased chromosome instability.

The above findings lead to the assumption that chromosome breakage is probably an ongoing process. The manifestations of chromosome breakage should therefore be apparent in most fast growing tissues. This seems to be true for the bone marrow, but other rapidly dividing tissues such as the gut and respiratory epithelium appear to remain unaffected by such aberrations, based on lack of respiratory or gastro-intestinal manifestations in affected homozygotes.

In conclusion, it appears that chromosome breakage may well occur in vivo but based on presently available evidence, it is still uncertain

as to whether this cytogenetic abnormality is primarily responsible for any of the clinical and haematological stigmata present in FA homozygotes. It is nevertheless tempting to speculate that chromosomal damage induced by breakage events could lead to abortive or differential replication of different cell types, resulting in growth retardation and aplasia. Alternately, stable structural changes could result, leading to clonal proliferation of cells which may be homozygous for certain genes such as pigmentary genes, or may have a selective growth advantage and would be considered as being of malignant potential.

5.3 ONCOGENIC IMPLICATIONS OF CHROMOSOME BREAKAGE IN FANCONI'S ANAEMIA

Garriga and Crosby (1959) were the first to note an apparent concentration of leukaemia cases in families with children affected by FA. However, prior to this, there had been sporadic reports in the literature of this association between FA and malignancy and it was known that leukaemia was not an uncommon occurrence in families of patients with 'aplastic anaemia' (eg. Reinhold *et al.*, 1952).

5.3.1 Incidence of malignancy in Fanconi's anaemia homozygotes

Little is known about the incidence and hypothesized predisposition to malignant transformation in FA. Besides poor documentation of cases, a contributory factor may well be that in FA any predisposition to malignancy may not be fully expressed because, until relatively recently, few patients survived for long following the onset of aplastic anaemia. Consequently, were a neoplastic cell-line to emerge and replenish the bone marrow, it would have to do so prior to the patient succumbing to haemorrhage or infection. According to Dosik *et al.* (1970), the incidence of acute non-lymphoid leukaemia in FA is approximately 8%. Contrary to the latter finding, a review of the literature by Polani (1976), showed that only about 1 in 50 FA patients (i.e. 2%) develop leukaemia. Alter *et al.* (1981) have however, stated that acute myelogenous leukaemia appears to

occur terminally in 5% to 10% of patients with FA. This discrepancy in reported incidences is probably due to the fact that the actual frequency of the FA gene is at present not accurately known, since many cases may be misdiagnosed.

It is interesting to note that the genes for two other classic chromosome breakage syndromes, viz. Bloom's syndrome (BS) and ataxia telangiectasia (AT), also appear to result in an increased predisposition to the development of malignancy. The frequency of neoplasia in AT is thought to be about 10%, while the estimate of cancer in Bloom's syndrome is approximately 21% (Spector et al., 1982).

An important unifying characteristic of the three main chromosome instability syndromes (viz. FA, BS and AT) must therefore be the observations that individuals affected by these disorders are cancer prone. However, the phenotypic specificity associated with the different genotypes may also extend to specific patterns of malignancy in these diseases. (See section 5.3.2) Quite clearly then, there does appear to be a relationship between chromosomal instability, whether induced genetically as in FA for example, or environmentally, and the aetiology of malignancy. (This postulated association is to be discussed in section 5.3.5.2.)

5.3.2 Types of malignant neoplasms observed in Fanconi's anaemia homozygotes

Fanconi's anaemia homozygotes are primarily at an increased risk for the development of acute non-lymphocytic leukaemia (ANLL). Following a review of approximately 300 FA cases, Alter and Potter (1981) (cited by Auerbach et al., 1982) noted that ANLL had developed in 23 (8%) of these cases. Patients belonging to both the early and late onset groups (Zakrzewski and Sperling, 1980a) were represented in those 23 cases. More often than not, the FA patients who survive their pancytopenia and develop ANLL, usually die within 1 to 3 months of diagnosis of leukaemia (Alter and Potter, 1981 - cited by Auerbach et al., 1982).

Although there are numerous reports in the literature of acute leukaemia in FA, only a select few of these will be described or referred to.

In 1955, Cowdell et al. described a late onset FA patient viz. a 27 year-old male, who developed ANLL as the first haematologic manifestation of FA. The first case in the early onset group to manifest leukaemia in the absence of diagnosed bone marrow aplasia was reported 27 years later by Auerbach et al. (1982). The latter authors described a 6 year-old female FA patient who developed acute myeloid leukaemia (AML) as the first haematologic manifestation of this disorder. The diagnosis of FA was confirmed by the hypersensitivity to DEB displayed by her lymphocyte and bone marrow chromosomes. Unexpectedly, this child remained in remission some 18 months after diagnosis, even though her management was complicated by unusually increased sensitivity to chemotherapeutic agents. Although remission was achieved, the patient's bone marrow cells continued to exhibit chromosomally abnormal clones, in addition to abnormal culture patterns and cell interactions. An unusually high frequency of 'spontaneous' chromosome breakage was found in her lymphocytes and bone marrow cells following treatment. During remission, her marrow cells were found to include non-specific marker chromosomes as well. These latter findings can most likely be ascribed to an increased sensitivity to the clastogenic effect of chemotherapeutic agents in FA. The doses used are known to cause minimal chromosome damage in most cancer patients (Shinzel and Schmid, 1976).

Skikne et al. (1978) carried out serial haematological investigations in 5 FA patients over periods of 6 months to 11 years. Leukaemic transformation occurred in two of these patients. Their one patient first presented with FA at the age of 9 years and developed acute myelomonocytic leukaemia some 9½ years following initial presentation. In another of the patients, bone marrow findings at age 16 years (i.e 10 years after diagnosis of FA) were indicative of acute myeloblastic leukaemia. Both patients died shortly after the diagnosis of leukaemia. At least 2 more of this series of patients are known to have died of leukaemia subsequent to publication of

Skikne et al.'s report (Prof. Renee Bernstein, 1985 - personal communication). The natural history in the above patients is therefore in stark contrast to the unusual cases of FA in which leukaemic transformation is the first haematologic manifestation of the disease.

The development of acute erythroleukaemia in FA homozygotes has been reported by Meisner et al. (1978), Prindull et al. (1979), Berger et al. '1980c) and Rotzak et al. (1982).

Nowell et al. (1984) described 2 young sisters with hypoplastic anaemia and increased chromosome fragility. No phenotypic characteristics of 'classic' FA were present and consequently, these sibs were diagnosed as having the Estren-Dameshek variant of FA (Estren and Dameshek, 1947). One sister was found to have a cytogenetically abnormal clone in her bone marrow and developed a dysmyelopoietic preleukaemia which progressed to acute non-lymphocytic leukaemia. This appears to be the first case in which the full triad of increased chromosome breakage, a chromosomally altered clone and progression to leukaemia has been noted in this FA variant. However, little uniformity exists in the diagnosis of so-called FA variants and it is probable that cases of FA not exhibiting classic stigmata of the disorder are not always defined as having the Estren-Dameshek variant of this disease.

Further cases of ANLL in FA patients have been described by Garriga and Crosby (1959), Bloom et al. (1966), Dosik et al. (1970), Schroeder et al. (1976b), Bourgeois and Hill (1977), Evans (1977), Berger et al. (1977) and Schroeder et al. (1979). Although this is not a comprehensive list of ANLL cases associated with FA, it is nevertheless obvious from these reports that acute myelomonocytic leukaemia is the most common form of ANLL seen in FA patients. The age of presentation and the clinical course both prior to and following leukaemia transformation appear to differ widely from patient to patient. Interestingly, there is an apparent lack of reports describing lymphoblastic leukaemias in FA patients.

Although acute leukaemia is by far the most common malignant neoplasm

observed in FA patients, a number of solid tumours of diverse origin have also been noted. These include squamous cell carcinoma of the oesophagus, carcinoma of the anus and hepatomas (Swift, 1976b). In a review of available FA literature, Evans (1977) noted 5 cases of hepatoma - many of this group had been subjected to androgen therapy, which is thought to have provoked tumour formation. (The role of androgen therapy in the development of FA-associated malignancies is described in section 5.3.5.1)

By way of comparison, it is interesting to make mention of the types of malignant neoplasms observed in the 3 other classic chromosome breakage disorders. Ataxia telangiectasia patients are primarily at an increased risk for the development of acute lymphatic leukaemia and lymphoma, as well as dysgerminoma, gastric carcinoma, glioma, medulloblastoma and chronic lymphocytic leukaemia (Swift 1976b, Swift et al., 1976; Ray and German, 1981; Spector et al., 1982). Various malignancies have been described in xeroderma pigmentosum homozygotes. These include basal and squamous cell carcinomas, malignant melanomas, ocular malignancies, acute lymphoblastic leukaemia, sarcoma of the testis and carcinoma of the tongue (Swift, 1976b; Swift and Chase, 1979). In Bloom's syndrome, leukaemia, usually of the acute myeloid type, is the predominant malignancy, but carcinoma of the tongue, lymphomas and tumours of the respiratory and alimentary tracts and the kidney have also occurred (Swift, 1976b; Ray and German, 1981).

Quite clearly then, a vast array of widely different malignant neoplasms have been reported to be associated with the classic chromosome breakage syndromes. The unifying features of these disorders are, by definition, increased chromosomal fragility, and an apparent genetic predisposition to the development of malignancy. Taking the latter facts into account, it would appear at first sight that there is a direct relationship between chromosomal breakage and neoplastic transformation. The validity of this assumption is evaluated in section 5.3.5.2, since it is of course possible that the phenotypic specificity which is a reflection of the different genotypes, may also extend to the specific patterns of malignancy which characterize these disorders.

5.3.3 The occurrence and possible significance of chromosomally abnormal clones in Fanconi's anaemia homozygotes

At the First International Workshop on Chromosomes and Leukaemia (1977), it was agreed to define a cytogenetically abnormal clone as 'two cells with the same extra chromosome or same structural rearrangement, or three cells within the same missing chromosome'.

Human tumour cells are frequently characterized by nonrandom chromosome abnormalities; these are thought to reflect the clonal evolution of cell populations in which certain cytogenetic changes are associated with the possible initiation of malignant transformation and may confer proliferative advantage on the already malignant cell (Mitelman and Levan, 1978; Klein, 1979; Mitelman, 1984).

In view of the increased chromosomal instability which characterizes FA homozygotes, the development of cytogenetically abnormal clones is to be expected. Unstable aberrations such as chromatid and isochromatid gaps and breaks, acentrics and complex exchange configurations would probably be inconsequential in terms of long-lived alterations to the genome, since cells bearing such abnormalities if not repaired, would be unable to undergo further mitosis and would ultimately be eliminated from the body. Of far greater potential significance for the development of malignancy, are the stable chromosome rearrangements such as translocations and inversions which result from chromosome breakage and reunion of broken ends. These stable rearrangements may result in such subtle changes in the genetic material of the affected cells, that survival will probably not be curtailed; growth may in fact be enhanced. These mutant clones may thus assume numerical predominance.

In line with the above-mentioned predictions, Auerbach et al. (1980) described the evolution of a cytogenetically aberrant clone observed in a diploid fibroblast culture from a patient with FA. This clone displayed an unusual proliferative advantage and had an increased growth potential when compared with diploid FA fibroblast cells from the same patient i.e. the 'parental' FA cells. It should be

remembered that 'parental' FA fibroblasts have been shown to have decreased growth rates, increased generation times and a decreased ability to sustain mitotic capability (Weksberg et al., 1979). Auerbach et al. (1980) confirmed that FA fibroblasts appear to have a lower growth potential than normal cells. It is possible that the unusual characteristics of the clone described by the latter authors are the result of a mutation to the wild-type allele at the FA locus. However, the retention of instability in the cytogenetically aberrant clonal cells indicates that such a reversal could not have taken place. Reports of abnormal bone marrow clones with proliferative advantage have also come from Meisner et al. (1978) and Berger et al. (1979).

Published reports of abnormal clones detected in FA patients are relatively common. Cytogenetically aberrant clones have been observed in PHA-stimulated lymphocytes (Schroeder et al., 1976a; Berger et al., 1977, Harnden, 1977; Zaizov et al., 1978; German and Warburton, unpublished results - quoted by Ray and German, 1981), dermal fibroblasts (Beard et al., 1973; Auerbach et al., 1980), and freshly aspirated bone marrow preparations (Hirschman et al., 1969; Crossen et al., 1972; Lisker and de Gutierrez 1974; Bargman et al., 1977; Berger et al., 1977, Bourgeois and Hill, 1977, Harnden, 1977; Meisner et al., 1978). An increased tendency to form chromosomally abnormal myeloid clones in the bone marrow is observed in both the anaemic phase (Hirschman et al., 1969; Crossen et al. 1972; Lisker and de Gutierrez, 1974; Pe[illegible]na et al., 1977; Zaizov et al., 1978; Meisner et al., 1978; Berger et al., 1980c) and the leukaemic phase (Berger et al., 1977; Bourgeois and Hill, 1977; Meisner et al., 1978; Obeid et al., 1980). Thus far, the presence of cytogenetically abnormal lymphoid clones in peripheral blood has not been reported in non-leukaemic FA patients. There also appear to be no reports of lymphoid leukaemias associated with FA. (See section 5.3.2)

In many of the clones reported in FA homozygotes, A-group and C-group chromosomes are frequently involved. However, the consistency of this involvement is far less than that found in AT clones which are usually characterized by the specific involvement of one, and

sometimes both, number 14 chromosomes (Hecht et al., 1973; Ray and German, 1981). A striking array of clones in AT with rearrangements involving chromosome 7, often in conjunction with chromosome 14, has also been recorded (Ray and German, 1981).

In a 6-year serial study of a male FA patient, Schroeder et al. (1976a) noted 5 different abnormal clones in PHA-stimulated blood lymphocyte cultures. Each clone was however, only present in a frequency of less than 1% and would quite possibly have been overlooked had serial chromosome analyses over several years, not been performed. Berger et al. (1977) also observed that some lymphocytic and bone marrow clones may be present in relatively low frequencies. These findings imply that clones with abnormal chromosome complements may occur with a far higher frequency than is currently recognized, but are simply not being detected because relatively few cells (usually at the most 50 to 100) are analyzed.

In the present study, 10 G-banded metaphases were analyzed in detail and 30 to 50 cells were screened for breakage - no abnormal clones were detected. (See section 4.2) Although this finding was expected, since none of the patients were diagnosed as having leukaemia or a myelodysplastic syndrome at the time of investigation, it is possible that some clones occurring at low frequencies may have been overlooked, because of the low number of cells analyzed.

Whatever the cause of the emergence of a chromosomally abnormal clone in FA homozygotes, the presence of such an aberrant cell-line is presumed to be of significance in the aetiology of cancer in these patients - otherwise clonal abnormalities would be as commonly detected in normal tissues. It is however, important (but usually impossible), to determine whether a transformed neoplastic clone was the particular one in which a particular chromosome rearrangement occurred. This has significant implications in assessing whether a cell with an abnormal chromosome complement should be considered as being malignant and the progenitor of a neoplastic stem-line.

Such a constant clone was serially traced by Saxon et al. (1979), who were able to follow an abnormal clone with a t(14;14) in

non-malignant T-lymphocytes through to a T-cell lymphoma in a patient with AT. On the other hand, Wake et al. (1982) noted a t(14;14) in the non-leukaemic cells of an AT patient, but a different clonal abnormality was seen in the leukaemic cells. This observation led to the conclusion that in the latter case, the malignant cells probably did not originate from the cells with the pre-existing t(14;14). The query that follows is whether stable clonal chromosome aberrations per se lead to cancer or whether the chromosome abnormalities found to characterize malignant neoplasms are merely epiphenomena. This question will be addressed in section 5.3.5.2.

5.3.4 Malignant disease in Fanconi's anaemia heterozygotes

Although affected homozygotes for the autosomal recessive, neoplasia-predisposing syndromes such as FA, ataxia telangiectasia, Bloom's syndrome, xeroderma pigmentosum, Werner's syndrome, dyskeratosis congenita and Chediak Higashi syndrome are rare, the clinically normal heterozygous carriers occur relatively more frequently. For example, Swift (1971;1976a) has estimated that FA heterozygotes probably occur at a minimum frequency of approximately 1 in 300 (based on New York State FA birth incidences from 1956 to 1967). The present study has indicated that the FA gene may be carried by at least 1 in 83 White Afrikaans-speaking South Africans. (See section 4.1.3.1) Since the homozygotes for these diseases are known to be at an increased risk of malignancy, it follows that the presumptive carriers may similarly be at a greater risk for acquiring cancer than the general population.

An initial study by Swift (1971) on the causes of death in 8 families of patients with FA, showed that heterozygotes for the FA gene appeared to have a risk of dying from malignant neoplasms about 3 times that of non-carriers. In the above study, all relatives (n=102), extending to first cousins once removed, were included, rather than only obligatory heterozygotes, so as to enable the assessment of a larger number of individuals with a wider distribution of ages. A malignant neoplasm was found to be the underlying cause of death in 27 cases, compared to the 17.4 deaths

expected ($p<0.05$). The estimated relative risk of malignancy compared to the general population was 2.6 for males and 1.1 for females.

In 1974, Swift et al. pointed out that the above results should be regarded with caution, since, among other possible factors, FA may be present primarily in a population sub-group or stratum in which malignant neoplasms are unusually frequent, independent of the FA gene. If this postulate was true, then the supposed increased risk of dying from malignant neoplasm might not be associated with the FA gene. To test this alternative hypothesis, data were clearly required on the illnesses and causes of death in 'non-blood' relatives.

Hill (1976) investigated the causes of death in 18 relatives of a child with FA; 10 deaths were found to be due to carcinoma of various organs. These data, although based on a small sample size, were regarded as being suggestive that heterozygotes for the FA gene are at an increased risk for cancer development.

In 1980, Swift et al. published the results of a study on 25 extended families of FA probands who were investigated for a possible excess of deaths due to malignancies. The sample comprised 601 male blood relatives, 605 female blood relatives, 265 male spouse controls and 268 female spouse controls. The non-blood relatives (spouses of aunts, uncles, first cousins as well as spouses of grandparents' siblings and probands' siblings) were included to provide a control population for comparison. This study differed from the earlier one in the number of families (25 compared to 8), in the greater diversity of ethnic origins and places of residence of the study subjects, in the use of spouse controls, and in the inclusion of only blood relatives whose probability of heterozygosity was at least 0.23 (compared to 0.125 in the previous study).

The above extended investigation of Swift et al. (1980) demonstrated no overall excess of cancers or cancer deaths for any age or sex category of blood relatives, and no statistical increase in frequency of specific types of malignancy among the obligatory heterozygotes. (As pointed out in section 5.3.2, acute leukaemias are the most

common neoplasias seen in FA homozygotes.) In fact, deaths from leukaemia among blood relatives of FA patients were fewer than expected. The FA blood relatives exhibited more cases and deaths from bladder, stomach and breast cancer than expected, but these differences were not statistically significant. An excess of deaths from lung and stomach cancer at an early age, was also noted among the FA blood relatives, but again this was not significant. However, among spouse controls, there were fewer deaths than expected from bladder, stomach and breast cancer, leading to the proposal that the expected numbers may be inappropriately high for this sample.

Swift et al. (1980) therefore concluded that since this study was larger and better controlled than the previous study (Swift, 1971), the more recent investigation demonstrating that presumptive carriers of the FA gene are not at an increased risk of malignancy, must supersede the earlier report. The question of predisposition to bladder, stomach and breast cancer among putative FA heterozygotes, remains unresolved and at this stage, only tentative conclusions should be drawn as to the possible association of the FA gene with these malignancies. Auerbach and Wolman (1978) have pointed out that a reliable test to identify FA heterozygotes would make a direct assessment of the frequency of malignant neoplasms in carriers of the FA gene possible.

The findings of a relatively recent study by Potter et al. (1983) were in concordance with those of Swift et al. (1980). The cancer risk in related family members (n=125) of 9 patients with FA was investigated. In total, 7 malignancies of diverse type were observed, as compared with the 10.4 expected. Furthermore, no relative developed acute leukaemia. These findings therefore, once again, provide little support for there being an excess of neoplasms in FA carriers.

Associations of the AT and XP genes with particular types of malignant neoplasms have also been examined (Swift 1976b; Swift et al. 1976; Swift and Chase, 1979). In 27 families of AT patients, a significant increase in deaths from malignant neoplasms was noted among blood relatives dying before age 75, and this was especially

striking in those relatives who died before age 45. From these data it was concluded that more than 5% of all persons dying from any malignancy before age 45 may carry the gene for AT. A review of the characteristics of the 27 AT families studied did not reveal any alternate explanation, such as geographic distribution or ethnic origin, for the greater than expected number of cancers in these families. An excess of skin cancers was noted in XP families. In the evaluation of the latter results, place of residence and occupation were taken into account. (It should be noted that both the AT and XP studies included spouse controls.)

In summary, to date, no significant association between an increased predisposition to malignancy and FA heterozygosity has been proven. By contrast, XP and AT relatives appear to exhibit significantly more malignant neoplasms than the general population. The association of neoplasia with the FA gene or any other gene in the category of chromosome breakage syndromes, should be directly measurable, when the heterozygote carriers can be unequivocally detected.

5.3.5 The aetiological basis of malignant transformation in Fanconi's anaemia

Retrospective studies have shown that individuals homozygous for the FA gene are quite clearly prone to the development of malignancies, usually in the form of acute leukaemias. (See section 5.3.2) As previously discussed, a number of other single gene disorders characterized by increased chromosome breakage and defective DNA repair, are also associated with a tendency toward neoplastic transformation. Despite the relative rarity of these syndromes, their association with an increased frequency of malignancy, leads to the assumption that unrepaired DNA damage has a high carcinogenic potential. Alternatively, it has been suggested that in the case of FA, androgen therapy may induce malignant transformation. This hypothesis will first be discussed, followed by an evaluation of the proposed link between chromosome fragility and malignancy. The latter postulate will be considered in the light of present evidence available with respect to the association of specific malignancies

with particular chromosome abnormalities, oncogenes and autosomal fragile sites.

5.3.5.1 The role of therapy in the induction of oncogenesis in Fanconi's anaemia

Currently, androgen therapy is administered to most FA patients for their bone marrow failure. It has been suggested that this treatment may be an important cause of cancer in these patients (Prindull et al., 1979, Obeid et al., 1980). In a series of 8 FA patients with leukaemia, quoted by Obeid et al., (1980), 4 of these patients are reported to have received therapy with oxymethalone prior to the development of leukaemia. Furthermore, hepatomas have been reported in at least 9 cases of FA (Bernstein et al., 1971; Johnson et al., 1972; Guy and Auslander, 1973; Cattan et al., 1974; Holder et al., 1975; Mulvihill et al., 1975; Sweeney and Evans, 1975; Mokrohisky et al., 1977). Several of these hepatoma patients had been treated with androgenic steroids, which appeared to promote the development of their tumours.

It should however, be noted that ANLL has been reported in at least 5 FA patients who did not receive androgen therapy prior to the development of malignancy (Cowdell et al., 1955; Bloom et al., 1966; Schroeder et al., 1976b; Auerbach et al., 1982). Consequently, Auerbach et al. (1982) proposed that androgen therapy may contribute to the frequency of malignant transformation in these patients simply by the resultant prolongation of their survival time.

It has been shown that even normal cells subjected to increased proliferative demands, are at a high risk for leukaemic transformation (Dameshek, 1967). Increased proliferation, which would usually be associated with an increased spontaneous mutation rate, occurs when hypoplastic FA marrows are treated with androgens (Meisner et al., 1978). Moreover, it follows that in FA, this increased proliferation would be accompanied by chromosome instability which is itself associated with increased mutation. This increased mutation rate would in turn favour the development of

mutant clones with a growth advantage. (This proposal will be more closely examined in section 5.3.5.2.)

Clearly then, androgenic steroid therapy may well play an important contributory, but not necessarily causative, role in the development of neoplasia in some cases of FA.

5.3.5.2 The significance of chromosome alterations in neoplastic transformation in Fanconi's anaemia

Over 70 years ago, Boveri (1914) proposed that malignant tumours arise as the result of a chromosome imbalance occurring in a somatic cell through a mitotic disturbance. The basic assumption implicit in this theory was that genetic mutation may lead to malignant transformation. This hypothesis is still held as a fundamental tenet of oncology. However, based on clinical and experimental evidence available in the past, the idea that such a mutation must necessarily translate into a microscopically visible chromosome change, met with strong opposition. The diversity of chromosome alterations observed in tumours were almost invariably interpreted as secondary epiphenomena, consequent to tumour formation. More recently, substantial advances in cytogenetic methods have led to the recognition of consistent abnormalities associated with specific human tumours, especially in the leukaemias and lymphomas.

At present, at least 22 specific chromosomal defects are known to be associated with over 30 distinct types of human cancer (Sandberg, 1980; Mitelman, 1983; Yunis, 1983). These abnormalities are primarily represented by either a reciprocal translocation or the loss of a specific chromosomal band (Yunis, 1983). With the advent of improved techniques for the culturing of solid tumours, it is probable that many more such associations will become evident.

Further interest in the genetic basis of human neoplasia has been generated by the recent molecular detection of human cellular oncogenes. These have now been mapped, with precise band assignment, to individual chromosomes. Some of the oncogene localizations

coincide with the specific breakpoints observed in particular tumours. (See section 5.3.5.2.1) Besides the identification of oncogenes and specific chromosome alterations, a number of heritable chromosomal fragile sites have also recently been defined. (See section 5.3.5.2.2) In several instances, a close correlation has been observed between the localization of these fragile sites and the breakpoints of chromosomal rearrangements associated with specific malignancies. These observed relationships between specific chromosomal alterations, the loci of cellular oncogenes and certain fragile sites, provides a link between cytogenetic observations and molecular biology, thus, leading to a more generalized, unified theory of oncogenesis.

5.3.5.2.1 Chromosome rearrangements and oncogenes associated with specific leukaemias and lymphomas

Many non-random, and in some instances specific, acquired chromosomal changes have been shown to be associated with particular types of human tumours, mostly leukaemias and lymphomas. These rearrangements have been extensively reviewed (Sandberg, 1980; Rowley, 1981; Mitelman, 1983; Rowley, 1983a; Yunis, 1983). This section will include a basic overview of this subject and a brief consideration of a few specific chromosome translocations found in leukaemias and lymphomas.

Chronic myelogenous leukaemia (CML)

The Philadelphia (Ph^1) chromosome is present in bone marrow cells of approximately 90% of patients with CML, and results from an apparently balanced translocation between chromosome 22 and usually chromosome 9, with breakpoints assigned to bands 9q34 and 22q11 (Rowley, 1973a). This breakpoint on chromosome 9 is in close proximity to the locus of the human cellular oncogene c-abl, which is homologous to the oncogene of the Abelson murine leukaemia virus (Heisterkamp et al., 1983). The characteristic 9;22 translocation results in the transfer of the c-abl oncogene from chromosome 9 to chromosome 22 (De Klein et al., 1982). At a molecular level, the

transposition of c-abl to the bcr region of chromosome 22 results in the production of a hybrid chromosome 22/c-abl fusion gene transcript and activation of this oncogene (Heisterkamp et al., 1985; Shtivelman et al., 1985).

Burkitt's lymphoma

In 1976, a translocation between chromosomes 8 and 14 was described in Burkitt's lymphoma by Zech et al. The associated breakpoints were defined as 8q24 and 14q32. Subsequently, a minority of cases have been reported with variant translocations between chromosome 8 and either chromosome 2 [t(2;8)(p13;q24)] (van den Berghe et al., 1979) or chromosome 22 [t(8;22)(q24;q11)] (Berger et al., 1979).

When the loci for the immunoglobulin (Ig) complex of genes were mapped, a remarkable concordance with the above-mentioned breakpoints was noticed (Erikson et al., 1981; Kirsch et al., 1982; Malcolm et al., 1981). Based on the latter observations, Klein (1981) postulated that particular genes regulating growth and differentiation could be activated by their translocation to a critical aberrant site, thus initiating cellular changes leading to malignancy. This hypothesis was later proven to be correct in the case of Burkitt's lymphoma, when it was shown that the cellular oncogene c-myc, the homologue of the transforming gene of the avian myelocytomatosis virus, is located at band 8q24, the breakpoint involved in all the Burkitt translocations (Dalla Favera et al., 1982b).

In the course of the usual 8;14 translocation, c-myc is transferred to chromosome 14 and thereby directly linked, head to head to the heavy chain constant region which is localized at 14q32 (Kirsch et al., 1982). Although the c-myc oncogene per se does not appear to be altered in this process, extensive sequence changes in a region 5' to the gene have been detected (Dalla Favera et al., 1983) and transcriptional activation of this gene has been observed in the malignant B-cell (Erikson et al., 1983).

Acute myeloblastic leukaemia (AML)

The FAB (French-American-British nomenclature) M2-subtype, or differentiated form of acute nonlymphocytic leukaemia was shown by Rowley (1973b) to be characteristically associated with a t(8;21)(q22;q22). The cellular homologue of the Moloney sarcoma virus oncogene, c-mos, is located at 8q22 (Neel et al., 1982). However, in the course of the 8;21 translocation, c-mos is not transposed to chromosome 21 (Diaz et al.,1985; Drabkin et al., 1985; Rosendorff et al., 1985). It therefore follows that either c-mos is not of importance in the aetiology of this malignancy or alternatively, if c-mos is significant in the pathogenesis of M2-ANLL, then it must be activated by some mechanism other than transposition (see review by Willecke and Schafer, 1984).

Acute promyelocytic leukaemia (APL)

Acute promyelocytic leukaemia (APL/M3-ANLL) is characterized by a reciprocal translocation between chromosomes 15 and 17, with the breakpoints assigned to bands 15q22 and 17q21 (Rowley et al., 1977; Sheer et al., 1985). The oncogene c-fes, the cellular homologue of the Snyder-Theilin feline sarcoma virus oncogene, maps to bands 15q24-25 (Heisterkamp et al., 1982; Dalla Favera et al., 1982a). Harper et al., (1983) have localized c-fes to 15q25-26. It is not as yet known whether c-fes is molecularly involved in the aetiology of APL. Another cellular oncogene, c-erb A, has been localized to the 17q21 breakpoint of chromosome 17 (Spurr et al., 1984). However, in the course of the 15;17 translocation, c-erb A is not transposed to chromosome 15 (Sheer et al., 1985). Thus, the molecular significance of this strategically positioned oncogene remains to be proven.

Interpretation

When considering examples such as the t(9;22) in CML and the t(8;14) in Burkitt's lymphoma, the conclusion may be reached that the activation of critical oncogenes through specific rearrangements results in tumourigenesis. However, great caution must be exercised before uncritically accepting such a generalization. It should be

borne in mind that the activation of human oncogenes through transposition has as yet not been demonstrated to be a general phenomenon, and even if it were, in vitro transformation assays cannot be considered as a direct reflection of the in vivo situation. Furthermore, the specificity of many of the chromosome alterations reported in malignancies is not absolute. For example, the t(9;22) found in 90% of CML cases, has also been reported in M1-ANLL, and in FAB-L1 and L2 acute lymphoblastic leukaemias (reviewed by Yunis, 1983). The Burkitt t(8;14) has additionally been found in cases of acute FAB-L3 lymphoblastic leukaemia and immunoblastic lymphoma (Ibid). However, it should be noted that these are B-cell tumours.

Therefore, with the evidence available at this stage, it is not known whether the acquired chromosome abnormalities associated with specific cancers, play a primary, causative role in oncogenesis. Most evidence suggests that at least two (or possibly more) events such as a mutation and a rearrangement, may be necessary for the natural progression of a malignancy (Cairns and Logan, 1983).

The above-mentioned specific chromosome abnormalities found in leukaemias and lymphomas serve as examples to illustrate the relationship between certain chromosome changes, which are brought about as a consequence of chromosome breakage and rearrangement, and oncogenesis. A review of the literature does not reveal any of the abnormalities described in this discussion to have been present in cases of FA which have undergone malignant transformation. However, according to Sandberg (1980), there is no reason to suspect that chromosome changes observed in the leukaemic cells of FA patients are not similar to those seen in other patients with the same type of leukaemias, but without the manifestations of FA.

5.3.5.2.2 The occurrence, localization and possible significance of fragile sites

In 1979, the following comprehensive definition of a fragile site was proposed by Sutherland: 'A fragile site is a specific point on a

chromosome which is liable to show the following features: (1) a non-staining gap of variable width usually involving both chromatids; (2) the site is always at exactly the same point on the chromosome in cells examined from any individual patient or kindred; (3) the site is inherited in a Mendelian dominant fashion, and (4) fragility must be evident by the production (under appropriate in vitro conditions) of acentric fragments, deleted chromosomes, triradial figures, and the like'. Under standard cell-culture conditions, some fragile sites are expressed, while others are not. However, all fragile sites are inducible under specified culture conditions such as thymidylate stress.

Since Dekaban reported the first fragile site in the long arm of a C-group chromosome in 1965, numerous reports have appeared of fragile sites on a variety of chromosomes. Lejeune (1968) was the first to show that such sites are heritable when he described a site at 2q1 in a woman and her daughter. At the Seventh Human Gene Mapping Workshop, (HGM VII), the 'Committee on Chromosome Rearrangements and Neoplasia and on Fragile Sites' accepted 50 specific breakpoints in chromosomal rearrangements that occur non-randomly in cancer cells (see section 5.3.5.2.1) and a total of 21 heritable fragile sites (de la Chapelle and Berger, 1984). The 21 established fragile sites belong to 3 classes based on their mode of induction: folate sensitive, distamycin A-inducible, and BrdU requiring fragile sites (Hecht and Glover, 1984).

The Xq27 fragile site is unique in that it is associated with an abnormal phenotype and mental retardation (Lubs, 1969; Giraud et al., 1976; Harvey et al., 1977). All the other fragile sites apparently have no clinical expression and according to Hecht and Hecht (1984a), may be regarded as 'cytogenetic markers in search of a biomedical meaning'. It should be noted that even homozygosity for some of these fragile sites appears to be harmless - homozygosity for the 10q25, 16q22 or 12p12 fragile sites is known to be associated with a normal phenotype (Berg et al.,1969; Schmid et al., 1980; Sutherland, 1981; Izakovic, 1984).

All of these established sites are relatively rare, except for the

10q25 fragile site induced by BrdU, which is present in approximately 2% to 3% of the general population and thus, by definition, may be regarded as a polymorphism (Hecht and Glover, 1984). Except for the fragile site 17p12 which was reported by Shabtai et al. (1982) and the 3p14 fragile site reported by Wegner (1983), identification of the great majority of the other accepted sites must be credited to Sutherland et al. (1983).

A new class of fragile sites were relatively recently described by Glover et al. (1984). These sites, known as 'common' fragile sites are induced by the addition of aphidicolin, an inhibitor of DNA polymerase alpha, to the culture medium, and include the 'hot spots' seen under conditions of thymidylate stress and in studies of spontaneous chromosomal damage. The aphidicolin-induced fragile sites were noted to occur frequently and may prove ubiquitous. All individuals studied exhibited aphidicolin sensitive fragile sites in bands 2q31, 3p14, 6q26, 7q32, 16q23 and Xp22. These sites were visible in a high proportion of lymphocytes from each indiviual. A number of other such sites were also noted, but were detected less often. Thus, the class of common fragile sites consists of 6 proven and 19 possible fragile sites.

While the fra Xq27 site is associated with mental retardation, the significance of all other fragile sites is unknown. Of course, it is possible that these may be neutral phenomena which are neither advantageous nor disadvantageous, and may never be found to be associated with disease. Alternatively, certain fragile sites may predispose to chromosome breakage. Chromosome breakage in meiosis would result in the production of chromosome rearrangements in gametes which would be expressed as constitutional rearrangements. Alternately, post-zygotic breakage could result in chromosome alterations which may either have no phenotypic manifestations, or these changes may have an oncogenic potential. These possibilities have been investigated and the data generated by these studies will be briefly discussed.

5.3.5.2.2.1 Fragile sites and their association with constitutional rearrangements

Hecht and Hecht (1984b) analyzed the locations of 278 breakpoints involved in chromosome rearrangements which were detected in amniocentesis. Of the 278 breakpoints, 59 (21%) were noted to be localized in bands containing fragile sites, compared to the expected 31 (11%). This difference proved to be highly significant ($p<0.001$). Of the 59 fragile sites in bands with breakpoints, 25 were folate sensitive, 1 was distamycin A inducible, 27 were common fragile sites and 6 were rare, possible sites.

In a similar study, Hecht and Hecht (1984c) examined 874 breakpoints found in spontaneous abortions, stillbirths and livebirths. In this series, 165 (18,5%) of the breakpoints coincided with fragile site locations, compared to an expected 98 (11%). Once again, there was a highly significant excess of breakpoints in fragile site bands ($p<0.001$).

The data presented in these 2 studies are clearly in accordance with the concept that fragile sites may well predispose to chromosome breakage and rearrangements in meiosis, and so may predispose to heritable chromosome rearrangements. In this context, it is of interest that Garcia-Sagredo et al. (1983) documented a case of a fra 16q22 carrier having a son with a t(1;16)(q32;q22).

5.3.5.2.2.2 Fragile sites and their association with cancer breakpoints and oncogenes

Mitelman (1984) has presented data which demonstrate that only a limited number of breakpoints are regularly involved in chromosomal alterations associated with human neoplasia. In addition, these breakpoints appear to cluster to relatively few chromosomal regions. Based on these findings, it was concluded that these regions probably contain genes of prime importance to cancer development. The remarkable concordance between the chromosomal location of human cellular oncogenes (c-onc) and the breakpoints

involved in certain consistent chromosomal rearrangements associated with various cancers, enhances this view (Rowley, 1983b). It follows that these cancer breakpoints and their associated oncogenes, may well also coincide with the locations of heritable fragile sites which are thought to predispose to chromosome rearrangements.

Hecht and Sutherland (1984) examined the locations of the 21 fragile sites and the 50 cancer breakpoints accepted by the Seventh Human Gene Mapping Workshop (de la Chapelle and Berger, 1984). Nine of the 21 fragile sites were noted to be located at or near a 'cancer' breakpoint. Statistical analysis of the data revealed a highly significant association between human fragile sites and cancer breakpoints ($p<0.001$). This association was not narrowly limited to one class of fragile site and appeared to extend to leukaemia, lymphoma and solid cancer breakpoints. Similarly, analysis of the common fragile sites (i.e. aphidicolin-induced) and cancer chromosome breakpoints indicated that at least 8 sites are in chromosome bands containing breakpoints leading to non-random chromosome rearrangements in cancer cells. This concordance was very unlikely due to chance alone ($p<0.01$). A significant association between fragile site location and chromosomal abnormalities has also been documented by Le Beau and Rowley (1984) as well as by De Braekeleer et al. (1985).

With respect to the possible association between oncogenes and fragile sites, the t(8;21)(q22;q22) characteristic of M2-ANLL, is intriguing since a fragile site and an oncogene, c-mos, have been localized to 8q22 (Neel et al., 1982). Although at least 10 autosomes carry both an oncogene and a fragile site, only in the case of chromosome 8 are they coincidental (Le Beau and Rowley, 1984 - findings based on 17 heritable fragile sites; Hecht and Sutherland, 1984 - based on 21 heritable fragile sites).

If the association between chromosomal rearrangements found in malignancy and heritable fragile sites is real and meaningful, then one might expect cancer patients with specific chromosomal changes to be carriers of fragile sites affecting the relevant chromosomes. Preliminary data suggest that this may in fact be so. Yunis (1983)

found a correlation between fragile sites and structural rearrangements in four patients. The non-malignant, PHA-stimulated lymphocytes of one of two patients with small-cell lymphocytic lymphoma and a t(11;14)(q13;q32) showed a fragile site at 11q13; a second patient with a malignant lymphoma and a t(12;14)(q13;q32) displayed a fra(12)(q13) in 13% of the normal lymphocytes; and two patients with acute myelomonocytic leukaemia (AMML) and an inversion of chromosome 16 [inv(16)(q13;q22)] also carried a fra (16)(q22) in 13% and 36% of the cells respectively. In another study of 3 patients with AMML and an inv(16) or del(16)(q22), only one patient exhibited a fragile site at 16q22 (Arthur and Bloomfield, 1983). Le Beau and Rowley (1984) reporting on 5 patients with AMML and an inv (16)(p13q22), observed a fragile site at 16q22 in 4 of the 5 patients, but not in 5 healthy controls. One might therefore predict that families whose members have a fragile site at 16q22 may be predisposed to developing AMML and having an inv(16) or del (16) in their malignant cells - such families are yet to be found.

The above-mentioned findings imply that fragile sites may play a role in oncogenesis. However, it should be remembered that the evidence for this role is circumstantial and is based largely on a statistically significant relationship between the location of the established and aphidicolin-induced fragile sites and cancer chromosome breakpoints at a cytogenetic level. A careful documentation of fragile sites in cancer is clearly indicated. This research should include fragile site studies in the normal and malignant cells of cancer patients, studies of cancer in families showing fragile sites and conversely, studies of fragile sites in families predisposed to cancer. Molecular studies will assist in elucidating the relationship between fragile sites and cancer breakpoints - coincident locations at a cytogenetic level may at a molecular level in fact be thousands of base pairs apart.

5.3.5.2.3 The association of Fanconi's anaemia breakpoints with cancer breakpoints, oncogenes and fragile sites

At the outset, it should be emphasized that it remains a matter of debate as to whether FA chromosome breakage sites are randomly or non-randomly distributed. (See section 1.6.1.2.3) Furthermore, there does not appear to be concordance between the findings of different research groups, when listing the preferred spontaneous breakage sites in FA. The present study did not include an analysis of breakage sites since the aim of the study was a quantitative rather than a qualitative assessment.

Of the 57 breakage sites identified by Taylor (1984) in 15 FA homozygotes and heterozygotes, 37 are also seen in leukaemia and lymphoma patients, but only 3 were observed at the sites of oncogenes, viz. 6q24-25 (c-myb); 8q22 (c-mos) and 15q25-26 (c-fes). An overlap was also noted between Fanconi's breaks and those in translocations associated with some solid tumours, viz. Ca bronchus; Ca ovary and retinoblastoma. However, FA patients do not appear to be at an increased predisposition to the development of these tumours. (See section 5.3.2) Interestingly, the most frequent break observed in the latter FA series was 11q13, which is also one of the 21 accepted fragile sites (HGM VII 1984 - de la Chapelle and Berger, 1984). Chromosome 8 at band q22 was also particularly prone to breakage - as previously stated, 8q22 is a cancer breakpoint [the t(8;21) associated with M2-ANLL] and is also a fragile site and the location of c-mos.

Von Koskul and Aula (1973) noted a clustering of breaks in FA homozygotes at bands 1p22, 3q27, 6p12 and 13q32. None of these sites coincides with those of the 21 accepted fragile sites or established common fragile sites. Berger et al. (1977; 1980c) have documented 2 cases of FA with leukaemia - in both cases, partial chromosome duplications were present and involved chromosome 3q at the same breakpoints, including 3q27 which is one of the preferential breakpoints reported by von Koskull and Aula (1973).

It is obvious that further investigations into FA breakpoints and cancer breakpoints in FA-associated malignancies is required before any conclusions can be drawn with respect to the putative association between FA breakpoints and cancer breakpoints, oncogenes and/or fragile sites.

5.4 CONCLUSION: THE CLINICAL SIGNIFICANCE OF CHROMOSOME BREAKAGE

As discussed in the preceding sections, it is evident that cytogenetic analysis, more often than not, reveals abnormalities in malignant cells. Human leukaemias, lymphomas and solid tumour tissues are usually characterized by abnormal mitotic figures, abnormal numbers of chromosomes per cell and abnormalities of chromosome structure which may in some instances be specific to the tumour type. As previously stated in section 5.3.5.2, in the past, most oncologists viewed these changes as epiphenomena of the uncontrolled cell growth that is known to characterize cancer, and thus as being of no aetiological importance in tumourigenesis. Today, following great strides in the field of molecular biotechnology and the resultant postulation of the oncogene concept, most observers are now of the opinion that an altered chromosomal complement may be of importance in the evolution of a tumour.

The significance of the chromosomal changes that are associated with the development of leukaemia, lymphomas and solid tumours, is difficult to evaluate convincingly and reliably because of some disturbing and confusing observations. The existence of clones of cells with abnormal chromosome complements in some individuals with chromosome breakage syndromes such as FA, AT, BS and XP, both before and after the clinical diagnosis of cancer, constitutes some of the strongest evidence presently available that chromosome changes play an aetiological role in neoplastic transformation. The correlation between defective DNA repair in these disorders and a greatly increased predisposition to the development of malignancy in homozygotes and possibly heterozygotes (see section 5.3.4), may once again be considered good evidence for the existence of a causal relationship between a change in DNA and the induction of cancer.

However, not all clinical conditions in which *in vitro* breakage of chromosomes has been observed have a propensity to develop neoplasia at a higher rate than would normally be expected. For example, Kostmann's agranulocytosis and glutathione reductase deficiency anaemia are characterized by increased chromosome fragility, but affected individuals have not been shown to be at a higher risk than the general population of developing cancer (Sandberg, 1980). Conversely, chromosome breakage has been observed in patients who presumably have normal DNA repair pathways and have diseases resulting from infectious agents or toxic substances, and in congenital disorders in which malignant transformation is not considered to be a significant risk (Ibid).

The existence of the chromosomal breakage disorders, though rare, is nevertheless of great interest since affected individuals have a distinctly higher predisposition to cancer or leukaemia development. The tumour specific chromosome changes that are often observed as integral phenomena of some malignant systems, must also be considered as evidence of the importance of chromosomal aberrations in neoplastic transformation..

In most chromosomal instability conditions, the characteristic cytogenetic changes have primarily been demonstrated in cultured blood lymphocytes. It follows that one of the prime concerns in assessing the relevance of chromosomal breakage to oncogenesis is correlating *in vitro* observations with the *in vivo* status of the chromosomes. In the relatively few cases in which the *in vivo* situation has been evaluated by examining bone marrow chromosomes, little deviation from the normal has usually been noted. There are however, some reports of bone marrow chromosomal instability, indicating that chromosome fragility is in all probability, inherent to these systems. (See section 1.6.1.4)

It is possible that the genetic defects characterizing these breakage disorders are manifold, with chromosome breakage being but a single entity comprising only a part of the phenotypic spectrum associated with such genetic anomalies. The 'stressful' conditions of *in vitro* culture may well result in an increased number of chromosome

aberrations, by comparison with that seen in bone marrow metaphases representative of *in vivo* conditions. In this respect, an analogy may be drawn with the situation pertaining to the *in vitro* manifestation of heritable fragile sites. The molecular mechanism involved in the expression of these fragile sites remains a mystery, but it is known that specific culture conditions are required for the *in vitro* detection of these sites. Although the *in vivo* status of these sites is not well documented, there is evidence suggesting that fragile sites may well predispose to chromosomal rearrangements at these sites, both during meiosis and mitosis. (See section 5.3.5.2.2)

Even though the degree of chromosomal disruption and rearrangement in cellular systems characterized by inherent chromosomal fragility may appear impressive, it is probable that this instability *per se* is not of direct aetiological importance in oncogenesis. Rather, chromosome instability may be vital in providing a predisposing background, and should be considered as a characteristic of cellular systems from which cancer is likely to emerge, but in which a clinical cancer only sometimes develops. Thus, the presence of increased chromosome breakage may create a vulnerable state in which further aetiological factors can precipitate neoplastic change.

Hecht and Hecht (1984a) have noted that at this point in time, the mechanism by which fragile sites might facilitate malignant transformation is conjectural. Taking the oncogene concept into account, they have proposed the following possible sequence of events: transmission of a fragile site, followed by breakage at that site and subsequent rearrangement of the chromosome, with simultaneous rearrangement of a critical oncogene at that site. Activation of the oncogene and production of an oncogene product would directly contribute to the development of cancer. Quite conceivably, a similar system may be operative in the chromosome breakage disorders. In the latter conditions, breakage and resultant chromosomal rearrangements may be taking place all the time, but only the formation of specific rearrangements at critical sites with resultant activation of relevant oncogenes would induce tumourigenesis. This hypothesis is in line with the finding that the

karyotypic changes observed in the leukaemic cells of subjects with chromosome breakage disorders are similar to those seen in other patients with the same type of leukaemia, but without any of the manifestations of the breakage syndromes (Sandberg, 1980).

A puzzling feature of the chromosome breakage syndromes is that although chromosome instability is evident at birth, and for that matter in utero, malignancy usually does not develop before the latter part of the first decade in life. Therefore, the breakage disorders should perhaps be regarded as preneoplastic states.

If carcinogenesis is seen as at least a 2-step process as suggested by Knudson (1983), then the formation of chromosomal rearrangements in FA, for example, may constitute but one of the required steps for oncogenesis. The period taken in order to achieve the required events may be equivalent to the time elapsed in these children prior to the development of malignancy. Clones of mutant cells that have a relatively poor or only slightly advantageous growth potential might remain occult for years, perhaps later acquiring secondary or tertiary changes and a resultant greater proliferative advantage.

Besides being at an increased risk for the development of malignancy, patients affected by the chromosome breakage disorders may also exhibit immunodeficiency. (See section 1.4) It is in fact well-established that a variety of immunodeficiency states are also associated with an increased tendency to malignancy (Klein and Klein, 1985). Polani (1976) proposed that chromosome fragility could affect the stability, viability and proliferation efficiency of the cells involved in immune-surveillance. This could in turn be responsible for a breakdown of the barrier against malignant invasion and for creating a relatively immunodeficient state. Therefore, the immunodeficiency associated with these disorders may in part, be responsible for the proneness to malignancy.

Thus far, the discussion has centred on implicating chromosome mutation in the conversion of normal cells to cancerous ones. It must however, be borne in mind that at the phenotypic level, the effects of mutation (i.e. a change in the DNA) in various cells will

fall roughly into two classes (1) those associated with malignant transformation, and (2) those that result in abnormal embryonic development, mental retardation and subfertility (reviewed by German, 1979b). In summary, German (1979b) has pointed out that in addition to being of oncogenic importance, 'chromosome imbalance is capable of disturbing cellular function, with clinical consequences ranging from mild mental retardation and anatomical malformation to profound retardation and anomaly incompatible with intrauterine life, and from mild subfertility to the complete absence of germ cells from the gonads'. The teratogenic effects of chromosome breakage pertaining to FA, have been discussed in section 5.2. (The association of reduced fertility with the FA gene in heterozygotes has not been documented.)

To sum up, there is little doubt that chromosome breakage may be of clinical significance, especially in relation to developmental malformations and probably in relation to cancer proneness. Therefore, the cytogenetic aberrations seen to be associated with the chromosome breakage disorders may give rise to cells with either a selective growth advantage (neoplasia) or to cells with an inability to proliferate and mature (aplasia). Overall, the significance of chromosome fragility in the pathogenesis of cancer is still poorly understood. In this respect, German (1973a) proposed that future research perspectives should include investigations into (a) whether the cell converting to cancer was the particular one in which a chromosomal rearrangement had previously occurred; (b) whether that cell was one of a non-malignant clone of cells displaying a rearrangement, but which, by virtue of a critical mutation, was predisposed to malignant transformation or (c) whether a transformed descendent of an original cell underwent a mutation and by virtue of this secondary change, became endowed with proliferative capabilities superior to those of both normal and transformed cells. These data are as yet, not available, but would certainly assist in the elucidation of the postulated relationship between mutagenesis and oncogenesis.

5.5 FUTURE RESEARCH PERSPECTIVE IN FANCONI'S ANAEMIA

'The scientific value of a discovery is judged by the amount of research it provides for others to do'.

Chargaff, 1970.

Even though they are rare, the chromosome breakage syndromes, including FA, are of great interest because they represent some of the best evidence presently available of a link between chromosomal instability and malignant transformation. As a working hypothesis, these disorders may be regarded as possibly being the phenotypic expression of genotypically defective DNA repair systems. It follows that the characteristic chromosome anomalies and associated cancers may thus arise from unrepaired or misrepaired DNA. The enzymatic defect need not, of course, be exclusively involved with DNA, but could conceivably also involve an enzyme directly or indirectly affecting the synthesis of those chromosomal proteins required for the maintenance of the structural integrity of the chromosomes. Nevertheless, the identification of the genetic defects associated with the chromosome breakage disorders appears to offer some hope of insight into the process of carcinogenesis. However, until the specific metabolic defects are conclusively elucidated, the inherited defects in these so-called 'preneoplastic states' should be regarded as having a facilitative rather than causative role in oncogenesis.

To date, the basic metabolic/enzymatic defect underlying the clinical syndrome of FA has not been identified. The diagnosis of FA homozygosity demands the consideration of both clinical and cytogenetic information. This diagnosis is complicated by the *in vitro* variation found in repeated lymphocyte cultures from the same patient, between patients from the same family and between different families. Since the mitotic index of FA cells is typically low, especially after exposure to DNA cross-linking agents, even small uncontrolled variations in cell culture conditions might cause disproportionate changes in the types of cells completing (in the case of 72-hour cultures) 2 cycles of replication prior to harvesting at metaphase. Such changes could account for some of the inter-study variations observed. Furthermore, the clinically unremarkable

heterozygotes cannot usually be cytogenetically identified, since individual carriers frequently have breakage values overlapping with those of normal controls, even when their cells are stressed, although collectively they exhibit significantly higher chromosome breakage than the general population.

Quite clearly then, a distinct need exists to identify the basic defect associated with FA. This would allow:

i. the establishment of an absolute and reliable test for the diagnosis of FA homozygosity and heterozygosity;
ii. the possible provision of a more effective therapy regimen for patients with this condition;
iii. an understanding of how this metabolic aberration predisposes to clinical malignancy;
iv. assessment of the possible association of the FA gene with specific neoplasms, with the aim of preventing the development of such malignancies or at least detecting them early at a prognostically good stage;
v. the accurate diagnosis of FA heterozygosity; and
vi. the determination of the frequency of the gene in different populations.

Should the actual defect prove elusive, the molecular cloning of the FA gene would allow for the unequivocal identification of this gene. If the gene were to be successfully cloned, it could be sequenced and the product of transcription could possibly be identified. Alternately, a restriction fragment length polymorphism (RFLP) closely linked to the FA gene could be employed in the detection of FA homozygotes and heterozygotes in informative families.

At present we have some data, mostly theoretical, on the significance and implications of chromosome damage. More experimental and epidemiological data on the apparent correlations between chromosome breakage and point mutation, somatic and germ-line damage and carcinogenesis are required. Future research must therefore concentrate on the identification of the FA gene mutation/s and the associated metabolic defect/s and the ramifications thereof. Until these findings become available, chromosome breakage studies

must still be regarded as a useful investigative tool for the reliable diagnosis of Fanconi's anaemia.

Appendices

APPENDIX A

TABULATION OF PERIPHERAL BLOOD LYMPHOCYTE CHROMOSOME BREAKAGE DATA FOR ALL INDIVIDUALS INCLUDED IN THE PRESENT STUDY

In order to interpret the tabulated peripheral blood lymphocyte chromosome breakage data, the following key should be applied:

KEY

1. Patient No. : 1 - 115
2. Sex : 1 = Male
   : 2 = Female
3. Age : in years
4. Ethnic Group : 1 = White
   : 2 = Black
   : 3 = Asian
   : 4 = Coloured
5. Control : same number assigned to patient(s) and simultaneously investigated control
6. Family : same number assigned to members of the same family
7. Class : 1 = homozygote
   : 2 = obligatory heterozygote
   : 3 = control
   : 4 = sib
   : 5 = features of FA
8. Parents : 1 = father
   : 2 = mother

A9 - J38 : number recorded aberrations and number of cells screened using the specified culture conditions:

S = spontaneous breakage

F = Hams F10 culture medium

M = M150 culture medium

D[0.01] = DEB at a concentration of 0.01 μg/ml

M[0.05] = MMC at a concentration of 0.05 μg/ml

+ = number of aberrations including gaps

- = number of aberrations excluding gaps

Number = number of cells screened using the specified culture conditions

	1.	Patient No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
	2.	Sex	2	2	1	2	1	2	1	2	1	1	1	2	2	2	2	1	1	2	2	1	2	2	2	2	1	2	1	1
	3.	Age	0.2	-	65	6.5	-	-	0.3	22	25	30	26	-	15	12	28	8	31	29	0.2	30	30	-	20	4	6	32	8	25
	4.	Ethnic Group	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	1	1	1	1	1	2	1	1	2	1
	5.	Control	1	2	3	4	5	5	0	6	7	4	8	9	9	10	11	0	12	13	14	14	14	13	15	16	17	17	18	19
	6.	Family	0	1	1	0	2	2	0	0	0	0	0	3	3	3	0	4	4	4	5	5	5	0	0	0	6	6	0	0
	7.	Class	5	3	3	5	2	2	1	3	3	3	3	2	4	1	3	1	2	2	1	2	2	3	1	5	1	2	3	3
	8.	Parents	-	-	-	-	1	2	-	-	-	-	-	2	-	-	-	-	1	2	-	1	2	-	-	-	-	2	-	-
A	9.	SP+	4	1	0	0	3	0	35	0	0	2	1	0	0	6	3	12	0	4	4	0	1	0	13	0	14	4	2	1
A	10.	SP-	4	1	0	0	2	0	26	0	0	2	1	0	0	3	1	9	0	3	4	0	0	0	11	0	9	1	2	0
A	11.	Number	50	30	30	50	27	29	30	30	30	50	30	30	30	30	50	50	50	50	30	30	30	50	30	30	50	50	30	50
B	12.	FD[0.01]+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	13.	FD[0.01]-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	14.	Number	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	15.	FD[0.1]+	-	-	-	0	-	-	-	0	-	0	-	7	0	-	1	247	2	3	-	-	-	1	17	-	258	6	-	1
C	16.	FD[0.1]-	-	-	-	0	-	-	-	0	-	0	-	7	0	-	1	200	1	1	-	-	-	1	15	-	166	6	-	0
C	17.	Number	-	-	-	50	-	-	-	30	-	50	-	30	30	-	50	50	50	50	-	-	-	50	30	-	50	50	-	50
D	18.	FD[0.2]+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	8	-	-
D	19.	FD[0.2]-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	6	-	-
D	20.	Number	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	50	-	-	-	-	-	-	-	-	50	-	-
E	21.	FD[0.4]+	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	4	6	-	-	-	1	-	-	-	-	-	-
E	22.	FD[0.4]-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	3	3	-	-	-	1	-	-	-	-	-	-
E	23.	Number	-	-	-	-	-	-	-	30	-	-	-	-	-	-	-	-	50	50	-	-	-	50	-	-	-	-	-	-
F	24.	FD[1.0]+	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F	25.	FD[1.0]-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F	26.	Number	-	-	-	-	-	-	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G	27.	FM[0.01]+	-	3	4	-	5	6	-	-	6	-	2	-	-	13	-	-	-	-	5	3	0	-	-	1	-	-	2	-
G	28.	FM[0.01]-	-	3	3	-	2	5	-	-	6	-	1	-	-	9	-	-	-	-	2	3	0	-	-	1	-	-	2	-
G	29.	Number	-	30	30	-	18	30	-	-	30	-	30	-	-	30	-	-	-	-	30	30	30	-	-	30	-	-	30	-
H	30.	FM[0.05]+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H	31.	FM[0.05]-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H	32.	Number	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	33.	SM+	-	-	-	-	3	3	-	-	3	-	-	-	-	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	34.	SM-	-	-	-	-	2	1	-	-	3	-	-	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	35.	Number	-	-	-	-	30	30	-	-	30	-	-	-	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-
J	36.	MM[0.01]+	-	-	-	-	14	7	-	-	7	-	-	-	-	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
J	37.	MM[0.01]-	-	-	-	-	11	4	-	-	7	-	-	-	-	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-
J	38.	Number	-	-	-	-	22	30	-	-	30	-	-	-	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	1.	Patient No.	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
	2.	Sex	2	1	1	2	1	2	1	1	2	1	2	2	1	2	1	2	1	1	1	2	2	1	1	2	1	1	2	1
	3.	Age	45	16	5	-	36	36	4	2	9	33	34	8	11	29	19	3	14	4	32	28	9	4	12	18	27	9	-	25
	4.	Ethnic Group	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	2	2	1	1	1	1	2	2	1	1	1	1
	5.	Control	20	20	21	6	20	20	20	20	20	17	22	22	22	23	24	25	26	18	27	27	28	28	8	29	30	31	32	33
	6.	Family	7	7	8	8	9	9	9	9	9	10	10	10	10	0	0	0	0	0	11	11	11	11	0	0	0	12	12	0
	7.	Class	2	5	1	2	2	2	1	4	4	2	2	1	1	3	3	3	5	5	2	2	1	1	1	5	3	1	2	3
	8.	Parents	2	-	-	2	1	2	-	-	-	1	2	-	-	-	-	-	-	-	1	2	-	-	-	-	-	-	2	-
A	9.	SF+	0	1	29	0	2	0	11	1	0	-	3	4	13	0	2	0	2	2	3	0	8	6	11	1	0	15	1	0
A	10.	SF-	0	1	25	0	2	0	8	0	0	-	1	3	7	0	0	0	2	1	2	0	7	6	10	1	0	12	1	0
A	11.	Number	30	30	50	30	30	30	30	30	30	-	50	16	50	30	50	30	30	30	30	30	30	30	30	50	50	50	30	30
B	12.	FD[0.01]+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
B	13.	FD[0.01]-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
B	14.	Number	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	30	-
C	15.	FD[0.1]+	-	-	244	3	-	-	-	-	-	5	5	148	91	-	0	-	-	-	6	2	-	-	-	2	1	93	-	-
C	16.	FD[0.1]-	-	-	202	2	-	-	-	-	-	4	3	92	54	-	0	-	-	-	3	2	-	-	-	1	1	75	-	-
C	17.	Number	-	-	30	30	-	-	-	-	-	50	50	30	30	-	50	-	-	-	30	30	-	-	-	50	50	36	-	-
D	18.	FD[0.2]+	-	-	-	-	-	-	-	-	-	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D	19.	FD[0.2]-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D	20.	Number	-	-	-	-	-	-	-	-	-	50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E	21.	FD[0.4]+	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	-	-	-	-	-	-	-	-
E	22.	FD[0.4]-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	-	-	-	-	-	-	-	-
E	23.	Number	-	-	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	30	30	-	-	-	-	-	-	-	-
F	24.	FD[1.0]+	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	1	-	-	-	-	-	-	-	-
F	25.	FD[1.0]-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	1	-	-	-	-	-	-	-	-
F	26.	Number	-	-	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	30	30	-	-	-	-	-	-	-	-
G	27.	FM[0.01]+	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	1	2	0	-	-	-	-	19	-	-	-	-	-
G	28.	FM[0.01]-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	0	1	0	-	-	-	-	14	-	-	-	-	-
G	29.	Number	-	-	-	-	-	-	-	-	-	-	-	-	-	30	-	30	30	30	-	-	-	-	5	-	-	-	-	-
H	30.	FM[0.05]+	-	-	-	-	8	5	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H	31.	FM[0.05]-	-	-	-	-	7	4	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H	32.	Number	-	-	-	-	30	30	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	33.	SM+	-	-	-	-	2	1	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	34.	SM-	-	-	-	-	1	0	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	35.	Number	-	-	-	-	30	30	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
J	36.	MM[0.01]+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
J	37.	MM[0.01]-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
J	38.	Number	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	1.	Patient No.	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
	2.	Sex	2	1	1	2	1	2	1	1	2	1	2	2	1	2	1	2	1	1	1	2	2	1	1	2	1	1	2	1
	3.	Age	45	16	5	-	36	36	4	2	9	33	34	8	11	29	19	3	14	4	32	28	9	4	12	18	27	9	-	25
	4.	Ethnic Group	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	2	2	1	1	1	1	2	2	1	1	1	1
	5.	Control	20	20	21	6	20	20	20	20	20	17	22	22	22	23	24	25	26	18	27	27	28	28	8	29	30	31	32	33
	6.	Family	7	7	8	8	9	9	9	9	9	10	10	10	10	0	0	0	0	0	11	11	11	11	0	0	0	12	12	0
	7.	Class	2	5	1	2	2	2	1	4	4	2	2	1	1	3	3	3	5	5	2	2	1	1	1	5	3	1	2	3
	8.	Parents	2	-	-	2	1	2	-	-	-	1	2	-	-	-	-	-	-	-	1	2	-	-	-	-	-	-	2	-
A	9.	SF+	0	1	29	0	2	0	11	1	0	-	3	4	13	0	2	0	2	2	3	0	8	6	11	1	0	15	1	0
A	10.	SF-	0	1	25	0	2	0	8	0	0	-	1	3	7	0	0	0	2	1	2	0	7	6	10	1	0	12	1	0
A	11.	Number	30	30	50	30	30	30	30	30	30	-	50	16	50	30	50	30	30	30	30	30	30	30	30	50	50	50	30	30
B	12.	FD[0.01]+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
B	13.	FD[0.01]-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
B	14.	Number	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	30	-
C	15.	FD[0.1]+	-	-	244	3	-	-	-	-	-	5	5	148	91	-	0	-	-	-	6	2	-	-	-	2	1	93	-	-
C	16.	FD[0.1]-	-	-	202	2	-	-	-	-	-	4	3	92	54	-	0	-	-	-	3	2	-	-	-	1	1	75	-	-
C	17.	Number	-	-	30	30	-	-	-	-	-	50	50	30	30	-	50	-	-	-	30	30	-	-	-	50	50	36	-	-
D	18.	FD[0.2]+	-	-	-	-	-	-	-	-	-	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D	19.	FD[0.2]-	-	-	-	-	-	-	-	-	-	[illegible]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D	20.	Number	-	-	-	-	-	-	-	-	-	50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E	21.	FD[0.4]+	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	-	-	-	-	-	-	-	-
E	22.	FD[0.4]-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	-	-	-	-	-	-	-	-
E	23.	Number	-	-	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	30	30	-	-	-	-	-	-	-	-
F	24.	FD[1.0]+	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	1	-	-	-	-	-	-	-	-
F	25.	FD[1.0]-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	1	-	-	-	-	-	-	-	-
F	26.	Number	-	-	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	30	30	-	-	-	-	-	-	-	-
G	27.	FM[0.01]+	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	1	2	0	-	-	-	-	19	-	-	-	-	-
G	28.	FM[0.01]-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	0	1	0	-	-	-	-	18	-	-	-	-	-
G	29.	Number	-	-	-	-	-	-	-	-	-	-	-	-	-	30	-	30	30	30	-	-	-	-	5	-	-	-	-	-
H	30.	FM[0.05]+	-	-	-	-	8	5	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H	31.	FM[0.05]-	-	-	-	-	7	4	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H	32.	Number	-	-	-	-	30	30	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	33.	SM+	-	-	-	-	2	1	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	34.	SM-	-	-	-	-	1	0	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	35.	Number	-	-	-	-	30	30	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
J	36.	MM[0.01]+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
J	37.	MM[0.01]-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
J	38.	Number	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	1.	Patient No.	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86
	2.	Sex	2	2	2	2	1	1	2	2	1	2	2	1	2	1	1	2	2	1	1	2	1	1	2	2	2	1	2	2	2	1
	3.	Age	8	0.03	4	-	3	3	39	6	-	-	13	0.05	40	16	25	25	18	9	24	-	7	11	24	25	30	-	-	-	24	25
	4.	Ethnic Group	2	2	2	2	1	1	2	2	1	1	1	2	2	2	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	3
	5.	Control	5	24	3	25	34	31	38	4	32	32	19	0	20	35	16	16	27	7	17	36	37	36	10	9	37	12	1	38	22	14
	6.	Family	0	0	13	13	21	21	14	14	15	15	15	0	0	0	16	16	0	0	0	17	17	17	0	0	0	0	20	20	0	0
	7.	Class	1	5	1	2	3	3	2	1	2	2	1	5	3	5	2	2	3	1	3	2	1	1	3	3	3	3	3	3	3	3
	8.	Parents	-	-	-	2	-	-	2	-	1	2	-	-	-	-	1	2	-	-	-	2	-	-	-	-	-	-	-	-	-	-
A	9.	SF+	4	4	9	0	0	0	2	10	1	2	11	1	1	2	0	1	0	6	1	6	4	8	0	0	0	0	0	1	-	0
A	10.	SF-	1	2	8	0	0	0	1	10	1	2	9	1	0	2	0	1	0	6	0	3	3	6	0	0	0	0	0	0	-	0
A	11.	Number	30	50	30	30	50	50	30	30	30	30	50	30	30	50	30	30	30	30	50	50	50	50	30	30	50	50	50	30	-	30
B	12.	FD[0.01]+	-	-	-	-	-	-	-	-	1	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	13.	FD[0.01]-	-	-	-	-	-	-	-	-	1	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	14.	Number	-	-	-	-	-	-	-	-	90	90	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	15.	FD[0.1]+	-	3	-	-	1	2	-	142	-	-	168	-	-	-	-	-	1	-	3	6	4	32	-	0	2	0	-	-	1	-
C	16.	FD[0.1]-	-	2	-	-	1	1	-	119	-	-	134	-	-	-	-	-	1	-	3	0	2	16	-	0	2	0	-	-	1	-
C	17.	mber	-	50	-	-	50	50	-	50	-	-	50	-	-	-	-	-	30	-	50	30	50	50	-	30	50	50	-	-	50	-
D	18.	[0.2]+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-	-	-	1	-	-	-	-
D	19.	FD[0.2]-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	1	-	-	-	-
D	20.	Number	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	50	-	-	-	-	-	50	-	-	-	-
E	21.	FD[0.4]+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	1	-	-	-	-
E	22.	FD[0.4]-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	1	-	-	-	-
E	23.	Number	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	30	-	-	-	-	-	-	-	-	50	-	-	-	-
F	24.	FD[1.0]+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-
F	25.	FD[1.0]-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-
F	26.	Number	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-
G	27.	FM[0.01]+	26	-	140	2	-	-	-	-	-	-	-	-	-	0	0	3	-	8	-	-	-	-	2	-	-	-	-	-	-	0
G	28.	FM[0.01]-	25	-	139	2	-	-	-	-	-	-	-	-	-	0	0	2	-	6	-	-	-	-	2	-	-	-	-	-	-	0
G	29.	Number	30	-	30	30	-	-	-	-	-	-	-	-	-	30	30	30	-	2	-	-	-	-	30	-	-	-	-	-	-	30
H	30.	FM[0.05]+	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H	31.	FM[0.05]-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H	32.	Number	-	-	-	-	-	-	-	-	-	-	-	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	33.	SM+	6	-	-	-	-	-	-	-	-	-	-	-	0	-	-		-	6	-	-	-	-	0	-	-	-	-	-	-	-
I	34.	SM-	5	-	-	-	-	-	-	-	-	-	-	-	0	-	-		-	6	-	-	-	-	0	-	-	-	-	-	-	-
I	35.	Number	30	-	-	-	-	-	-	-	-	-	-	-	30	-	-	-	-	30	-	-	-	-	30	-	-	-	-	-	-	-
J	36.	MM[0.01]+	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	166	-	-	-	-	7	-	-	-	-	-	-	-
J	37.	MM[0.01]-	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	..	-	161	-	-	-	-	3	-	-	-	-	-	-	-
J	38.	Number	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	26	-	-	-	-	30	-	-	-	-	-	-	-

	1.	Patient No.	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115
	2.	Sex	2	1	1	2	2	1	1	2	2	1	2	2	2	2	2	2	1	2	1	2	1	2	1	2	2	1	2	2	2
	3.	Age	20	20	51	50	3	32	10	24	27	16	0.9	0.5	18	18	–	–	7	5	41	35	15	6	13	23	18	5	19	5	4
	4.	Ethnic Group	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	4	1	1	1	1	1	1	1	2	1	2	2
	5.	Control	2	39	39	39	40	41	41	0	15	36	34	35	11	29	28	5	21	26	17	17	17	17	17	32	16	30	39	23	33
	6.	Family	0	18	18	18	0	0	0	0	0	0	0	0	0	0	19	19	0	0	22	22	22	22	22	0	0	0	0	0	0
	7.	Class	5	4	2	2	3	3	5	3	3	3	1	3	5	3	3	3	3	3	2	2	4	1	4	3	3	5	3	1	5
	8.	Parents	–	–	1	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	2	–	–	–	–	–	–	–	–	–
A	9.	SF+	0	1	0	3	1	1	0	0	1	2	9	0	1	2	1	2	1	0	6	4	1	16	2	0	0	0	0	7	0
A	10.	SF-	0	1	0	2	1	1	0	0	1	1	5	0	1	2	1	1	0	0	4	1	0	10	2	0	0	0	0	7	0
A	11.	Number	30	30	30	30	30	30	30	30	30	50	50	50	50	50	30	30	50	30	50	50	50	50	50	30	30	50	30	30	29
B	12.	FD[0.01]+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–
B	13.	FD[0.01]-	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–
B	14.	Number	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	30	–	–	–	–	–
C	15.	FD[0.1]+	–	–	–	–	0	–	–	–	1	4	233	–	3	0	–	–	1	–	6	8	5	55	4	–	–	0	–	–	–
C	16.	FD[0.1]-	–	–	–	–	0	–	–	–	1	2	194	–	2	0	–	–	0	–	*	6	3	41	3	–	–	0	–	–	–
C	17.	Number	–	–	–	–	50	–	–	–	30	50	50	–	50	50	–	–	50	–	50	50	50	12	50	–	–	50	–	–	–
D	18.	FD[0.2]+	–	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	18	5	–	–	–	–	–	–	–	–	–
D	19.	FD[0.2]-	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	13	1	–	–	–	–	–	–	–	–	–
D	20.	Number	–	–	–	–	–	–	–	–	–	50	–	–	–	–	–	–	–	–	50	50	–	–	–	–	–	–	–	–	–
E	21.	FD[0.4]+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
E	22.	FD[0.4]-	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
E	23.	Number	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F	24.	FD[1.0]+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F	25.	FD[1.0]-	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F	26.	Number	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
G	27.	FM[0.01]+	1	8	6	6	–	–	–	–	–	–	–	2	–	–	–	3	–	0	–	–	–	–	–	–	1	–	4	15	–
G	28.	FM[0.01]-	1	7	5	6	–	–	–	–	–	–	–	2	–	–	–	3	–	0	–	–	–	–	–	–	1	–	4	15	–
G	29.	Number	30	30	30	30	–	–	–	–	–	–	–	50	–	–	–	30	–	30	–	–	–	–	–	–	30	–	30	30	–
H	30.	FM[0.05]+	–	25	4	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	17	–	–
H	31.	FM[0.05]-	–	24	4	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	17	–	–
H	32.	Number	–	29	9	30	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	30	–	–
I	33.	SM+	–	1	2	1	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	0	–	–
I	34.	SM-	–	1	1	1	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	0	–	–
I	35.	Number	–	30	30	30	–	–	–	–	–	–	–	–	–	–	–	30	–	–	–	–	–	–	–	–	–	–	30	–	–
J	36.	MM[0.01]+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	25	–	–	–	–	–	–	–	–	–	–	–	–	–
J	37.	MM[0.01]-	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	24	–	–	–	–	–	–	–	–	–	–	–	–	–
J	38.	Number	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	6	–	–	–	–	–	–	–	–	–	–	–	–	–

APPENDIX B

CHROMOSOME ABERRATION SCORING SCHEDULE

CASE (CONTROL:

Culture medium	
DEB concentration	
MMC concentration	
Chromatid gap	
Isochromatid gap	
Chromatid break	
Isochromatid break	
Acentric fragment	
Chromatid deletion	
Isochromatid deletion	
Dicentric	
Triradial	
Quadriradial	
Complex exchange	
Ring	
Endoreduplication	
Other	
Total Breaks (+ gaps)	
Total Breaks (- gaps)	
Total Unstable Cells	
Total Cells	
% Unstable Cells	
Breaks / Cell (+ gaps)	
Breaks / Cell (- gaps)	

APPENDIX C

COLCHICINE

Powder is stable indefinitely.

Stock solution :

2.5 mg Colchicine is added to 100 ml dist. water (stable for at least 7 to 10 days in solution).

Add 0.1 ml stock solution/5ml culture i.e. final concentration = 0.5 μg Colchicine/ml.

DIEPOXYBUTANE (DEB)

Stock solution

MW = 86.09

1 litre = 1.12 kg

Dilution of DEB to final concentration of [0.1μg/ml]

i. Add 0.1 ml stock DEB to 9.9 ml sterile dist. water - Soln. A. i.e. 112 000 μg in 10 ml.

ii. Add 0.1 ml Soln. A to 9.9 ml sterile dist. water - Soln. B. i.e. 1120.0 μg in 10 ml.

iii. Add 1 ml Soln. B to 9 ml sterile dist. water - Soln. C. i.e. 112μg in 10 ml = 11.2 μg in 1 ml.

For a final concentration of [0.1 μg/ml], add 0.05 ml Soln. C (= 0.5 μg) to each 5 ml culture.

Storage of DEB:

DEB has a half-life in aqueous solution of 4 days. Therefore, the DEB solution must not be stored and should always be freshly made up just prior to use.

GIEMSA STAIN

Commercially prepared Giemsa solution :

i. Filter newly opened bottle prior to initial use.

ii. Re-filter if sediment reforms.

HYPOTONIC SOLUTION

0.05 M KCl : 0.94 g KCl/250 ml dist. water.

0.075 M KCl : 1.40 g KCl/250 ml dist. water.

MEDIUM

Can be stored at 4°C for up to 2 to 3 months.

1. Hams F10 + l-glutamine (1x) commercially prepared liquid medium. To the commercial medium add:-
   a. 0.25 ml crystapen per 500 ml medium. (Crystapen 500mg/vial diluted in 2.5 ml sterile dist. water). Discard remainder of vial.
   b. 0.25 ml Novostrep per 500 ml medium. (Novostrep 1g/3ml vial). Discard remainder of vial.
   c. 0.8 ml phenol red per 500 ml medium. (0.5g phenol red in 100 ml dist. water; the solution is filtered through a 0.22 μm pore size millipore filter).

2. M150 medium (equivalent to TC 199 commercially prepared liquid medium). Obtainable from media department of National Institute of Virology (NIV), Johannesburg - already has antibiotics and pH indicator added.

MITOMYCIN C (MMC)

Dilution of MMC to final concentration of [0.01 μg/ml]:

i. To each 2 mg vial of MMC, add 2 ml sterile saline - Soln. A.
ii. Add 0.1 ml Soln. A to 9.9 ml sterile saline - Soln. B.
iii. Add 1 ml Soln. B to 9 ml sterile saline - Soln. C.

For a final concentration of [0.01 μg/ml], add 0.05 ml Soln. C (= 0.05μg) to each 5 ml culture.

Storage of MMC:

MMC solutions should preferably be freshly made up just prior to use, or may be stored in a light-proof box at 3° to 5°C for approximately 1 week.

PHOSPHATE BUFFER (pH 6.8)

Dissolve: 27 g potassium dihydrogen orthophosphate (KH_2PO_4)
23 g sodium hydrogen phosphate. $12H_2O$ ($Na_2HPO_4.12H_2O$)
in 4.5 l dist. water.

PHOSPHATE BUFFERED SALINE (PBS, pH 7.3)

Oxoid PBS tablets (Dulbecco-A) : 1 tablet/100 ml dist. water.

PHYTOHAEMAGGLUTININ (PHA)

PHA (WELLCOME)

Add 5 ml sterile dist. water per vial.
Use 0.05 ml diluted PHA per 5 ml culture.

TRYPSIN

Stock solution :

2.5 g Trypsin is added to 200 ml dist. water (or PBS).

Store deep-frozen in 2.0ml aliquots.

TRYPSIN - EDTA

Working solution :

To 20 ml dist. water, add 0.1 g Trypsin plus 0.05 g EDTA.

Dissolve as thoroughly as possible.

Add 80 ml PBS.

Pre-warm to 37^{o}C.

Use 3 ml working solution per 5 ml culture.

APPENDIX D

SOURCES OF REAGENTS

Acetic acid - glacial	Merck
Alcohol - absolute	SAIMR
Bromide photographic paper	Agfa/Ilford
Colchicine	Merck
Culture vessels	Falcon
Diepoxybutane (DEB)	Merck
Dimethylsulphoxide (DMSO)	BDH
EDTA	Merck
Film - Technical Pan	Kodak
Foetal calf serum	M A Bioproducts
Giemsa stain	Clinical Sciences
Glass slides	Menzel-Gläser
Hams F10 medium	Gibco
Human AB serum-pooled	SAIMR
Hypam rapid fixer	Ilford
Lithium heparin tubes	Vacutainer
M150 medium	NIV
Methanol	Merck
Mitomycin C (MMC) - crystalline	Sigma
Penicillin - 'Crystapen' 600 mg	Glaxo
Phenol red - water soluble	BDH
Phosphate buffered saline tablets	Oxoid
Phytohaemagglutinin	Wellcome
Pipettes - sterile; disposable	Sterilin
Potassium dihydrogen orthophosphate	Merck
Potassium chloride	SAARCHEM
Promicol ultrafine grain developer	Maybaker
PQ universal developer	Ilford
Saline - sterile	Baxter
Sodium hydrogen phosphate $\cdot 12\ H_2O$	Merck
Streptomycin - 'Novo-Strep' 1g/3ml	Novo
Thymidine - $[^3H]$	Amersham
Trypsin	Difco
Water-sterile; distilled 5 ml ampoules	Bovit

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