INVESTIGATION OF THE CELL CYCLE RESPONSE OF NORMAL AND FANCONI'S ANAEMIA FIBROBLASTS TO NITROGEN MUSTARD USING FLOW CYTOMETRY

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SUMMARY
Cell survival has been measured in normal and Fanconi's anaemia (FA) human fibroblasts after treatment with the bifunctional alkylating agent, nitrogen mustard (HN2). Two FA cell lines exhibited 6- to 10-fold greater sensitivity than the normal cell line.

Flow cytometry was used to investigate the effects of HN2 on cell cycle progression of normal and FA cells. After 0.1 µg/ml HN2 (surviving fraction, s.f. = 0.8) normal cells exhibited an S phase accumulation within 6 h, followed by a transient G2 delay. At higher doses of HN2, the S phase delay became more pronounced and there was considerably greater accumulation of cells in G2. HN2 at 0.01 µg/ml (s.f. = 0.8) induced no detectable S or G2 delay in FA cells. A higher dose, 0.1 µg/ml (s.f. = 0.13 and 0.29), again induced no S phase delay, but a gradual accumulation of cells in G2 was observed up to 78 h after treatment.

The presence of an S phase delay in normal cells after HN2 treatment may be important in allowing time for DNA repair before completion of DNA synthesis. The absence of such a delay in FA cells suggests that an inability to delay S phase traverse in response to DNA damage from bifunctional alkylating agents may contribute to the sensitivity of FA cells to such drugs.

INTRODUCTION
Fanconi's anaemia (FA) is one of a group of 'cancer prone' genetic disorders. FA patients run a high risk of malignant disease in addition to suffering a range of congenital abnormalities (German, 1972; Fanconi, 1967; Swift, 1976). Cells cultured from FA individuals exhibit a longer generation time, more specifically extended S and G2 phases (Weksberg et al. 1979; Sasaki, 1975), reduced probability of successful cell division (Elmore & Swift, 1976) and a degree of chromosomal instability, manifest as a high incidence of spontaneous chromosome breakage and micronucleus production (German, 1972; Sasaki, 1975; Sasaki & Tonomura, 1973; Heddie et al. 1978).

The enhanced sensitivity of FA cells to DNA crosslinking agents is well-documented and reduced survival, increased chromosome damage and increased mitotic delay after a variety of bifunctional alkylating agents have been reported (Weksberg et al. 1979; Ishida & Buchwald, 1982; Fornace, Little & Weichselbaum, 1979; Fujiwara, Tatsumi & Sasaki, 1977; Latt et al. 1975; Kaiser et al. 1982; Sasaki, 1975). The response to monofunctional alkylating agents is comparable to that of normal cells (Weksberg et al. 1979; Kano & Fujiwara, 1981; Ishida & Buchwald,
W. Dean and M. Fox (1982) although Auerbach & Wolman (1976) and Sasaki (1975) have reported increased chromosome damage after treatment with ethyl methanesulphonate (EMS) and decarbomyl mitomycin C (DCMMC), respectively.

Attempts to identify the basic defect that leads to the FA phenotype have tended to focus on the ability or otherwise of FA cells to remove induced DNA crosslinks. Such work and investigations of other endpoints thought to reflect repair capabilities have produced conflicting evidence. For example, reduced nicking of DNA strands either side of one arm of mitomycin C (MMC)-induced crosslinks by FA cells has been reported by Fujiwara et al. (1977), using alkaline sucrose gradient sedimentation and hydroxyapatite chromatography of DNA from treated cells. However, Fornace et al. (1979), using alkaline elution, observed no difference between FA and normal cells in their ability to remove MMC-induced crosslinks.

The rate of loss of endonuclease-sensitive pyrimidine dimer sites from FA cells after treatment with ultraviolet irradiation has been reported to be normal (Smith & Patterson, 1981; Ahmed & Setlow, 1978), and normal rates of excision and rejoining of u.v.-induced damage have been observed by Fornace et al. (1979) and Fujiwara & Tatsumi (1977). Poon, O'Brien & Parker (1974) on the other hand have shown normal excision of u.v.-induced DNA damage but a slower rate of strand rejoicing in FA cells. Fornace et al. (1979) showed that FA cells rejoined X-ray-induced single-strand breaks at a normal rate as measured by the alkaline elution assay. In contrast, studies by Coquerelle & Weibezahn (1981) have indicated a slower rejoicing of X-ray-induced double-strand breaks in two of four FA strains tested.

Investigations of unscheduled DNA synthesis by measuring the incorporation of \[^{3}H\text{thymidine}\] into nuclei after treatment with MMC, nitrogen mustard (HN2) and u.v. have consistently shown similar levels in FA and normal cell lines (Sasaki & Tonomura, 1973; Fujiwara et al. 1977; Poon et al. 1974; Ahmed & Setlow, 1978). The induction of sister chromatid exchanges (SCEs) by MMC has also been reported to be normal in FA cells, by Novotna, Goetz & Surkova (1979). Other reports, however, suggest reduced (Latt et al. 1975) or elevated (Kano & Fujiwara, 1981) levels of SCE induction in FA cells after MMC.

In a recent study, Kaiser et al. (1982) examined the effects of MMC on the rate of progress of normal and FA cells through the cell cycle. A more marked accumulation of cells in \(G_2/M\) was observed in FA than in normal cells. Unfortunately, no survival data were provided but thymidine incorporation by FA cells was markedly reduced over a long period and after 48 h they were incorporating no \[^{3}H\text{thymidine}\] possibly indicating considerable cell death.

Previous studies on the effects of bifunctional alkylating agents on human cells have revealed a consistent inhibition of DNA synthesis as measured by incorporation of \[^{3}H\text{thymidine}\], accompanied by a tendency for cells to be delayed in \(S\) phase (Levis, Danieli & Piccinni, 1965; Crathorn & Roberts, 1965, 1966; Roberts et al. 1971; Roberts, Sturrock & Ward, 1975; Murnane, Byfield, Ward & Calabro-Jones, 1980). It is interesting that MMC was not observed to induce \(S\) phase delay in either normal or FA cells (Kaiser et al. 1982), but it is possible that if samples had been taken at more frequent intervals an \(S\) phase delay may have been detected prior to the \(G_2/M\) block.
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With the above apparently conflicting data in mind and taking into account the fact that the cytokinetic effects of bifunctional alkylating agents on human cells have not been fully examined, we undertook the present investigation of the effects of HN2 on human cells using detailed flow cytometric analysis. Drug concentrations were chosen that permitted high levels of survival and therefore would allow meaningful discussion of the fate of living cells. Furthermore, as FA cells appear to be sensitive to the cytotoxic effects of HN2, analysis of their cytokinetic responses may provide further clues to the nature of the cellular defect responsible for the FA phenotype.

MATERIALS AND METHODS

Cell culture

Three human fibroblast cell lines were used and all work was carried out on cells between passages 4 and 18; 16MA, derived from the foreskin of a normal 4-year-old male was kindly donated by Mrs C. Blease of these laboratories. The two Fanconi's anaemia cell strains, GM1746 and GM2053, were obtained from the N.I.G.M.S. Human Genetic Mutant Cell Repository, Camden, N.J., U.S.A.

All cells were grown routinely as monolayers in 75 cm² plastic tissue-culture flasks (Nunc Products) in Eagle's minimum essential medium (Flow Laboratories Ltd), supplemented with 0.2% sodium bicarbonate, 2 mM-L-glutamine, 100 units/ml streptomycin sulphate (Dista products Ltd), 250 units/ml benzylpenicillin (Glaxo Laboratories Ltd) and 10% foetal bovine serum (FBS; Flow Laboratories Ltd). Cells were harvested using 0.1% trypsin (Worthington Biochemical Corporation) in PBS, and cell numbers were determined using a Coulter counter. All cultures were regularly checked for mycoplasma contamination by the method of Chen (1977) and were consistently found to be mycoplasma-free.

Cell survival assay

Cell survival was established using a method adapted from that of Arlett, Harcourt & Broughton (1975). 16MA cells were harvested and irradiated with 5860 rad from a caesium source while in suspension in growth medium, before being plated out at 5 × 10⁴ cells in 8 ml medium per plate to provide a feeder layer.

After 24 h the medium on the irradiated feeder cells was replaced with 8 ml containing 500 viable cells harvested from exponentially growing cultures, HN2 (Boots Co. Ltd) was administered from freshly made filter-sterilized stock solutions, after a further 24 h. HN2 has a relatively short half-life (Fox & Scott, 1980) and the activity of remaining drug is likely to be negligible within ~1 h. Plates were incubated in gas boxes in a humidified 5% CO₂ atmosphere at 37 °C for 2–3 weeks, with one change of medium. Colonies were fixed with formal saline and stained with 0.04% Crystal Violet. Colonies containing greater than 50 cells were scored.

Treatment of cells for cell cycle analysis

Cells were plated at a density of 5 × 10⁴ per plate and incubated for 2–3 days to establish exponentially growing populations prior to treatment. Before exposure to HN2 and at appropriate intervals afterwards, cells from duplicate dishes were harvested by trypsinization and suspended in 10 ml phosphate-buffered saline (PBS). Preparation of cells for flow cytometry (FCM) analysis and propidium iodide staining (Calbiochem-Behring Corporation) has been described previously (Graham & Fox, 1983). Fluorometric measurement was carried out using a Biophysics Cytofluorograph 4800A with an argon ion laser exciting at 488 nm.

Data were assimilated on flexible computer discs and DNA histograms were analysed using a Hewlett Packard 9845A microcomputer and a program based on the methods of Dean & Jett (1974) and Fried (1976), which calculates the proportions of cells in G₁/G₀, S and G₂/M.
RESULTS

The mean population doubling time for exponentially growing 16MA fibroblasts was 29.9 h, whereas doubling times for the FA cell lines, GM1746 and GM2053, were considerably greater: 89.6 and 76 h, respectively. These values can be compared with reports for FA fibroblasts of 45.5 h by Weksburg et al. (1979) and 30.3 h by Elmore & Swift (1976).

Plating efficiency (p.e.) was calculated as the percentage of cells plated that formed...
colonies on untreated plates; 16MA had a mean p.e. of 22.3%; GM1746 and GM2053 both had lower p.e. values of 7.3% and 10.5%, respectively.

**Effect of HN2 on survival**

The survival of the three cell lines after exposure to HN2 is shown in Fig. 1. In all cases, exponential cell killing was observed. The $D_0$ (lethal dose) of GM1746 is approximately 0.05 μg/ml HN2, i.e. this cell line is 10 times more sensitive than 16MA, $D_0 = 0.5 \mu g/ml$. GM2053 is slightly less sensitive than GM1746 with a $D_0$ equal to 0.08 μg/ml. These data are consistent with the reported sensitivity of FA cells to other bifunctional alkylating agents (see Introduction).

**Effect of HN2 on the cell cycle**

The proportions of cells in the various phases of the cell cycle were measured over a period of 78 h in untreated exponentially growing 16MA cultures (Fig. 2A). The proportion of cells in $G_1$ phase rose gradually and there was a concomitant fall in the proportion of cells in $S$ and $G_2/M$ during the period of the experiment, probably due to the approach of confluency. The effect of exposure of 16MA cells to 0.1 μg/ml HN2 (s.f. = 0.8) is shown in Fig. 2B. Six hours after treatment there was a rise in the proportion of $S$ phase cells, which was accompanied by a corresponding decrease in the proportion of cells in $G_1$ and a slight reduction in the number of cells in $G_2$. The

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**Fig. 2.** The proportion of 16MA normal fibroblasts in various phases of the cell cycle over 78 h after treatment with HN2. Data at each time-point are derived from duplicate DNA histograms: A, controls; B, 0.1 μg/ml; C, 0.5 μg/ml; D, 1.0 μg/ml. $G_1$ (●); $S$ (×); $G_2$ (○).
Fig. 3. Representative DNA histograms showing cell cycle distributions of 16MA (A–D), GM1746 (E–H) and GM2053 (I–L) cells after treatment with HN2. Histograms are derived from up to 10,000 cells. Times are shown in hours.

Fig. 4. The proportion of GM1746 FA fibroblasts in various phases of the cell cycle over 78 h after treatment with HN2. Data at each time-point are derived from duplicate DNA histograms: A, control; B, 0.01 μg/ml; C, 0.05 μg/ml; D, 0.1 μg/ml. G1 (○); S (×); G2 (□).
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Proportion of cells in S phase declined after approximately 12 h, at which time there was a corresponding rise in the proportion of cells in G2, indicating that the cells delayed in progression through S phase had passed in a synchronous fashion into G2. By 36 h the proportions of cells in the different phases were similar to those in untreated cultures.

Treatment of cultures with 0.5 µg/ml HN2 (s.f. = 0.37) resulted in a greater perturbation of progression through the cell cycle (Fig. 2c). There was a more marked accumulation of cells in S phase, which was again accompanied by a fall in the proportion of cells in G1. There was also a slight but immediate fall in the proportion of cells in G2 phase. The rise in the proportion of S phase cells was again followed by a rise in the proportion of cells in G2 at 18 h. By 36 h, approximately 60% of the cell population was in G2, and the proportion had declined only slightly by 78 h. There was only a small rise in the proportion of G1 phase cells, indicating reduced transit of cells from G2 through mitosis to G1. Exposure to 1.0 µg/ml HN2 (s.f. = 0.1) resulted in a more marked alteration in the cell cycle progression (Figs 2d, 3a–d). There was again a rise in the proportion of S phase cells accompanied by a fall in both G1 and G2 cell numbers. This fall in G2 content, preceding the fall in G1, is probably due to the passage of cells through mitosis into G1 without replacement from S phase due to

Fig. 5. The proportion of GM2053 FA fibroblasts in various phases of the cell cycle over 78 h after treatment with HN2. Data at each time-point are derived from duplicate DNA histograms: a, control; b, 0.1 µg/ml. G1 (●); S (×); G2 (○).
the $S$ phase block. The evacuation of the $G_2$ compartment must, however, be inhibited within 6 or 9 h because the $G_1$ cell numbers also fall, indicating that more cells are leaving $G_1$ than are entering it from $G_2/M$. Thus at early times after treatment with 1.0 $\mu$g/ml HN2, a substantial block in $S$ phase traverse is followed within 6 h by a $G_2$ block. By 18 h, the proportion of cells in $S$ phase had reached almost 60% and then had begun to fall slowly over the next 36 h. This gradual exit of cells from $S$ phase was accompanied by a gradual but marked increase in the proportion of cells in $G_2$. The $G_2$ compartment remained elevated for the duration of the experiment after this dose.

Fig. 4 shows a similar experiment on the FA cell line, GM1746. In untreated cells (Fig. 3A) the proportion of cells in $G_1$ phase was considerably higher, and that in $G_2$ and

![Graph showing growth of cell populations](image-url)

Fig. 6. Growth of cell populations shown in Figs 2–4 after treatment with HN2. Each point is the mean of duplicate samples. Control (○); 0.01 (▲); 0.05 (■); 0.1 (□); 0.5 (□); 1.0 (△); A, 16MA; B, GM1746; C, GM2053.
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Figs 6b, c
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S considerably lower than in the normal cell line. This indicates that the increased population doubling time for GM1746 is largely due to an extended duration of G₁ phase.

Exposure to 0·01 μg/ml HN2 (s.f. = 0·8) produced little perturbation in the cell cycle distribution of GM1746 cells except for a very slight reduction in G₁ and a slight increase in G₂ content relative to the control (Fig. 4A, B). An increase in the dose of HN2 resulted in a further fall in the proportion of G₁ cells and a gradual increase in G₂ content, but no alteration in the proportion of cells in S phase (Figs 4C, D, 3E–H). This suggests that cells are passing unhindered from G₁ through S and into G₂, but that there is some delay of cells in G₁.

Since it was possible that we had not continued sampling long enough to observe a block because of the long cycle time of these cells, the experiment was repeated on the FA cell line GM2053, which has a slightly shorter doubling time, sampling was also continued over a longer period. The proportion of cells in G₁ in untreated cultures (Fig. 5A) was slightly less than that of untreated cultures of GM1746, consistent with the slightly shorter doubling time. Treatment with 0·1 μg/ml HN2 (s.f. = 0·29) resulted in a pattern similar to that seen for GM1746 (Figs 3I–L, 4D). A slight rise in the proportion of cells in G₂ was observed, which had almost returned to control levels by 125 h.

Effect of HN2 on growth rate

Since it was possible that data presented in Figs 2A, 4A and 5A could be observed if cells were not growing, cell numbers were determined at all time-points at all doses of HN2. Fig. 6A–C shows the growth characteristics of the cell cultures used for the flow cytometry. In all cases, control cultures were obviously proliferating and the slopes of the lines reflect the doubling times of the particular cell lines. The data in Fig. 6 also show that the growth of cells was inhibited by HN2 and that growth was resumed by the end of the experiment after using lower drug concentrations.

DISCUSSION

The cytokinetic responses of 16MA fibroblasts to HN2 treatment have two important features. First, the most immediate effect is an S phase delay, evident at all drug concentrations tested, and apparent almost immediately after drug treatment. Second, there is an accumulation of cells in G₂ phase. However, at the lower dose (Fig. 2A) this is probably almost entirely due to the synchronous passage of cells delayed in S phase into G₂ phase and at this dose there may be no real G₂ block. At the higher doses, the increase in the proportion of cells in G₂ is contributed to by exit of cells from S phase, but there is also a real block operating on cells in G₂. We have observed similar responses to HN2 in two normal human lymphoblastoid cell lines (unpublished data).

The observation of a prolongation of S phase as described is consistent with the known inhibitory effects of HN2 on DNA synthesis. However, the eventual passage of cells into G₂ indicates that the cells are capable of overcoming this inhibition. After 0·1 μg/ml HN2, the majority of cells appear to be delayed in transit through S phase but do not show a significant G₂ block, suggesting that the damage responsible for the delay in S was successfully overcome or repaired. At the higher doses, the presence
of $G_2$ delay suggests that damage was not fully repaired during the $S$ phase delay and that either a $G_2$ delay is required for further repair activity or cells with damaged DNA are incapable of progression and die during $G_2$ and $M$. This is reflected in the reduced s.f. values at these higher doses.

The effects of HN2 on the cell cycle kinetics of FA cells are strikingly different from those described for normal cells. The most important feature is the absence of any perturbation of $S$ phase transit time in either GM1746 or GM2053 after any of the drug concentrations used. In addition, the $G_2$ delay apparent at later times appears less severe. The existence of an $S$ phase delay in normal but not in FA cells suggests that its absence may be a feature of the FA phenotype. There appears to be a parallel, therefore, between FA and another genetic disorder, ataxia telangiectasia (A-T), which has been the subject of much research due to its extreme radiosensitivity (Taylor et al. 1975). As with FA, there are conflicting reports of defects in ability to remove DNA lesions in A-T cell strains (Paterson et al. 1976; Hariharan, Eleczko, Smith & Paterson, 1981). In addition, several reports have shown that DNA synthesis in A-T cells is less sensitive to inhibition by X-rays (De Wit, Jaspers & Bootsma, 1981; Edwards & Taylor, 1980; Houldsworth & Lavin, 1980; Painter & Young, 1980) and a reduced X-ray-induced mitotic delay relative to normal cells has been demonstrated (Zampetti-Bosseler & Scott, 1981; Scott & Zampetti-Bosseler, 1982). These findings have been interpreted by Painter (1981) as indicating the existence of radioresistant DNA synthesis in A-T cells, which results in their failure to delay cell cycle transit, with the implication that in normal cells delay allows time for repair before completion of DNA synthesis and mitosis. In line with this interpretation, we suggest that the HN2-induced $S$ phase delay in our normal fibroblasts is a positive aid to survival, as shown by the absence of such a delay in the more sensitive FA cells. We also suggest that the $G_2$ delay observed in a consequence of the induced damage and probably results from an accumulation of dead or dying cells. It therefore follows that FA cells may be sensitive to HN2 and perhaps other cross-linking agents, because of an inability to respond to drug treatment by delaying $S$ phase transit; failure to delay thus results in fixation of damage and consequent cell death during $G_2$. As the FA cells are cycling so slowly, those damaged in late $S$ may enter $G_1$ and disintegrate before cells treated in early $S$ reach $G_2$, hence no marked accumulation in $G_2$ is observed.

The results of Kaiser et al. (1982) do not necessarily contradict this hypothesis. Firstly, MMC was used and not HN2. Secondly, the drug concentrations used may have been more toxic in terms of s.f. than those used in the present study. Indeed, if the drug concentration was very high this could explain the marked inhibition of DNA synthesis seen in FA cells. Thirdly, the FA strain used may have been cycling faster, which would emphasize the observed $G_2$ delay. Differences in cell cycling times could significantly influence the manifestation of drug-induced cycle perturbations and must be considered when interpreting such data. Finally, the absence of an $S$ phase delay in the normal cell lines may be due to sampling only at 24 and 48 h, and not at intermediate times. Our own data would not have revealed the $S$ phase delay had we sampled at these times only. The absence of a marked accumulation of normal cells in $G_2$ may also be due to the times chosen for sampling.
The data indicate the importance of frequent sampling to ensure that any perturbations in the cell cycle of treated cells, however temporary, will be detected. Furthermore, previous reports concerning cell cycle responses to alkylating agents have tended to emphasize the appearance of a G2 block, possibly because of the extent and size of the phenomenon, although an S phase delay is also observed, albeit of lesser magnitude (Barlogie & Drewinko, 1978; Barlogie, Drewinko, Göhde & Bodey, 1977; Layde & Baserga, 1964; Göhde et al. 1979; Rao, 1979). It is important, therefore, to note the temporal relationship of such cell cycle delays and not their magnitude, in order to attempt to interpret the mechanisms involved.

In spite of much effort over the years there is little evidence of a consistent correlation between differential rates of repair and cellular sensitivity. Investigations using mouse L5178Y cells differentially sensitive to X-rays, u.v. and MMC revealed no difference in repair replication or in the case of X-rays, number of initial or residual DNA single-strands breaks (Fox & Fox, 1973a,b,c; Hesslewood, 1978). Similar work in Yoshida cells differentially sensitive to u.v., MDMS and HN2 did show reduced unscheduled DNA synthesis (UDS) in the sensitive cell lines (Fox & Fox, 1973b,c), and Portman, Fox & Boyle (1977) reported reduced excision of u.v.-induced dimers by sensitive Yoshida cells, although the differences were not large enough to account for the considerable differences in cellular sensitivity. More recently, Walker tumour cells differentially sensitive to crosslinking agents have been reported not to differ in the extent of their reaction with protein, RNA or DNA, or in the kinetics of formation and loss of DNA/DNA or DNA/protein crosslinks (J. J. Roberts, 1982, personal communication).

Thus increased cellular sensitivity in a number of cases does not relate directly to reduced ability to remove lesions from DNA and other reasons for the sensitivity must be considered. The answer may ultimately lie in the structural conformation and functional state of chromatin, which quite possibly could affect the ability of a cell to recognize DNA damage and hence delay during S phase. Painter (1982) suggested that the cellular sensitivity of A-T cells to X-rays may be due to altered chromatin structure, which renders the cell unable to identify X-ray-induced DNA damage. Further evidence was presented by Ishiwata & Oikawa (1982), who reported that alterations to chromatin structure interfere with excision of abnormal bases from DNA of human lymphoblastoid cells. The accessibility of the damage to damage-repair enzyme complexes may be an important determinant of the efficiency with which lesions are removed.

At present the molecular mechanisms underlying the ability of a cell to delay in traverse through S phase are unknown. The data presented here are consistent with the interpretation that FA cells lack the ability to recognize damage and lend support to the hypothesis that delay in normal fibroblasts has a protective function.

REFERENCES

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(Received 24 May 1983—Accepted 8 June 1983)