Methotrexate-induced morphological changes mimic those seen after heat shock

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Summary

The survival of cells cultured in medium containing the chemotherapeutic drug methotrexate (MTX) is related directly to drug concentration. Changes in DNA resulting from a severe imbalance in the cells' nucleotide pools are thought to account for this cytotoxicity. We have attempted to clarify the gross biochemical changes that might lead to cell death. DNA strand breaks occur in cells treated with high concentrations of MTX but it is not clear that these are sufficient to account for cytotoxicity at lower doses. We observed dramatic changes in cytoskeletal morphology. Gross reorganization of the cytoskeleton is shown by immunolabelling but is highlighted dramatically when cells are lysed to leave 'nucleoids'. The nature of the changes seen in MTX-treated cells is characteristic of the cells' general stress response, seen originally following heat shock. This study shows that other factors, such as changes in cytoskeletal function, must be considered together with any contribution from DNA damage, in order to account for the lethal effects of MTX.

Key words: methotrexate, nucleoids, cytoskeletal organization, DNA lesions, heat shock.

Introduction

Methotrexate (4-aminopteroyl glutamic acid; MTX) is a folic acid analogue widely used as a chemotherapeutic agent. It binds to, and inhibits, the enzyme dihydrofolate reductase (Futterman, 1957) with subsequent perturbations in intracellular pools of reduced folates (Tattersall et al. 1974). Since these folates are required for the synthesis of purine and pyrimidine nucleotides, DNA synthesis ceases in treated cells (Kornberg, 1980).

The accompanying biochemical effects leading ultimately to cell death are still unclear. Goulian et al. (1980) reported the misincorporation of deoxyuridine into DNA of MTX-treated human lymphoblastoid cells. He postulated that a subsequent futile cycle of deoxyuridine misincorporation and excision could result in DNA strand breaks and therefore be a mechanism of cytotoxicity. However, we were unable to find deoxyuridine incorporation in two different human cell lines after a variety of MTX treatments (Fraser & Pearson, 1986). Observations including: (1) the time-dependent increase in detectable single-strand breaks in cells cultured with MTX (Li & Kaminskas, 1984; Lorico et al. 1988); (2) the MTX-induced stimulation of ADP-ribosyl transferase (Prise et al. 1986), an enzyme known to be activated by DNA strand-breaks (see Gaal & Pearson, 1985, for review); and (3) the fragmentation of DNA in cells with increased intracellular dUTP pools (Ingraham et al. 1986), imply some correlation between MTX cytotoxicity and DNA strand breaks.

One technique that may be used to measure DNA strand breaks is the nucleoid sedimentation method of Cook & Brazell (1975, 1976). Cells are lysed directly on top of sucrose density gradients in buffer containing non-ionic detergent and 2 M salt, to leave 'nucleoids', which contain supercoiled DNA but little nuclear RNA and protein (Cook & Brazell, 1975, 1976, 1978). This supercoiled DNA is protected from shear by the 'nucleoid cage' formed during lysis as cytoskeletal elements collapse onto the nuclear lamina. The sedimentation velocity of nucleoids is dependent on the degree of DNA supercoiling. Introducing single-strand breaks into the DNA causes a relaxation of supercoiling and consequent reduction in sedimentation velocity. A quantitative relationship between the number of single-strand breaks introduced by γ-irradiation and sedimentation was established by Dur-
Materials

All reagents used were Analar grade (BDH) or the purest available. Methotrexate, (+)-Amethopterin, was from Sigma. [6-3H]thymidine (sp. act. 26 Ci mmol−1) and [2-14C]thymidine (sp. act. 51 mCi mmol−1) were purchased from Amersham International.

Methods

Colony-forming assays. HeLa cells were grown in 25 cm culture dishes and treated with MTX. The cells were harvested with trypsin and diluted to a cell concentration of 2×10^6 cells ml^{-1}. A 1 ml sample of each solution was then added to sterile 5 cm Petri dishes already containing 5 ml of warm growth medium. After 14 days growth at 37°C in an atmosphere of 5% CO_2 the medium was removed and the colonies were fixed with 5 ml of formal saline for 20 min at room temperature. They were then washed twice with distilled water, stained with Giemsa and counted. A collection of 50 or more colonies was counted as one colony.

Nucleoid sedimentation analysis of neutral sucrose density gradients. Our procedures were based on methods described by Cook & Brazell (1975, 1976). Cells were grown in the presence of either [6-3H]thymidine (26 Ci mmol⁻¹, 1 μCi ml⁻¹) or [14C]thymidine (51 mCi mmol⁻¹, 0-05 μCi ml⁻¹). Both cell lines were grown in the presence of radiolabel for 24 h then chased in fresh, unlabelled medium for 16 h before being used for experiments.

Density gradients (15% to 30% (w/v) sucrose gradient (4-6 ml) over an 80% (w/v) sucrose shelf (0-4 ml)) were prepared and fractionated as described (Prise et al. 1986). Gradients were centrifuged at 20°C for 30 min at 2827 gₑₑₑ (HeLa) or for 45 min at 11 308 gₑₑₑ (CCRF cells) in an SW 50-1 rotor in a Beckman L8-M ultracentrifuge.

The migration ratio is the distance sedimented (fraction number) by the nucleoid peak: total gradient distance (total number of fractions). The relative migration ratio is this value relative to that of control nucleoids sedimenting in the same rotor. Control nucleoids, by definition, have a relative migration ratio of 1-0.

Cell encapsulation and lysis. Cells were encapsulated (0-5×10^6 cells ml⁻¹) in 0-5% agarose as described by Cook (1984), the beads filtered through monofilament nylon filters of 100 μm mesh (R. Cadisch and Sons, London N3 2JW) and incubated for 2 h at 37°C; cell culture medium was used throughout. Beads were recovered, washed in PBS and the cells were lysed by adding NaCl and Triton X-100 to final concentrations of 2 M and 0.5%, respectively.

Fluorometry. The ethidium-binding capacity of encapsulated and lysed cells was determined on ice-cold samples as described (Cook & Brazell, 1978). Encapsulated and lysed cells were washed with 0.5 M-NaCl, 10 mM-Tris HCl (pH 8.0), 1 mM-EDTA to remove the detergent. Samples were γ-irradiated as appropriate and ethidium was added to each sample shortly prior to measurement of fluorescence using a Shimadzu RF-540 spectrofluorophotometer. For best results the final sample (1 ml) should contain about 0.5 ml of beads with 2.5×10^5 cells.

Antibody probing of cytoskeletal proteins. HeLa cells grown as monolayers on glass were fixed sequentially in methanol and acetone at 4°C for 10 min and washed in 5 mM-Tris-buffered saline (TBS) at pH 7.6 for 5 min, twice. The fixed cells were incubated for 60 min with mouse monoclonal antibodies specific for different cytoskeletal elements (Amersham International) diluted 1 in 250 with TBS. Normal mouse immunoglobulin was used as a control. Slides were washed in TBS 3× and rabbit anti-mouse monoclonal antibody conjugated to FITC (Cappel) diluted 1 in 25 with TBS applied for 60 min. Alternatively, rabbit anti-mouse monoclonal antibodies conjugated to alkaline phosphatase (Dako Ltd) diluted 1 in 30 with TBS was applied for 60 min. Slides were washed 3× in TBS and incubated in alkaline phosphatase reaction mixture (50 ml veronal acetate buffer (pH 9.2), 25 mg naphthol-AS-MP salt, 12 mg Levamisole and 25 mg Fast Red TR) for 15 min. Nuclei were counterstained with haematoxylin.

Electron microscopy. Nucleoids were spread following Kleinseid’s procedure as described in detail by McCready et al. (1979).

TEM of HeLa cells grown as monolayers on glass was performed as follows. Slides were rinsed in PBS, fixed in 4% buffered formal glutaraldehyde, rinsed, transferred to Palade’s osmium tetroxide for 15 min, rinsed and stained with haematoxylin. Samples were dehydrated in a graded ethanol series and equilibrated with Epon. After polymerization, a strip of resin was cut from the slide with a scalpel, lifted off and then glued to a dummy block and 60-80 nm sections were picked onto copper grids and stained with uranyl acetate and lead citrate. Samples were examined using a Joel JEM 100S electron microscope.

Results

Methotrexate toxicity to HeLa cells was assessed by a colony-forming assay (Fig. 1). The drug kills HeLa cells in a dose-dependent fashion (where the dose is a product of the drug concentration and exposure time). Any cellular damage caused by exposure to low concentrations of MTX (1 μM or 10 μM) for only 1 h was sub-lethal. Loss of colony-forming ability was evident in each case at longer drug-exposure times. The surviving fraction was still some 35% after cells had been exposed to 1 μM-MTX.
Fig. 1. Effect of methotrexate on colony-forming ability. HeLa cells were exposed to MTX for different times and drug concentrations, shown in the figure. The surviving fraction was calculated as the ratio of the number of colonies in drug-treated cells to that of control cells. Experimental points are means ± S.D. from five culture dishes.

for 24 h, but no colonies were observed after exposure for this length of time to the higher drug concentrations used. Complementary colony-forming assays of CCRF cells in soft agar, according to the methods of Imamura & Moore (1968), consistently resulted in no colony growth. Taylor et al. (1985), however, encountered the same problem with CCRF-CEM cells, a cell line related to our own. Crude toxicity analysis via Trypan Blue exclusion showed the drug to be similarly toxic to both HeLa and CCRF cells at high doses (100 μM for 24 h) with approximately 40% of cells deemed 'dead' by staining. In control cultures 2-5% of cells did not exclude Trypan Blue. These results demonstrated the relative sensitivities of different methods of determining cytotoxicity (see Drewinco et al. 1979).

Nucleoid sedimentation analysis of the two cell lines is shown in Fig. 2. In both cases a slower sedimentation rate was observed after 1 h exposure to the most toxic MTX concentration (100 μM). The observed shifts in nucleoid sedimentation represent about 300 breaks/genome in HeLa cells and 600 in CCRF cells (Durkacz et al. 1981). In contrast, nucleoids from cells exposed to the drug for longer showed an increase in sedimentation rate (Fig. 2). The extent and time at which this occurred varied; CCRF nucleoids sedimented at relative migration rates of 1.3 and 1.8 after 12 h and 24 h drug-exposure, respectively. HeLa cell nucleoids, by comparison, sedimented at control rates after 12 h exposure and only reached a relative migration ratio of 1.2 after 24 h with the drug. Using CCRF cells we established that the response was drug dose-dependent. At 1 μM-MTX faster nucleoid sedimentation was first observed after 6-12 h drug exposure, while with 10 μM-drug treatment it was found after 3-6 h treatment. The acid-precipitable material visible at the top of the CCRF gradients (Fig. 2A) was rarely observed when HeLa cells were centrifuged, and then only in small amounts; probably because of the relative fragility of their nucleoid cages (discussed by Cook, 1984). The amount of this material was not increased by MTX treatment of CCRF cells.

Proteinase K digestion of isolated HeLa cell nucleoids prior to loading onto linear sucrose gradients reduced their sedimentation velocity. This decrease was greater in nucleoids from MTX-treated cells (100 μM for 24 h), suggesting that the faster sedimentation was a protein-
mediated phenomenon. In view of this we decided to examine the morphology of nucleoids prepared from MTX-treated cells. CCRF cells were used because their greater range of sedimentation velocity indicated that any morphological changes would be more marked.

Electron microscopy

Electron microscopy of control nucleoids (Fig. 3A) showed these to be generally diffuse, irregularly shaped structures with large bundles of DNA fibres connecting different nucleoid bodies. Their appearance was characteristic of cells that yield weak cages upon lysis. In contrast, nucleoids from MTX-treated cells (100 μM for 24 h) were more compact, reasonably uniform in size and regularly shaped (Fig. 3B). In control cells the DNA in the skirt was generally diffuse with large tracks of parallel fibres (indicating DNA damage), whereas nucleoids from MTX-treated cells usually had discrete skirts of intact, supercoiled DNA. Background DNA (areas of the grid remote from any nucleoid bodies) was clearly visible in controls but insignificant in the drug-treated samples.

In view of the highly toxic conditions used to create these clear changes in nucleoid morphology we also examined nucleoids from cells exposed to less toxic doses of MTX. Conditions chosen were those that routinely produced nucleoids that sedimented either more slowly, or faster than controls.

Nucleoids sedimenting more slowly than controls (cells were exposed to 100 μM-MTX for 1 h)

These nucleoids were very heterogeneous, displaying a wide range of condensation. About 40% were similar to controls (Fig. 4A), 30% had a condensed appearance similar to that seen commonly after treatment with high doses of MTX (Fig. 4B), while others had very diffuse cages, which were not seen in control nucleoids (Fig. 4C). Since nucleoid sedimentation rate is influenced by the different structures present (irrespective of their heterogeneity, nucleoids tend to sediment as a discrete band, with a rate that reflects the composition of the population; Charles & Cleaver, 1982), we assume that the slower sedimentation is due to the presence of the latter population (Fig. 4C). Occasionally, 'hybrid' nucleoids containing areas of condensation in an otherwise very diffuse structure were seen and harness-shaped structures with a fibrillar appearance were evident in many nucleoids (highlighted in Fig. 4C).

Nucleoids sedimenting faster than controls (cells exposed to 1 μM-MTX for 24 h)

These nucleoids were of a uniform shape with well-formed cages (Fig. 5A). The DNA skirt was distinct and contained superhelical DNA fibres (Fig. 5B). Little damaged DNA was seen in the background. These nucleoids were less condensed than those from cells treated with 100 μM-MTX for 24 h, but consistent with the observed rate of sedimentation.

Analysis of DNA integrity

Nucleoid sedimentation provides a sensitive and accurate estimate of DNA integrity only when other features of nucleoid structure are invariant. Our data clearly show that whether the DNA is relaxed or supercoiled cannot be
determined against the background of other changes in nucleoid structure that follow MTX treatment.

DNA integrity can be inspected directly but with lower sensitivity. Gross single-stranded nicking of DNA in MTX-treated cells was suggested by the alkaline-elution method (Li & Kaminskas, 1984). In our hands this technique revealed a low level of DNA breaks, detectable only after the highest MTX concentration used. The accumulation of breaks reported by Li & Kaminskas (1984) and more recently by Lorico et al. (1988) was not observed. Also we did not find DNA breaks using an alkaline unwinding procedure, under conditions where breaks introduced by known DNA-damaging agents were detected readily (Collins et al. 1981).

A similarly sensitive alternative to these alkaline techniques uses ethidium bromide, which binds preferentially to superhelical DNA and fluoresces accordingly (Cook & Brazell, 1978). However, because CCRF nucleoids are quite fragile they are easily damaged when manipulated in solution. To overcome this we have used the encapsulation procedure described by Cook (1984). Cells are encapsulated in agarose microbeads by emulsification in liquid paraffin. After lysis the encapsulated nucleoids can be manipulated freely without damage.

Table 1 shows the effect of growth in MTX on the integrity of DNA in encapsulated CCRF cells. The data are expressed as ratios of the ethidium bound by two identical samples, the DNA in one of which was fully relaxed by γ-irradiation. When the DNA is undamaged initially this ratio falls in the range 1-24-1-30. Variation within this range is due primarily to the background fluorescence from ethidium bound to RNA. The experimental samples from cells subjected to various doses of MTX fell within this range. MTX had no effect on the

![Fig. 4. Extreme examples of nucleoid morphology. Nucleoids from cells grown with MTX (100 μM for 1 h) were prepared and inspected after spreading. The morphology of the nucleoid cage varies dramatically (A–C). Bar, 3 μm; A–C are at the same magnification. The arrowhead in C is referred to in the text.](image-url)

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<th>Time of treatment (h)</th>
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CCRF cells were incubated with methotrexate (100 μM) for various times. Cells were encapsulated, lysed and washed. Half of each sample was treated with γ-irradiation (220 J kg−1) and the fluorescence of matched samples (+ and − γ-irradiation) was determined after adding ethidium (8 μg ml−1). Average values from three to five experiments are shown.

Table 1. Methotrexate-induced DNA-damage in CCRF cells

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integrity of encapsulated nucleoid DNA as judged by this technique. If breaks were present their detection was limited by the sensitivity of the assay, i.e. the minimum number of breaks detectable is about 1000 genome.

**DNA domain size and superhelical density**

Ethidium binding can also be used to probe the size and superhelical density of a cell's looped DNA domains (Cook & Brazell, 1978). Changes in domain size or superhelical density could result if protein denaturation modifies DNA/protein interactions in cells subjected to trauma. The size distribution of DNA domains in MTX-treated and control CCRF cells is shown in Fig. 6. The characteristic shape of these curves illustrated the size distribution of the DNA loops in nucleoids, which reflect the chromatin domains found inside the cell (unpublished observations). The average loop size in CCRF nucleoids, estimated from a standard curve of DNA circles of known size (D.A.J., unpublished) was $120 \times 10^3$ base pairs. Little difference in domain size was seen when cells were treated with low or high doses of MTX prior to analysis (Fig. 6B).

The fluorescence of ethidium bound to DNA may also be used to estimate superhelical density, as intact, quasi-circular DNA (loops) bind ethidium differently from the way their relaxed counterparts do (Cook & Brazell, 1978). The point at which relaxed and intact DNA loops bind the same amount of ethidium (i.e. the crossover point in Fig. 7) is salt-dependent and reflects the superhelical density of the intact DNA. Fig. 7 shows that there was no significant difference in the superhelical density of nucleoids from CCRF cells and CCRF cells treated with 100 $\mu$M-MTX for 24 h. In 0.5 M-NaCl the crossover point occurred at an ethidium concentration of about 0.5 $\mu$g ml$^{-1}$.

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**Fig. 5.** Fast-sedimenting nucleoids from cells treated with a low dose of methotrexate. Nucleoids from cells grown with MTX (1 $\mu$M for 24 h) were prepared and inspected after spreading (A). DNA in the skirt is clearly supercoiled (B). Bars: A, 3 $\mu$m; B, 0.3 $\mu$m.

**Fig. 6.** The DNA domains of CCRF cells. DNA of nucleoids from cells grown without (A) or with (B) MTX (■, 100 $\mu$M for 1 h; ▲, 100 $\mu$M for 24 h) was nicked by $\gamma$-irradiation (0–50 J kg$^{-1}$) and the amount of DNA remaining supercoiled estimated from bound ethidium. All DNA domains were relaxed after irradiation with a dose of 250 J kg$^{-1}$.
Methotrexate is undoubtedly cytotoxic, but our experiments indicate that, while there may be some DNA damage, no gross rearrangement of chromatin occurs.

The most dramatic MTX-induced changes involve the cytoskeletal elements that collapse in vitro to make the nucleoid cage. MTX appears to initiate substantial changes in the stability of the cytoskeleton. At low doses this is weak and diffuse but as the drug dose increases a dramatic stabilization or 'fixing' of the cytoskeleton is seen.

Immuno-visualization of the cytoskeleton of HeLa cells grown in MTX supported these observations. In control cells, fluorescein-conjugated second antibody and immunoalkaline phosphatase-staining revealed a fine reticulated network of vimentin filaments spreading from the perinuclear region to the cell periphery (Fig. 8A). A negative control revealed no staining. HeLa cells treated with low doses of MTX exhibited contraction of vimentin filaments towards the perinuclear region, especially in the centrosomal region of the cytoplasm (Fig. 8B,C). At high doses (Fig. 8D), perinuclear capping of the vimentin network resembled that seen after heat shock (Fig. 8E). With increasing MTX concentration patchy staining of vimentin within the nucleoplasm became apparent (Fig. 8D). This nucleoplasmic staining had a granular appearance and was most marked in HeLa cells treated with 100 μM-MTX and stained with immunoalkaline phosphatase (results not shown), in clear contrast to controls, in which virtually no staining was seen within nuclei. Microtubules (Fig. 8G–J) and actin filaments (results not shown) appeared to be unaffected by MTX treatment.

Further changes in nuclear morphology were revealed by transmission electron microscopy (Fig. 9). After treatment with MTX HeLa cell nuclei were generally condensed with reticulated nucleoli. Their chromatin appeared more dispersed than in controls. Short filamentous bundles were observed after MTX treatment but were absent in controls (Fig. 9A).

Cytoskeleton reorganization was suggested by the appearance of condensed clusters of intermediate filaments in the perinuclear region (Fig. 9B) and there were various changes in the detailed morphology of mitochondria; of particular note was the appearance of fine 'myelinated-like' whorls. Previous studies have shown a relationship between mitochondrial distribution and intermediate filament integrity (Summerhayes et al. 1983) and an effect of MTX on mitochondrial morphology and function (this correlates with the loss of clonogenic ability in L1210 cells) has also been described (Bernal et al. 1982).

**Fig. 7.** The superhelical density of CCRF nucleoid DNA. Nucleoids from cells grown without (A) or with (B) MTX (100 μM for 24 h) were prepared. Half of each sample was treated with γ-irradiation (250 J kg⁻¹) and the fluorescence of matched samples (+ (○) and − (●) γ-irradiation) containing different concentrations of ethidium was measured.

**Cytoskeletal changes in vivo**

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**General stress responses**

The morphological changes that follow MTX treatment are similar in many respects to those seen in rat fibroblasts following heat shock (Welch & Suhan, 1985). Furthermore, heat shock stabilizes the nucleoid cage of freshly isolated lymphocytes, which are otherwise unmanageable (Jackson, 1986a). Similarities in nucleoid morphology can be seen by comparing Fig. 10, which shows nucleoids following heat shock, with Fig. 5. In the heat-shock HeLa nucleoids (Fig. 10B,C) the cytoskeletal elements were condensed. After severe heat shock, DNA in the nucleoid skirt was diffuse and poorly spread (compare Figs 10D and 5B). Dense nodules were seen on fibres emanating from the nucleoid bodies (Fig. 10D).

In spite of these visually dramatic morphological changes there was little difference in the polypeptide pattern of nucleoids isolated either before or after heat shock. The only clear difference was the appearance of the major heat-shock proteins with molecular weights of about 70×10³ (Fig. 11, compare lanes 1 and 2). The polypeptide pattern of cells grown at elevated temperature is shown for comparison (Fig. 11, lane 3); nucleoids contained 7% of the radioactivity found in an equal number of cells. Similar morphological changes associated with growth in MTX were not accompanied by the appearance of heat-shock proteins (results not shown), or the sharp decrease in the rates of transcription and translation that followed heat shock. When CCRF cells were labelled for 2 or 24 h with [³⁵S]methionine in medium containing 10 or 100 μM-MTX the amount of radioactivity incorporated into protein was 90 and 75% of that found in controls, respectively.

Condensed skeletal elements do not seem to alter the state of the chromatin. As with MTX, nucleoids isolated after heat shock had undamaged DNA loops. At higher magnification, DNA in the skirt of Fig. 10B was clearly supercoiled and the ethidium-binding experiment illust-
Fig. 8. Cytoskeletal changes induced by methotrexate. HeLa cells were grown on glass coverslips for 24 h. The following treatments were performed before fixing: A, G, controls, no treatment; B, H, 1 μM-MTX added for 6 h; C, I, 10 μM-MTX added for 6 h; D, J, 100 μM-MTX added for 6 h; E, K, heat shock for 10 min at 42°C; F, L, lysed by immersing in 2 M-NaCl, 10 mM-Tris-HCl, pH 8.0, 10 mM-EDTA, 0.5% Triton X-100 for 10 min at 0°C. Cells were fixed and stained with mouse monoclonal antibodies to vimentin (A–F) or β-tubulin (G–L). Bar, 5 μm.

The observed behaviour of nucleoids from MTX-treated HeLa and CCRF cells gives yet another example of variation in the way different cells respond to MTX. It is known, for example, that both folate and deoxyribonucleoside triphosphate pools vary widely in different cell lines treated with MTX and that there is no correlation between levels of folate or nucleotides and intrinsic cell sensitivity to the drug (Tattersall et al. 1974). The nucleoid structures seen do not simply reflect cell synchrony created by MTX. Two factors exclude this interpretation: (1) 1 μM-MTX is sufficient to synchronize cells (Comings & Kakefuda, 1968; Williams & Ockey, 1970). Therefore, the differential response in nucleoid sedimentation rate seen at higher MTX concentrations would not be expected. (2) Various structures seen in nucleoids from cells exposed to MTX were not represented in the control cell population, which includes nucleoids from cells in each phase of the cell cycle.

Fig. 4 shows the range of nucleoid structures obtained from MTX-treated cells and emphasizes the fact that nucleoid sedimentation is not necessarily linked to conformational changes within the DNA domains. The sequence of cytoskeletal changes that gives rise to these different nucleoids is suggested by the occurrence of occasional 'hybrid' nucleoids containing areas of condensation in an otherwise diffuse structure. Brief exposure to MTX apparently disorganizes the cytoskeleton in a

Fig. 9. Methotrexate treatment induces formation of filamentous bundles. HeLa cells were grown in medium containing MTX (10 μM for 24 h) and changes in morphology were visualized by electron microscopy of stained sections. A. Predominantly nuclear, shows the development of actin-like rods in the nucleus (arrow). Note also the dispersion of chromatin and the early segregation of the granular and dense fibrillar components of the nucleolus. B. Cytoplasmic region, shows dense clustering of intermediate filaments in the centrosomal region of the cytoplasm (arrows). Electron-dense microvesicular bodies are noted (arrowheads). Bars, 0.2 μm.

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manner that profoundly affects the nucleoid cage, which is very diffuse. Subsequent changes, particularly those involving collapse of the vimentin network, yield stabilized nucleoids after prolonged MTX treatment. The relationships between MTX, nucleoid stability and cytoskeletal disorganization are intriguing. It may be that the changes that yield condensed nucleoids are late events of programmed cell death. However, a variety of treatments are known to result in collapse of intermediate filaments without serious side-effects (Schliwa, 1986). Nucleoid stability must reflect the state of the cytoskeleton prior to cell lysis. Vimentin and actin filaments are the major structural components of the nucleoid cage. If either network is collapsed onto the nucleus prior to cell lysis condensed or stabilized nucleoids result. It is clear from Fig. 8 that the components of the cytoskeleton behave differently in cells treated with MTX. The same is true in numerous other situations (Thomas et al. 1981; Zieve & Roemer, 1988). This differential behaviour is accentuated in spread cells (Fig. 8) or nucleoids prepared from cells bound to a substratum (Fig. 8F and L, and other results not shown). When nucleoids are prepared from HeLa cells growing in suspension or lymphoid lines the nucleoid cage is formed by uniform collapse of the cytoskeleton, which naturally forms a shell around the nucleus. Under these circumstances prior collapse of part of the cytoskeleton can have a profound effect on nucleoid morphology (Jackson, 1986a); this is particularly evident in lymphoid lines, which naturally form poor nucleoid structures (Figs 3, 4).

The relationship between nucleoid morphology and MTX-induced DNA damage requires clarification. Breaks were clearly present in DNA from control cells while the DNA skirt of nucleoids from MTX-treated cells showed less evidence of DNA damage. This is in conflict with the observed accumulation of DNA damage when cells were grown in MTX (Li & Kaminskas, 1984; Lorico et al. 1988). This discrepancy may reflect intrinsic biochemical variabilities (such as different rates of DNA repair) but could be a consequence of the different experimental protocols used. In fact, while nucleoid sedimentation remains the most sensitive method of measuring DNA strand breaks the data presented here show that this method is only valid when DNA damage is the principal variable.

In an attempt to resolve the DNA damage issue we chose to use spectrofluorometry (Cook & Brazell, 1978), using the modification described by Cook (1984). Cells grown in high doses of MTX (100 \(\mu\)M for 24 h) had a substantial proportion of their DNA domains intact. Our analysis indicated that MTX nicked less than 5% of the cells' DNA domains (equivalent to giving encapsulated nuclei a dose of \(\gamma\)-irradiation of about 100 rads). Colony-forming assays suggested more dramatic DNA damage. For example, 6 h at 100 \(\mu\)M- MTX gave about 2% viable cells, equivalent to treating cells with a dose of about 750

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**Fig. 10.** HeLa nucleoids following heat shock. Nucleoids were prepared from cells treated as follows: A, incubated in medium at 37°C, control; B, incubated in medium at 42°C for 15 min, standard heat shock; and C, incubated at 45°C for 15 min, severe heat shock, and visualized after spreading. Characteristic features seen in the nucleoid skirt after severe heat shock are shown at higher magnification (D). Bars: C, 3 \(\mu\)m; D, 0.3 \(\mu\)m; A–C are at the same magnification.

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Fig. 11. The effect of heat shock on nucleoid protein composition. HeLa cell proteins were labelled (see Materials and methods) for 1 h in medium containing 10 nCi ml\(^{-1}\) L-\(^{35}\)S\)methionine and nucleoids were prepared. The heat-shock treatment was 42°C for 15 min. The gel shows nucleoids from untreated cells (lane 1), nucleoids from cells labelled after heat shock (lane 2) and total cell proteins labelled after heat shock (lane 3). Proteins were denatured and separated in a 10% discontinuous polyacrylamide gel (Laemmli, 1970). About 10\(^{5}\) and 2.5\(\times\)10\(^{5}\) cts min\(^{-1}\) were loaded in lanes 1 and 2 and lane 3, respectively. An autoradiograph (1 week exposure) of the dried gel is shown.

Cells grown in medium containing MTX exhibited a number of clear morphological changes (Figs 8, 9). Some of these contribute to the characteristic appearance of nucleoids from MTX-treated cells (discussed above). Recently, other compounds that might alter nucleic acid metabolism have been reported to result in cytoskeletal reorganization: (1) the intermediate filaments of cells grown in cordycepin collapsed into juxtanuclear caps within 15 min of adding this nucleoside analogue (Zieve & Roemer, 1988); (2) the sedimentation behaviour of nucleoids from fluorodeoxyuridine-treated HeLa cells led Synzynys et al. (1987) to propose some gross reorganization of chromatin domains; however, changes in nucleoid structure akin to those described here are more likely to account for these observations.

Similar morphological changes are seen in cells or nucleoids following heat shock (Welch & Suhan, 1985; Jackson, 1986a) and these are reflected by changes in nucleoid sedimentation (Simpson et al. 1987). The heat-shock response was originally visualized as modifications in the puffs of salivary gland chromosomes from Drosophila grown at elevated temperature (Ritossa, 1962). The classical response is characterized by the stress-related expression of a group of proteins with exceptional evolutionary conservation (Lindquist, 1986). A number of unrelated compounds are now known to induce a generalized response to stress or trauma that closely mimics that following heat shock (reviewed by Lindquist, 1986). The biochemical responses to many different stimuli are clearly related: the conditioning heat treatments of thermotolerance experiments can be replaced by treatment with a wide variety of agents e.g. ethanol, hypoxia, cadmium, arsenite), which have the common property of inducing heat-shock proteins at physiological temperatures. In spite of this the mechanism of induction and primary biochemical function of the stress response remains obscure. One possible role of the major heat-shock proteins is to assist reassembly of denatured proteins or protein complexes (Pelham, 1986). This is supported by the recent observation that constitutively expressed heat-shock proteins are involved in the translocation of proteins across intracellular membranes (Deshais et al., 1988; Chirico et al., 1988). The heat-shock proteins are thought to operate by relaxing the tertiary structure of translation-incompetent conformations (Deshais et al., 1988). They may have a general role in protecting or reactivating exposed hydrophobic sites (Chirico et al. 1988) and hence may operate to permit recovery of protein complexes that denature under conditions of stress.

Methotrexate-induced contraction of the intermediate
filament network is characteristic of the stress response, which may be induced by such widely ranging treatments as heat, amino acid analogues and heavy metals (Welch & Suhan, 1985; Thomas et al. 1981). While all of these treatments induced heat-shock protein synthesis, MTX does not. Damage caused by MTX may be insufficiently severe to induce heat-shock protein synthesis, the activity of pre-existing, constitutively expressed heat-shock proteins being adequate to counteract its effects. Alternatively, the type of damage caused by MTX might initiate other responses rather than triggering the mechanism(s) that induces heat-shock protein synthesis. Interestingly, a study of chemically induced thermotolerance (Haveman et al. 1986) has suggested that enhanced synthesis of heat-shock protein is not an essential prerequisite for the development of thermal resistance. Why these characteristic changes are associated with such a wide range of insults is an intriguing question. The answer might turn out to reflect complex interactions between skeletal networks that interconnect all compartments of the cell (Schlwa, 1986, Jackson, 1986b). Indeed, the cytoskeleton may be a particularly sensitive target for many types of insult or stress: contraction of the vimentin network might be part of a 'reporter' system to monitor cytoskeletal malfunction and, in turn, reflect the general well-being of the cell.

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References


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