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ENHANCEMENT OF METHOTREXATE-INDUCED GROWTH INHIBITION, CELL KILLING AND DNA LESIONS IN CULTURED L5178Y CELLS BY THE REDUCTION OF DNA REPAIR EFFICIENCY

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Abstract—Acute treatment of L5178Y cells by methotrexate (MTX) caused concentration-dependent post-treatment growth inhibition and cell killing. The effects were potentiated in the presence of caffeine (CAF). At the same experimental conditions the CAF-dependent increase in mature and newly formed DNA lesions was found. The results suggest that even short MTX treatment can cause DNA lesions which are normally, at least partially, repaired. By the reduction of DNA repair efficiency with CAF, these lesions can be expressed what finds its reflection in the enhancement of MTX cytotoxicity.

The main target of methotrexate (MTX) is the enzyme, dihydrofolate reductase. Inhibition of this enzyme by MTX leads to inhibition of new DNA synthesis by the restriction of supply of dTTP and of purine nucleotides. It is considered that the resulting deficiency in the availability of one or more deoxynucleotide precursors can cause damage to the newly synthesized DNA. MTX is not considered, however, to be compound which by itself damages mature DNA by a direct attack. Nevertheless, MTX-induced progressive accumulation of ss-breaks has been described in mature DNA which was synthesized in cells before drug exposure [1]. It has been proposed that this effect is due to MTX inhibited DNA repair of normally occurring ss-breaks in mature DNA which are formed irrespective of the treatment with this drug. The defective repair is caused by the restriction of supply of dTTP and of purine nucleotides. Leucovorine, D,L-N^5-formyl-tetrahydrofolic acid, prevented MTX from inducing DNA lesions [2]. This result was considered as providing support for the proposal that MTX can induce DNA lesions by interfering with nucleotide biosynthesis. Moreover, it has been recently reported that MTX caused not only ss-, but also ds-, breaks in mature DNA [3, 4]. Occurrence of ds- breaks cannot be explained by the random presence of ss- breaks close to complementary DNA strands since a much larger number of ss- breaks would be required. The mechanism by which this DNA fragmentation occurs was not explained by the authors, but the fact that when protein synthesis was inhibited by cyclohexamide MTX-induced DNA damage was no longer seen suggested the involvement of a protein in the formation of DNA breaks.

On the other hands, it has been recently suggested that the prolonged inhibition of DNA synthesis without the inhibition of protein synthesis, with consequent unbalanced growth of the cell, leads to overproduction and abnormal accumulation of numerous hydrolases, among them endonucleases, with their progressive leakage through the cell membranes and resulting damage to various cell constitutents [5]. Involvement of DNA nucleases in this process can lead to the progressive digestion of cellular DNA with resulting DNA breakage [6]. Since the observed DNA fragmentation in MTX treated cells increased with the increasing time of MTX treatment [1–4] and the involvement of protein synthesis was noted [3, 4], the possibility of DNA breakage in the consequence of progressive unbalanced growth of the cell should be, in our opinion, taken into consideration. Circumvention of MTX-induced blockage of DNA synthesis by leucovorine [2] or DNA/protein imbalance by cycloheximide [3, 4] can prevent the development of the unbalanced growth with its DNA-damaging consequences and other cytotoxic events.

The present study was undertaken to get some further insight into the possible relationship between MTX cytotoxicity and DNA lesions in cultured L5178Y cells under the conditions of acute MTX treatment. Relatively short times of exposure to MTX were used in an attempt to eliminate the development of unbalanced growth and secondary metabolic disturbances resulting from prolonged exposure of the cells to exogeneous MTX. Cell response to MTX treatment was compared in the presence and absence of caffeine (CAF), known to impair DNA repair and used to enhanced the expression of DNA lesions [7–10]. It was previously reported that CAF used simultaneously with MTX can potentiate MTX-induced cell growth inhibition, but no explanation was offered of the synergistic effect of these two compounds [11].

The experimental design of the present study was as follows: the cells at the log phase of growth were exposed for 1 or 3 hr to 0.005, 0.05, 0.25 or 0.5 mM

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† Abbreviations used: MTX, methotrexate; CAF, caffeine; ss-, single strand; ds-, double strand; dThR, thymidine.

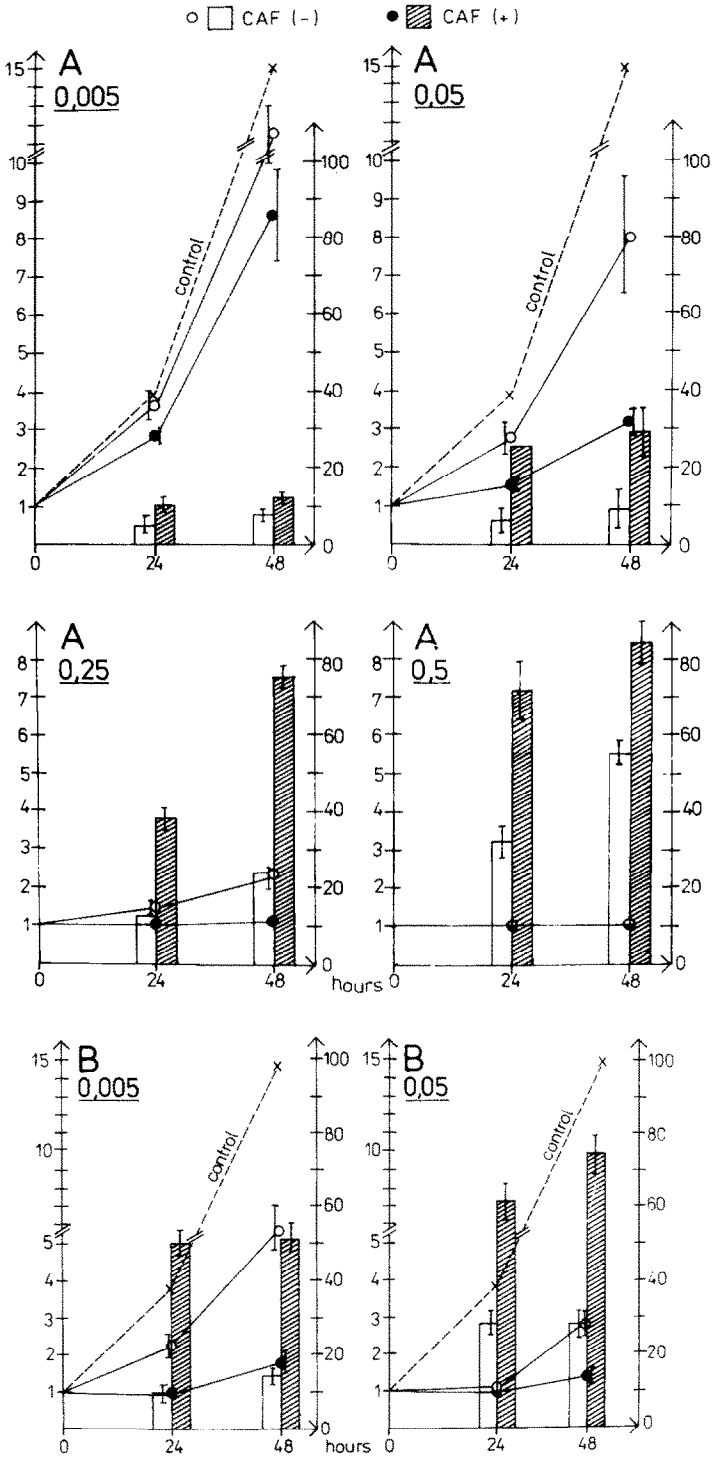

Fig. 1. Effect of 0.75 mM caffeine on post-treatment reproduction and survival of MTX-pretreated cells as a function of MTX concentration. Left ordinate (curves): relative rate of reproduction. Right ordinate (columns): per cent of dead cells. Abscissa: time of incubation after the end of MTX treatment (hr). To obtain normalized values of the rate of cell reproduction for a given experiment total cell numbers at indicated time were divided by corresponding cell numbers at time zero (about 10^5 cells per ml of suspension). Cells were pretreated with indicated concentration of MTX (mM) for 1 hr (A) or 3 hr (B) in the presence or absence of CAF, then washed and resuspended in a MTX free medium with or without CAF, respectively, for a further 48 hr. All experiments were repeated 2–4 times and the data were averaged. Bars denote ranges.

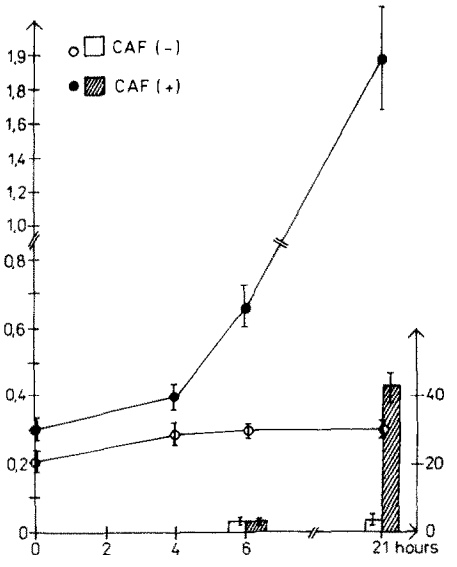

Fig. 2. Effect of 0.75 mM caffeine on the breakage of parental DNA and on cell survival in MTX pretreated cells as a function of time of post-treatment incubation. Left ordinate (curves): ss-/ds- DNA ratio. Right ordinate (columns): per cent of dead cells. Abscissa: time after the end of MTX treatment. Cells prelabelled with [^{3}H]dThR were treated with 0.05 mM MTX for 1 hr in the presence or absence of CAF then washed and resuspended in a MTX free medium with or without CAF, respectively, for a further 21 hr. At the indicated time of post-treatment period the cells were harvested and subjected to hydroxyapatite chromatography assay of ss- and ds- DNA content. Each value represents the mean from two parallel cultures. Bars denote ranges.

MTX in the presence or absence of CAF, then collected by centrifugation and resuspended in an MTX-free medium with or without CAF, respectively. CAF was applied at the 0.75 mM concentration which in our previous experiments was non-toxic for untreated L5178Y cells but significantly enhanced hydroxyurea [12] and unbalanced growth-induced [6] DNA breakage and cell killing. The following parameters were compared at various times after the end of MTX treatment: the rate of recovery of cell reproduction, cell survival and the frequency of ss-breaks in mature and newly formed DNA. The analysis of the frequency of ss- DNA breaks was done by the DNA strand unwinding method [13] in which, after treatment of labelled cells with alkali, the ss- and ds- DNA fractions are separated on hydroxyapatite columns and their proportion is estimated. Strand separation is considered to begin independently at each break on the DNA molecule and the total rate of strand separation is thus initially proportional to the number of breaks. It is known, moreover, that DNA-damaging action of several agents can result also in alkali labile regions which are converted to strand breaks during the alkali treatment [14]. Thus references in the text to strand breaks refer both to strand breaks and to alkali labile regions as in Refs. 1–4 and 15.

The chosen concentrations of MTX induced small to complete post-treatment growth inhibition after 1 hr of treatment in the absence of CAF.

MATERIALS AND METHODS

Cell culture. The L5178Y murine lymphoblasts (derived from ascites bearing DBA/2 mice) were grown in Eagle's MEM with 10% of calf serum. Cells were grown in suspension at 37° at an initial concentration of about 1×10^5 cells/ml.

Cell viability was assessed by Trypan blue exclusion test.

Drugs and reagents. MTX (Lederle) solution in Eagle's medium was prepared just before use. [^{3}H]dThR sp. act. 30 mCi/mmol and [^{14}C]dThR sp. act. 50 mCi/mmol were obtained from Amersham. All reagents were of analytical grade.

Estimation of ss- and ds- DNA fractions by hydroxyapatite chromatography. To estimate the proportion of ss- and ds- fractions in parental DNA the cells at log phase of growth were labeled for about two generation times (22 hr) at 37° with [^{3}H]dThR and, after the labeled medium had been discarded, washed twice in Eagle's medium and incubated for a further 2–3 hr in unlabeled medium. The cells were then treated with MTX and after indicated times of post-treatment incubation lysed. Alkaline lysis was performed as in Ref. 13: 1×10^5 cells were mixed with 1 ml of the lysing solution (0.02 M NaOH, 0.98 M NaCl). After 30 min of lysis at 20° in the dark the solution was neutralized with 0.02 M NaH_2PO_4, sonicated and made up to 0.4% SDS. Each lysate was adsorbed onto fresh hydroxyapatite in water-jacket columns maintained at 65°. The retained material was eluted with potassium phosphate buffer at pH 7 of increasing concentration (0.01–0.125–0.5 M) with 20% formamide [16]. Radioactivity of 0.125 M (ss- fraction) and 0.5 M (ds- fraction) eluates was measured. For liquid scintillation counting the 0.5 M eluates were diluted to 0.125 M concentration. All samples were counted after mixing with equal volume of Insta-gel.

To compare the ss-/ds- ratio in parental and newly formed DNA after MTX treatment, the cells prelabeled with [^{3}H]dThR for about two generation times were treated with MTX under specified conditions and then, after washing, resuspended in an MTX-free medium supplemented with [^{14}C]dThR. After the indicated time the cells were lysed as described above and their ss- and ds- fractions in parental ([^{3}H]-radioactivity) and in newly formed ([^{14}C]-radioactivity) DNA were assayed with hydroxyaptite chromatography analysis.

RESULTS

As shown in Fig. 1A, 1 hr treatment of L5178Y cells with 0.005, 0.05, 0.25 and 0.5 mM MTX resulted in a concentration-dependent post-treatment cell growth inhibition and in a decrease in cell survival. Three hours treatment with MTX enhanced both effects in comparison with 1 hr treatment (Fig. 1B). The presence of CAF significantly potentiated MTX cytotoxicity.

Preliminary experiments on the effect of 1 hr pretreatment with 0.05 mM MTX on mature DNA

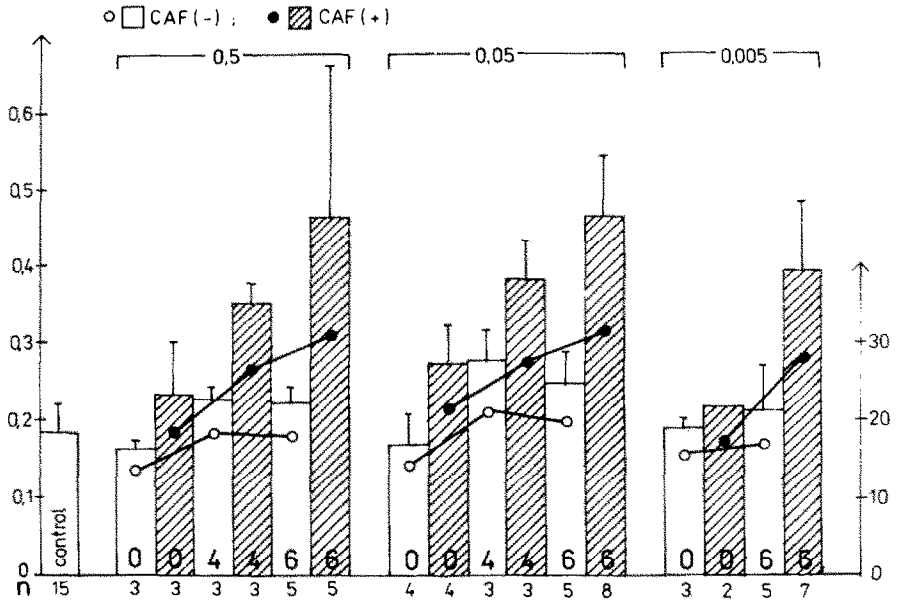

Fig. 3. Effect of 0.75 mM caffeine on the breakage of parental DNA in MTX pretreated cells as a function of MTX concentration and of time of post-treatment incubation. Left ordinate (columns): ss-/ds- DNA ratio. Right ordinate (curves): per cent of ss- DNA fraction. Cells prelabelled with [^{3}H]dThR were pretreated with 0.5, 0.05 or 0.005 mM MTX for 1 hr in the presence or absence or CAF, then washed and resuspended in a MTX free medium with or without CAF, respectively, for a further 6 hr. At indicated time (0, 4 or 6 hr) the cells were harvested and subjected to hydroxyapatite chromatography assay of ss- and ds- DNA content. Each value represents the mean from indicated number of cultures (N). Bars denote SD.

breakage as a function of post-treatment time revealed (Fig. 2) that during the first 6 hr the extent of breakage in the absence of CAF tended to increase, then remained at the same level up to 21 hr of incubation. In the presence of CAF the extent of DNA breakage at the 0 and 4 hr time points was slightly higher than that in its absence. Thereafter, the accumulation of breaks sharply and progressively increased. The highest level of DNA breaks at 21 hr time point correlated with a significant decrease in cell survival. It is therefore possible that this DNA damage occurred as a consequence of cell death. In an attempt to avoid the secondary effects of cell death on cellular DNA, in the next experiments the post-treatment period was limited to 6 hr.

As shown in Fig. 3, the extent of parental DNA breakage after 1 hr pretreatment with various concentrations of MTX was, in the absence of CAF, negligible. The presence of CAF caused slight but reproducible progressive accumulation of breaks with the maximal difference between CAF treated and untreated cells after 6 hr of post-treatment incubation (i.e. from about 6% of average net increase in ss- DNA fraction at the 0 time point to about 12% at the 6 hr time point). The influence of CAF at corresponding time points was similar irrespectively of MTX concentration employed.

Figure 4 represents a comparison between the ss-/ds- ratios in parental and newly formed DNA of the same cells pretreated for 3 hr with MTX. The results indicate that in the untreated cells the levels of ss-fraction were similar to each other in both kinds of DNA (about 17% on average) and independent from the presence of CAF. The results on parental DNA breakage in MTX treated cells indicate again that acute MTX treatment in the presence of CAF produced slight but reproducible DNA lesions (about 10% net increase in ss- fraction) which do not depend on MTX concentration. The proportion of ss- fraction in newly formed DNA of MTX-treated cells increased in the absence of CAF on the average to about 18 and 25% over control cells in 0.005 mM and 0.05 mM MTX treated cells, respectively. In the presence of CAF the average net increase in ss-fraction was about 40 and 48%, respectively.

DISCUSSION

CAF is believed to impair DNA repair. The mechanism by which CAF acts on this process is suggested to be twofold: direct inhibition of DNA repair [7, 9] and reducing the mitotic delay associated with DNA damage, therefore not allowing enough time for DNA repair to take place [8, 10]. The lethal effect of CAF in irradiated cells was considered to be the result of increased expression of potentially lethal DNA lesions [17].

Several workers have suggested that a DNA lesion can be a primary cause of cell killing, and the repair of cell damage is causally related to cell repair [18]. In view of this opinion, CAF-dependent cytotoxic effects of MTX could be the reflection of MTX-induced DNA lesions which are normally, at least partially, repaired but can be expressed by the reduction of DNA repair efficiency. Gradual increase in post-treatment cell growth inhibition and

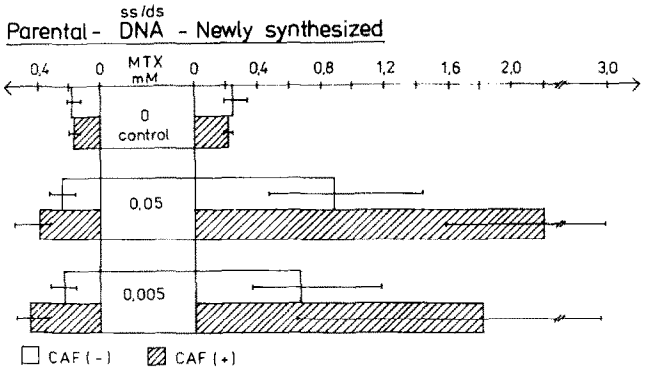

Fig. 4. Effect of 0.75 mM caffeine on ss-/ds- ratio in parental and newly synthesized DNA of cells after MTX treatment. Cells prelabelled with [^{3}H]dThR were treated with 0.05 or 0.005 mM MTX for 3 hr in the presence or absence of CAF, then washed and resuspended in a MTX-free medium supplemented with [^{14}C]dThR with or without CAF, respectively, for a further 5 hr then subjected to hydroxyapatite chromatography assay of [^{3}H] and [^{14}C] ss- and ds-DNA content. Each value represents the mean from 2–5 (control) or 3–4 (MTX-treated) cultures. Bars denote ranges.

cell killing with the increasing concentration of MTX suggests that the increasing proportion of cells sustained DNA damage.

If, however, MTX exposure induces DNA lesions in both newly formed and parental DNA then the question on the mechanism of this damage arises. Formation of lesions in the newly formed DNA could be explained by the known mechanism of deficiency in the availability of deoxyribonucleotide precursors during DNA synthesis. Progressive formation of mature DNA lesions during treatment with antimetabolites such as MTX [1–4], hydroxyurea [19] or 5-fluoropyrimidines [20], having in common the ability to reduce intracellular levels of nucleotides, was suggested to be due to reduced efficiency of repair of spontaneous DNA lesions which appear independently of the drug treatment. On the other hand the experiments from this laboratory suggested a causal relationship between antimetabolite-induced DNA breakage, DNA/protein imbalance and DNAse overproduction occurring in consequence of prolonged blockage of DNA synthesis [6]. Nevertheless, having considered some observations suggesting free radical-generating ability of MTX at non-enzymatic conditions [21], in human leucocytes [22], erythrocytes [23] and erythrocyte ghosts (unpublished), which could be prevented by free radical scavengers [21, 22], the possibility of free radical-mediated mechanism of MTX-induced, particularly acute, DNA lesions should not be overlooked. The overall mechanism of MTX-induced DNA damage could be in such case multifactorial as it is hypothesized for another antimetabolite, hydroxyurea, suggested to cause DNA lesions through the interference with free radical reactions [12, 24, 25].

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