Discussion

Expansion of intracellular folate pools by folinic acid exposure can decrease tumor cell sensitivity to MTX probably because high cellular folate contents decrease the capacity of the cells to metabolize MTX to polyglutamate derivatives. Indeed, MTXPGs are thought to be very important to the antitumor activity of the parent drug by adding important new characteristics to MTX [9]. Associations between impaired capacity to produce MTXPGs, decreased intracellular retention of the metabolites and decreased MTX sensitivity have been made previously in a number of experimental models [9]. Our findings extend earlier reports of the inhibition of MTXPG formation in cells by increased folate pools [2, 3] by showing that decreased metabolism leads to impaired cytotoxicity. However, the mechanism underlying the inhibition of MTXPG formation by intracellular folates remains uncertain. Possible sites of interaction between folates and antifolates leading to decreased MTX metabolism include membrane transport and folylpolyglutamate synthetase. Since MTX uptake was identical under both folate repletion states examined, folates and MTX probably interacted at the enzymatic level. The folinic acid refed cells had 10-fold higher folate levels than the folic acid refed cells with a similar distribution of reduced folates under both conditions. Reduced folates are better substrates for folylpolyglutamate synthetase than MTX and can decrease MTXPG synthesis *in vitro* when both substrates are coincubated [10] and possibly *in vivo* as suggested by our experiments. Since high-dose MTX therapy is followed clinically by rescue with folinic acid, it would be important to know what effect this folate exposure has on intracellular tumor cell folate pools. If the latter remained elevated until subsequent MTX administration, they might decrease MTX metabolism and cytotoxicity during subsequent MTX therapy.

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Hydroxyurea, methotrexate and adriblastine can mediate non-enzymatic reduction of nitroblue tetrazolium with NADH which is inhibited by superoxide dismutase

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Free radical mediated reactions are known to induce a number of types of cell damage [1]. Among them are highly degradative processes of lipid peroxidation exerted primarily within plasma membranes. Increasing number of drugs are suggested to cause their toxic effects through the interference with free radical reactions. Among anticancer agents quinone containing anthracycline antibiotics are known to exert their cytotoxicity through generation of free radicals and their side toxic actions *in vivo* have been attributed to their ability to induce peroxidative injury [2]. However, several non-specific, possibly peroxidative, toxic effects induced by treatment with anticancer agents unrelated structurally to anthracycline antibiotics, have been reported recently.

Although it is generally believed that the primary mechanism of HU cytotoxicity is the inhibition of ribonucleotide reductase, an additional mechanism of its toxicity is considered [3, 4]. This is based on the hypothesis of DeSesso [5] formulated to explain the teratogenic action of this drug and rapid cell killing and suggesting that it may exert side toxic action through free radical reactions. The hypothesis has been supported by several observations indicating that HU* treatment can induce changes in osmotic fragility, methemoglobim formation and sensitivity to oxidant stress in erythrocytes [6], suppress phagocytic activity [7] and migration rate (I. Szczepańska, J. Malec; unpublished) of leucocytes and impair lysosomal stability [8, 9]. Moreover, the exposure of L5178Y lymphoblasts to higher concentrations of HU induced X-ray-like post-treatment giant cell formation and blockage of DNA synthesis [10]. All these toxic effects could be substantially prevented by radical scavengers, among them tocopherol—the most potent physiological membrane antioxidant. Despite these effects suggesting peroxidative mechanism of HU-induced cell damage, our attempts to detect malondialdehyde formation in HU-treated cells were unsuccessful.

MTX is a recognized antifolate antagonist. However, there were observed several MTX-induced toxic effects which are difficult to explain by the antifolate mechanism. They were: occurrence of methemoglobim formation in intact erythrocytes [11] and diene conjugation in erythrocyte ghosts (W. M. Przybyszewski and J. Malec; unpublished), suppression of phagocytic activity [12] and of migration rate of leucocytes which could be prevented by

* Abbreviations used: HU, hydroxyurea; MTX, methotrexate; 5-FU, 5-fluorouracil; ADB, adriblastine; SOD, superoxide dismutase; NBT, nitroblue tetrazolium.

tocopherol (I. Szczepańska and J. Malec; unpublished). The administration of MTX to healthy rats produced an increase in lipoperoxide levels in liver and plasma and a decrease in glutathione peroxidase activity in erythrocytes [13]. Similar effects in healthy rats produced also another antimetabolite—5-FU [13]. Rats which received vitamin E supplementation concomitantly with 5-FU treatment had liver and plasma lipoperoxide levels which were significantly lower than those which had received only the anticancer drug.

In view of the above observations which suggest a possibility of peroxidative toxicity of HU, MTX and 5-FU, we decided to see whether oxygen free radicals can be generated by these drugs in non-enzymatic reactions. For comparison, ADB, an anthracycline antibiotic, was tested under the same conditions. The inspiration of this work was the study on the production of oxygen free radicals by membrane damaging agent—phenazinemethosulphate. Intracellular [14] and enzyme free [15] production of oxygen free radicals by this agent was shown by the reduction of NBT. NADH was one of the hydrogen donors able to react with phenazinemethosulphate and O_2 in order to generate oxygen free radicals in SOD dependent reaction.

Solutions of MTX (Lederle), HU (Polfa), 5-FU (Fluka), ADB (Farmitalia) and SOD (Calbiochem) were prepared in 0.9% saline immediately before use. The reaction was carried out in 0.017 M sodium pyrophosphate buffer, pH 8.3 at 37°, according to [15]. The reaction mixture contained 300 μM NBT, 468 μM NADH and indicated drug. The reduction of NBT was followed spectrophotometrically at 560 nm. All assays were performed in duplicate in at least 3 experiments. The reduction of NBT with NADH alone under these conditions was very slight and its level has been subtracted from the values shown.

As shown in Fig. 1 the addition of HU, MTX and ADB to NBT–NADH system provoked the reduction of NBT, indicating that each drug was acting as an electron carrier; however, with a great difference in the concentration, 5-FU at concentration up to 10^{-3} M was, in this system, inactive. In order to test for the participation of O_2^- in this system, the effect of SOD on the reduction of NBT was examined. In the preliminary trials the effect of various concentrations of SOD on the NBT reduction in the presence of HU and NADH was determined (Fig. 2). About 95% inhibition was attained at SOD concentration 5 units/ml. Further increasing of enzyme concentration up to 10 units/ml did not increase the inhibition. Figure 3 indicates

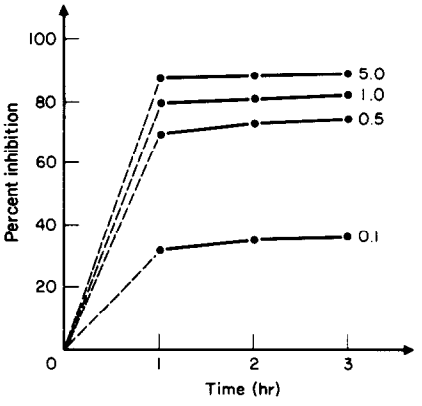

Fig. 2. The effect of SOD on the rate of reduction of NBT in the presence of NADH and hydroxyurea (10 mM). Numbers indicate SOD concentration in units per ml. Reaction conditions as in the text.

that SOD exerted similar inhibitory effects on HU-, MTX- and ADB-mediated reduction of NBT. Heat-inactivation of SOD abolished its inhibitory action. However, when the levels of NBT reduction in the absence of SOD and in the presence of inactivated SOD are compared some differences between particular drugs are visible. While in HU-supplemented samples inactivated SOD stimulated NBT reduction by about 45%, in the case of MTX the difference between SOD-untreated and inactivated SOD-treated samples was negligible, if any. The simulatory effect of inactivated enzyme in ABD-supplemented samples did not exceed 10%. As an explanation of the stimulatory effect of inactivated SOD we take into consideration the effect of metal ion in the enzyme molecule. The stimulatory effect of transitional metal ions on the free radical generation is well known [1]. The lack of similar stimulation in the NBT–NADH–MTX system may indicate, therefore, that the mechanism of HU and MTX action in this system is not the same. In the last experiments we tested the effect of hydroxylamine on the NBT reduction. The question stemmed from the hypothesis of DeSesso [5] who suggested

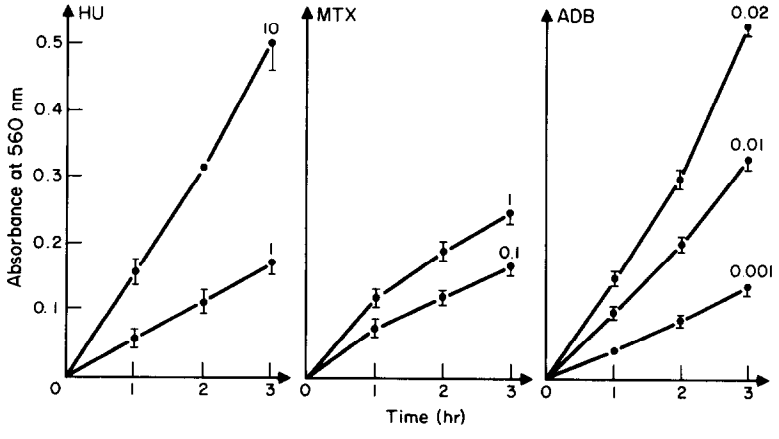

Fig. 1. The effect of hydroxyurea, methotrexate and adriblastine on the rate of reduction of NBT with NADH. Numbers indicate drug concentrations expressed in mM. Bars denote SD. Reaction conditions as in the text.

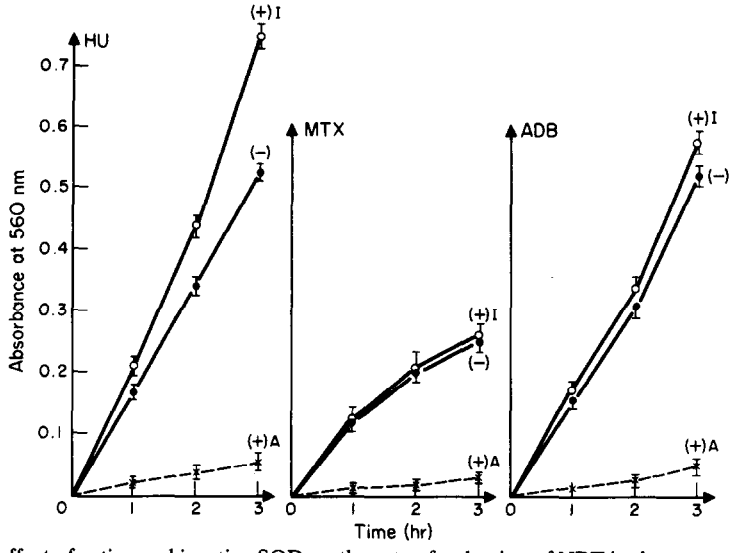

Fig. 3. The effect of active and inactive SOD on the rate of reduction of NBT in the presence of NADH and hydroxyurea, methotrexate or adriblastine. Bars denote SD. (−): SOD absent; (+): SOD present at concentration 10 units per ml. A—enzyme added in the active form. I—enzyme added in inactive form. Inactivation of the enzyme was performed by boiling for 5 min. Concentrations of drugs: HU—10 mM; MTX—1 mM; ABD—0.02 mM. Reaction conditions as described in the text.

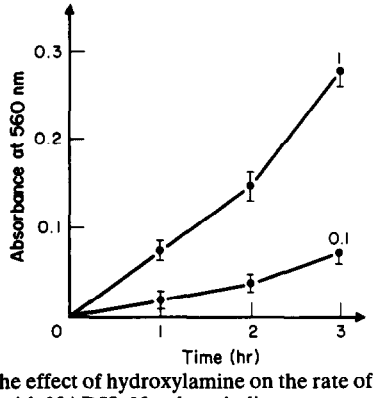

Fig. 4. The effect of hydroxylamine on the rate of reduction of NBT with NADH. Numbers indicate concentrations of hydroxylamine in mM. Bars denote SD. Reaction conditions as described in the text.

that radical reactions of HU are initiated by its hydroxylamine group. Figure 4 indicates that hydroxylamine mediated the reduction of NBT with NADH at the concentration close to that of HU. The reaction was also inhibited by SOD (data not shown).

Summing up, we have found the non-enzymatic, SOD-dependent, reduction of NBT with NADH mediated by HU, MTX and ABD, that suggested the generation of oxygen free radicals under these conditions. This is a further support for a hypothesis on free radical mediated HU toxicity. The mechanism by which MTX can generate oxygen free radicals remains to be elucidated as well as any biological and clinical relevance of this observation, so more than minimal concentration of MTX active in NBT reduction (above 10^{-4} M) markedly exceeded therapeutic concentration of this drug (10^{-5} M during high-dose therapy) [16]. It seems, though, that this matter requires further investigation.

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