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SEQUENTIAL COMBINATION OF METHOTREXATE AND 1-β-D-ARABINOFURANOSYLCYTOSINE SHOWS SYNERGISTIC EFFECT ON THE GENERATION OF DNA STRAND BREAKS IN A HUMAN PROMYELOCYTIC LEUKEMIA CELL LINE

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It has recently been stated that MTX can cause DNA strand breaks. Ara-C, on the other hand, inhibits DNA repair synthesis after UV irradiation or exposure to chemical agents. Therefore, we investigated the accumulation of DNA strand breaks in a human promyelocytic leukemia cell line, HL-60, treated with methotrexate (MTX) and 1-β-D-arabinofuranosylcytosine (Ara-C). The sequential treatment with MTX (0.01-10μM) then Ara-C(10μM) had a synergistic effect on the formation of DNA strand breaks, which was dependent on MTX concentration. On the other hand, when Ara-C preceded MTX, no such synergism was observed. The addition of both thymidine(10μM) and hypoxanthine(100μM) to this system, but not thymidine or hypoxanthine alone, abolished the synergism. Pretreatment with MTX augmented the generation of 1-β-D-arabinofuranosylcytosine 5'-triphosphate and the incorporation of Ara-C into DNA. However, these augmentation did not necessarily correlate with the amount of strand breaks. Whatever the underlying mechanism of this synergism is, our present data provide one possible biochemical basis for sequential MTX and Ara-C therapy.

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ANALYSIS OF MOLECULAR STRUCTURE OF RAT PHOSPHORIBOSYLPYROPHOSPHATE SYNTHETASE GENES.

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Phosphoribosylpyrophosphate (PRPP) synthetase catalyzes the reaction: Rib-5-P+ATP ⇌ PRPP+AMP. The mammalian enzyme exists as a complex aggregate and a 34 kDa component has been identified as the catalytic subunit. A mutant enzyme in man with hyperactivity leads to elevated purine biosynthesis and eventually to gout. For further study of this enzyme, gene analysis is essential. We screened a cDNA library from rat Yoshida ascites sarcoma cells, using oligonucleotide probes based on the partial amino acid sequences of PRPP synthetase from rat liver. Two distinct clones were obtained and nucleotide sequencing revealed that both clones encode 317 amino acids. The deduced amino acid sequences of the two differ only by 13 residues, whereas the nucleotide sequences are relatively divergent (81 % identity in the coding regions). These results and amino acid sequencing data suggest the presence of two distinct isoforms of the enzyme subunit (PRS I and II, JBC 262:14867,1987). To analyze the genomic gene structure, a rat genomic EMBL3 library was screened using PRS I and II cDNA probes and 16 clones were obtained, 15 of which were related to PRS I sequence and the other to PRS II. Studies of 7 overlapping PRS I-related clones showed the PRS I structural gene to span about 20 kb. Four other PRS-related clones were quite distinct from the above 7, regarding restriction maps. Thus the presence of additional gene or pseudogene loci for this enzyme is suggested, in analogy to the four loci in humans (HGM 9:739,1987).

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ESTIMATION OF THE IN VITRO AND IN VIVO INHIBITORY EFFECTS OF ANTIFOLATES UPON THYMIDYLATE SYNTHASE (TS) IN WHOLE CELLS.

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The ability of an antifolate to generate polyglutamates [via folyl polyglutamate sythetase (FPGS)] is an important determinant of its activity as this results in retention of the antimetabolite within the cells and may increase its affinity for the target enzyme. The estimation of TS activity in intact cells can be used to evaluate the retention of 5,8-dideazafolic acid (DAF) analogues by monitoring [^{3}H]2'-deoxyuridine metabolism in cells following the attainment of a state of dynamic equilibrium. CB3717 (2-amino 10-propargyl DAF) is an inhibitor of TS which has demonstrable clinical efficacy but also exhibits unpredictable toxicities. An in vitro structure activity study, with variations at positions 2 and 10, gave results which ranged from well retained (e.g. 2-methyl, 10-H) through intermediate (e.g. CB3717) to essentially unretained (e.g. 2-methoxy, 10-propargyl). Retention within cells was found to correlate with FPGS substrate activity but independent of both isolated TS inhibition and in vitro cytotoxicity. In mice it is possible to estimate the effect of analogues upon TS in ascitic tumour cells which are totally resistant to CB3717 due to the high circulating thymidine. CB3717 exhibited modest inhibition of TS in vivo with only 43% inhibition 2hrs after the i.v. administration of the maximum tolerated dose (200 mgs/kg). Modification at the 2 position had a dramatic effect such that the dose required to generate retained metabolites which inhibited TS activity by 50% after 2hrs, was >>500, 240, 27, and 2 mgs/kg for 2-methoxy, CB3717, desamino (2-H), and 2-methyl analogues respectively. Measuring the drug/target interaction in intact cells has allowed us to identify analogues which are considerably more potent in vivo TS inhibitors.

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AN ANALYSIS OF 5' REGULATORY SEQUENCES AND EFFECT OF THE MoMSV ENHANCER ON EXPRESSION OF THE APRT GENE.

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The transcriptional unit of the CHO APRT gene was analyzed by deletional mutants at the 3' and 5' end of the gene, and by deletions in the intronic regions. Two sequences 5' to the transcription start site are involved in gene regulation. One of these is a putative Spl binding region. The other region, within 52bp of the transcription start site, as measured by DNA footprinting experiments contains a second protein binding site of 22 bp conserved in both mouse and CHO aprt genes, suggesting that it could be functionally important. This region is being analyzed by in vitro mutagenesis. The aprt structural gene and promoter was fused to the Moloney Murine Sarcoma Virus (MoMSV) LTR. These sequences contain the viral promoter and enhancer signals. The 5' non-coding region of the aprt gene supresses activity of the Mo-MSV LTR. Deletion of the native aprt promoter allows Mo-MSV enhancer activity when the cells are grown in sodium butyrate. Under such conditions aprt enzyme activity is enhanced 20-30 fold in both transient expression assays, and in stable transformants. The overproduction of APRT has no obvious detrimental effect on cell growth.

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IN VITRO MUTAGENESIS OF THE CHO ADENINE PHOSPHORIBOSYL TRANSFERASE GENES.

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The mRNA of the APRT gene appears to be constitutively expressed and, thus, enzyme levels will be directly affected by mutations which alter translational efficiency. Accordingly, we have generated mutants altering the ATG start codon by in vitro mutagenesis of the CHO apt gene (the 2.8 kb bam HI-XbaI fragment) on an M13 vector. Mutagenesis was performed by the use of dut$^-$ung$^-$ E. coli strains as described by Kunkel, et. al. (Methods in Enzymology, 1987). Mutant genes are transferred to an Epstein-Barr virus derived plasmid (p220.2). These plasmids grow episomally in human cell lines. We have also generated APRT- mutants in the HL-60 myelocytic leukemic cell line, which will be used as recipients for the plasmid. In addition to increasing our knowledge of translational efficiency of non-cannonical start codons in eucaryotes, such a system can be used as a mutagen test system for potential mutagens and carcinogens, and also to analyze functional regions of the APRT protein.

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FUNCTIONAL CHARACTERIZATION OF ECTO-5'-NUCLEOTIDASE (ECTO-5'-NT) POSITIVE AND NEGATIVE HUMAN LYMPHOCYTES.

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Human T and B lymphocytes, separated into ecto-5'-NT positive and negative populations with goat anti-5'-NT antibodies and the fluorescence-activated cell sorter, were characterized in functional assays. Ecto-5'-NT$^-$ T cells proliferated as well as, or better than, ecto-5'-NT$^+$ T cells after stimulation with phytohemagglutinin or in a mixed lymphocyte reaction. Ecto-5'-NT$^-$ T cells also provided more help for pokeweed mitogen (PWM)-stimulated immunoglobulin synthesis than ecto-5'-NT$^+$ T cells. Therefore, the inability of ecto-5'-NT deficient lymphocytes from patients with immunodeficiency diseases to respond in these assays cannot be attributed solely to their reduced ecto-5'-NT activity. In contrast, ecto-5'-NT$^+$ T cells proliferated in response to ten-fold lower doses of phorbol esters than ecto-5'-NT$^-$ T cells, suggesting that ecto-5'-NT$^+$ T cells may utilize unique biochemical pathways of cellular activation or may have a preferential ability to respond to certain stimuli.

Ecto-5'-NT positive and negative B cells were tested for the ability to synthesize IgM and IgG after stimulation with PWM and Epstein Barr virus. Although both groups of cells synthesized IgM, the synthesis of IgG was restricted to the ecto-5'-NT$^+$ subpopulation. These data provide the first direct evidence that ecto-5'-NT is a marker for the functional maturation of human B cells and demonstrate that ecto-5'-NT is different from other human B cell surface antigens such as IgD, Leu 8, and HB-4 which are lost as B cells mature.