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PROTECTION AGAINST CYTOTOXICITY OF ENDOGENOUS COPPER IN THE REQUIREMENT FOR MERCAPTOETHANOL BY A LYMPHOMA IN PRIMARY CULTURE

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SUMMARY: A specific copper chelator, 2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonic acid substituted for mercaptoethanol to support growth of a L1210 lymphoma in primary culture. Added Cu^{++}, but not Zn^{++} or Fe^{++} interfered with growth promotion by the chelator. It also can protect an established L1210 culture, which does not require mercaptoethanol, from cytotoxicity of two bis-thiosemicarbazones. Since these are known to require copper for cytotoxicity, the results indicate that 2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonic acid acts by removing a source of endogenous copper in the tissue culture medium which prevents growth of the primary culture.

INTRODUCTION: Mercaptoethanol is an essential growth-promoting additive for several lymphoid cell lines, both primary and established [1]. A wide range of human tumors also require ME for growth in primary culture and this thiol has become a standard medium additive for such growth of human tumor cells [2,3]. It is neither a requirement for most tumors in established culture nor for normal tissue in primary culture. Recently, Ishii and coworkers [4] have reviewed the ME requirement in culture and presented evidence that dependent cells are defective in cystine transport, requiring the mixed disulfide of ME and cysteine for uptake of cysteine through the amino acid L transport system [5]. However, Broome and coworkers [6] have indicated that ME may overcome the autoinhibitory activity of small peptides. Peptides have been reported to promote transition metal transport and copper toxicity [7]. A role of endogenous copper in establishment of the ME requirement is indicated by our observation that a specific copper chelating agent, BCS, [8,9], supports growth of ME requiring L1210 cells.

ABBREVIATIONS: ME, mercaptoethanol; BCS, bathocuproinedisulfonic acid, disodium salt, systematic name, 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline-disulfonic acid, disodium salt.

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MATERIALS AND METHODS:

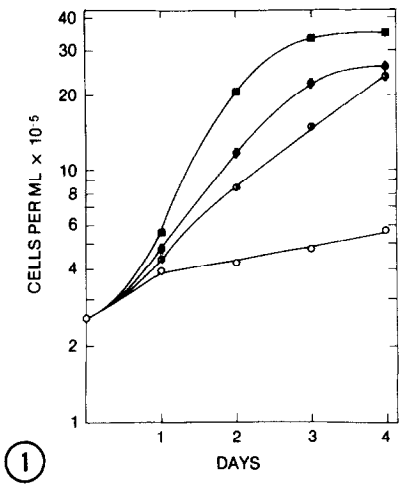
Materials. All cells were grown in RPMI 1630 medium (K. C. Biologicals, Lenexa, KA) containing 16.5% heat inactivated fetal bovine serum (Flow Laboratories, McLean, VA) and 40 μg/ml Gentamicin (Schering Corp, Kenilworth, NJ). 2-Mercaptoethanol (Sigma Chemical Co., St. Louis, MO) was used at a final concentration of 50 μM. The water soluble disodium salt of BCS was obtained from the G. Frederick Smith Chemical Co., Columbus, OH, and the bis-thiosemicarbazones, 3-ethoxy-2-oxobutyraldehyde-bis-thiosemicarbazone and 3-ethoxy-2-oxobutyraldehyde-bis-(N^4-methylthiosemicarbazone) were obtained from Research Organics, Cleveland, OH. These were dissolved in dimethylformamide (DMF), sequanol grade, (Pierce Chemical Co., Rockford, IL).

Tissue Culture.

Primary Culture. The in vivo L1210 line was routinely passaged as a free cell suspension by i.p. injection of 10^5 cells into DBA/2 female mice. Prior to use in primary culture, 10^5 cells were injected i.p. into male CDF_1 mice. These were sacrificed on days 6 or 7 and cells removed from the peritoneal cavity by flushing with 6 to 10 ml of the medium (less ME or BCS) used for growing the primary culture. The cells were washed three times by gentle centrifugation and resuspension to remove ascitic fluid and red blood cells, pooled, adjusted to a density of 2.5 x 10^5 cells per ml as determined with a Coulter counter, model ZBI, and 7 ml of the cell suspension were added to 25 cm^2 Corning flasks. ME and BCS were introduced as serial dilutions in water (10 μl/ml). Cells were grown at 37° in tightly stoppered flasks.

Established Culture. The L1210 established culture line, which does not require ME for growth, was used to evaluate the effect of BCS on the cytotoxicity of the bis-thiosemicarbazones. It was maintained in RPMI 1630 medium containing 16.5% heat inactivated FBS without antibiotics. Cells were harvested at mid-log phase (8-10 x 10^5 cells per ml), washed three times in fresh medium and resuspended at 1 x 10^5 cells/ml as determined by a Coulter Counter, model ZBI. Seven ml of the cell suspension were added to 25 cm^2 Corning flasks, BCS was introduced in water solution (10 μl/ml) and the bis-thiosemicarbazones as serial dilutions in DMF (1 μl/ml). Cells were grown at 37° in tightly stoppered flasks.

RESULTS AND DISCUSSION: BCS at several concentrations supported the growth of a primary culture of L1210 cells in the absence of mercaptoethanol (Fig. 1). Evidence that the support of growth by BCS was indeed due to the removal of copper was obtained in two ways. 1. Addition of other essential ions, Fe^{++} as ferrous ammonium sulphate or Zn^{++} as zinc acetate to a final concentration of 50 μM did not interfere with the growth promotion supported by 50 μM BCS. Copper ion however, added as copper sulphate, completely blocked such growth at final concentrations of 50 to 10 μM. 2. BCS protected cells of an established culture against the cytotoxic action of bis-thiosemicarbazones (Fig. 2). Since these compounds are dependent upon their copper chelates for cytotoxicity [10,11], our observations indicate that endogenous copper in the tissue culture medium is available for inhibition of growth of the primary



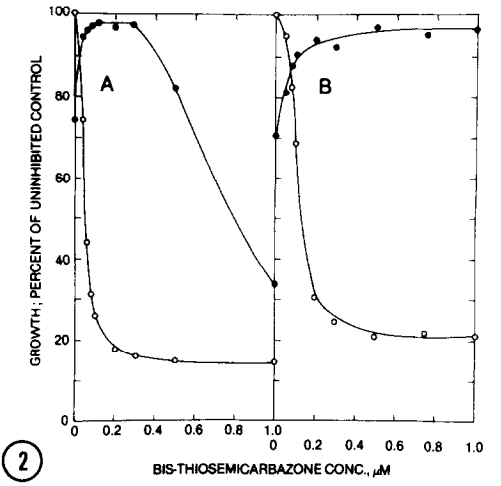


Fig. 1. Support of growth of L1210 cells in primary culture by mercaptoethanol or bathocuproinedisulfonic acid. Cells were incubated in medium, as described in Materials and Methods, with mercaptoethanol, 50 μM (■) or bathocuproine-disulfonic acid, sodium salt, at a concentration of 100, 50, 25, 10 μM (◖), or 5 μM (◑). Growth in absence of these additions is indicated by (○).

Fig. 2. Protection of toxicity of bis-thio-semicarbazones to L1210 cells in established culture by bathocuproinedisulfonic acid. Cells were incubated for 48 hrs with the indicated concentrations of two bis-thiosemicarbazones, (A), 3-ethoxy-2-oxobutyraldehyde-bis-(thiosemicarbazone) or (B), 3-ethoxy-2-oxobutyr-aldehyde-bis-(N^4-methylthiosemicarbazone). Open circles, bis-thiosemicarbazones alone; filled circles, in the presence of bathocuproinedisulfonic acid, sodium salt, 50 μM, added concurrently. Mercaptoethanol was neither required nor added to support growth of this established culture.

culture. The relationship between depressed cystine uptake [4,5] and susceptibility to copper-mediated cytotoxicity by ME requiring cells, may be related to the catalysis by copper ion of sulfhydryl group oxidation.

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