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Differential In Vitro Sensitivity of Marrow Erythroid and Granulocytic Colony Forming Cells to Chloramphenicol

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The effects of chloramphenicol (CAP) and thiamphenicol (TAP) on mouse and human in vitro erythroid (CFU-E) and granulocytic (CFU-C) colony forming units have been studied. Both drugs inhibited CFU-E growth in a concentration-dependent, stereospecific, manner. Complete inhibition of human CFU-E growth was observed at a CAP concentration of 10 μg/ml while a concentration over 50 μg/ml was required to inhibit CFU-C growth. Furthermore, whereas the inhibition of CFU-C growth could be blocked in vitro by high colony stimulating factor concentrations, inhibition of CFU-E growth was not affected by increased erythropoietin (ESF) levels. The lack of protection by ESF may account for the apparent vulnerability of erythroid cells to CAP in vivo.

Key words: marrow erythroid colonies, chloramphenicol, thiamphenicol

INTRODUCTION

Chloramphenicol (CAP) is commonly associated with dose dependent, reversible suppression of erythropoiesis and, less frequently, granulopoiesis and thrombocytopoiesis (1–3). Evidence indicates that this effect results from inhibition of mitochondrial protein synthesis (4–6). The biochemical mechanism underlying the greater susceptibility of erythroid cells to CAP is uncertain. Recent studies suggest that this may be related to CAP-induced suppression of ferrochelatase activity and a block in the last step of heme synthesis (7). Whatever the mechanism, it must ultimately be reflected in suppression of cellular proliferation.

Previous studies of the effects of CAP on the granulocytic colony forming unit (CFU-C) (8, 9) have demonstrated that therapeutic concentrations of the drug inhibit colony growth and that inhibition can be largely blocked by increasing the level of colony

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stimulating factor (CSF) in the medium. These observations suggested that the level of CSF in the cell milieu in vivo may be an important determining factor in the occurrence and/or severity of CAP-induced granulocytopenia (9).

In the present study we have examined the in vitro effect of CAP and its analogue, thiamphenicol (TAP), on mouse and human erythroid colony formation in relation to the concentration of erythropoietin (ESF). For comparison we have also studied the effects of these drugs on mouse and human CFU-C.

MATERIALS AND METHODS

Crystalline CAP and its L-threo isomer were obtained through the courtesy of Dr. Robert Hans, Parke Davis and Co., Detroit, Mich. Crystalline TAP was a gift from Dr. Della Bella, Zambon, S.p.A., Milan, Italy. Each drug was dissolved in balanced salt solution (Microbiological Associates, Bethesda, Md.) to give a concentration of 0.5 mg/ml. Thus, when 2 μl of the solution are added to 1 ml of cell suspension in tissue culture medium, a final drug concentration of 1 μg/ml is obtained. These drugs were tested over a range of 0–60 μl/ml.

Preparation of Cells

Marrow cells were harvested from the femurs of LAF_1 male mice (20 gm weight; Jackson Laboratories, Bar Harbor, Me.) by flushing the marrow cavity with 1–2 ml of ice-cold α-medium (Flow Laboratories, Rockville, Md.) containing 2% fetal calf serum FCS; Rehatuin, Reheis Chemical Corporation, Kankakee, Ill.). The cells were then dispersed by gentle passage 2 to 3 times through a 22-gauge needle. Human marrow cells from the posterior iliac spine of hematologically normal subjects were aspirated into α-medium containing 50 units/ml of preservative-free heparin. The human marrow cell suspension was first centrifuged at 400 $\times$ g for 10 min at 4°C, the buffy layer resuspended in heparin-free α-medium with 2% FCS, and it was centrifuged again. The number of murine or human nucleated cells was determined by hemocytometer counting and the final concentration of trypan-blue dye-excluding cells adjusted to 2×10^6 cells/ml.

CFU-E

Colonies of hemoglobin synthesizing cells were grown in plasma clots using a modification of the method of Stephenson et al. (10). A cell concentration of 2×10^5 cells/ml was employed. The ESF was from sheep plasma and obtained commercially. (Step III; Connaught Laboratories, Willowdale, Ont.). Drugs in appropriate concentrations were added along with 0.1 ml of citrated bovine plasma (Grand Island Biological Co., N.Y.) and the cell-medium suspension pipetted into microwells (Cocke Engineering, Alexandria, Va.) in 0.1 ml aliquots. At least 6 wells were established for each data point. Cultures of mouse marrow were harvested after 48 hours of incubation while human marrow cell cultures were harvested at day 7. The clots obtained from individual microwells were spread on glass slides, fixed with 5% glutaraldehyde, dried and then stained with a benzidine stain followed by a hematoxylin counterstain. Aggregates of 8 or more hemoglobin-containing cells were counted as colonies. With this technique, the number of colonies is linearly related to both the number of nucleated cells plated and the concentration of ESF.

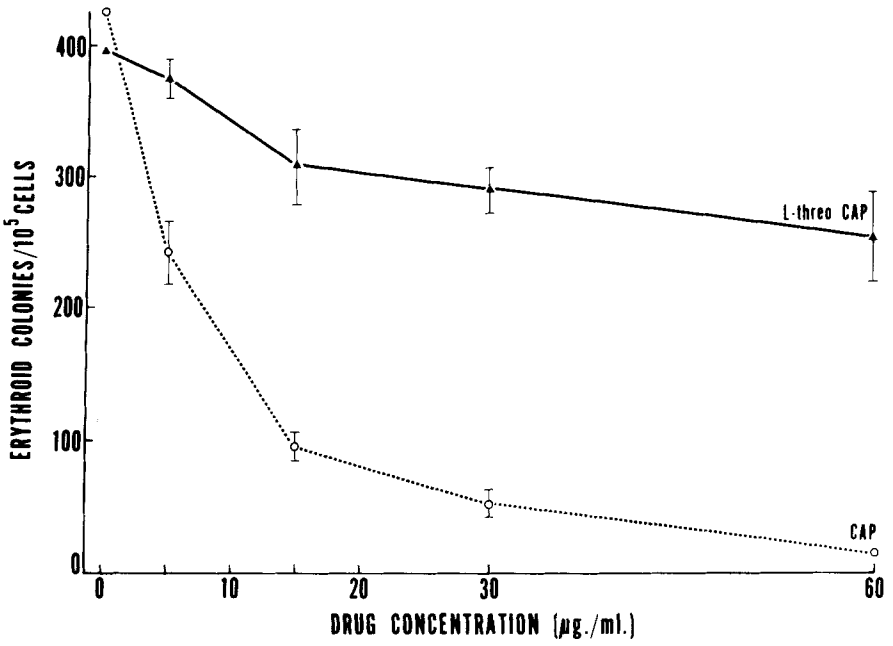

Fig. 1. Effect of increasing concentration of CAP and its L-threo isomer on mouse marrow CFU-E growth. Erythropoietin was used at a level of 0.15 µ/ml. Each point represents the mean of 6 values ± SD as indicated under "Methods".

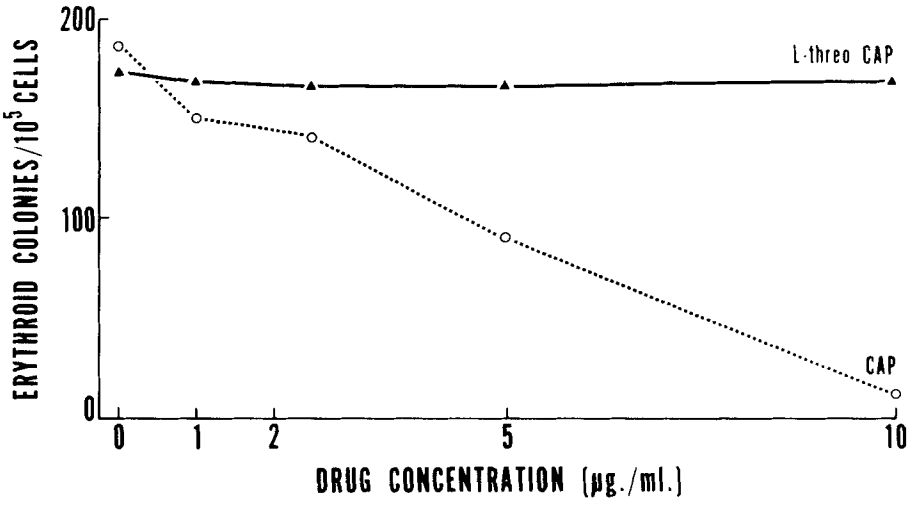

Fig. 2. Effect of increasing concentrations of CAP and its L-threo isomer on human CFU-E growth. Erythropoietin was used at a level of 0.15 µ/ml.

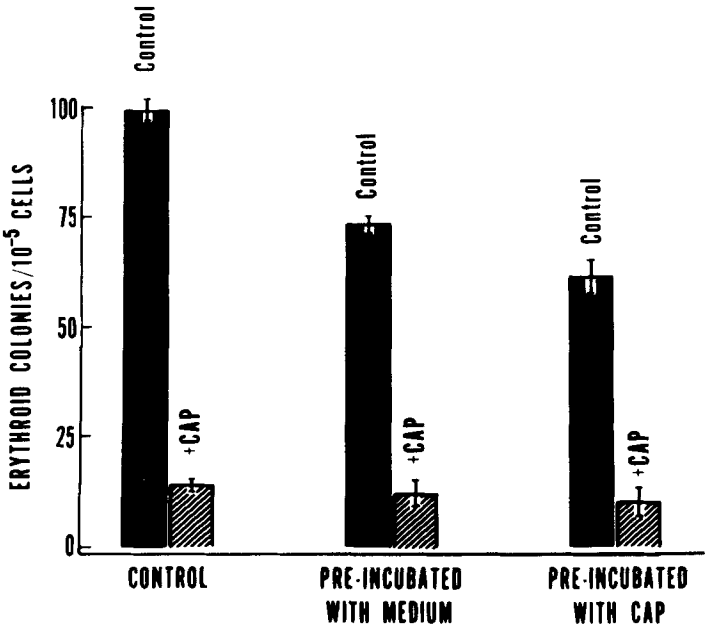

Fig. 3. Reversibility of the CAP effect. In this experiment mouse marrow cells were preincubated for 4 hrs in the presence of CAP (15 μg/ml) or medium without drug. The cells were then washed and plated with and without CAP in the presence of ESF, 0.15 μ/ml.

CFU-C

The assay for CFU-C was carried out as described previously (11). All assays were done in triplicate. For mouse marrow, the source of CSF used was serum-free lung conditioned medium prepared from endotoxin-treated mice (8). For human marrow the source of CSF was prepared as follows (12). Lung tissue obtained at autopsy was cut into 2–3 mm slices and incubated under sterile conditions in serum-free Dulbecco's modified Eagle's medium for 48 hours. The medium was then harvested, centrifuged to eliminate tissue debris, treated at 56°C for 30 min. and dialyzed against distilled water.

RESULTS

Effect of CAP on CFU-E Growth

The effect of increasing concentrations of CAP on mouse marrow CFU-E growth is illustrated in Figure 1. Inhibition was observed at drug concentrations as little as 5 μg/ml and became nearly complete at 60 μg/ml. The biologically inactive L-threo isomer of CAP exerted comparatively little inhibition. A similar experiment with human marrow is illustrated in Figure 2. Here complete inhibition was observed at a CAP concentration of 10 μg/ml indicating that human CFU-E are more sensitive to the drug under the culture conditions described. The L-threo isomer had virtually no effect.

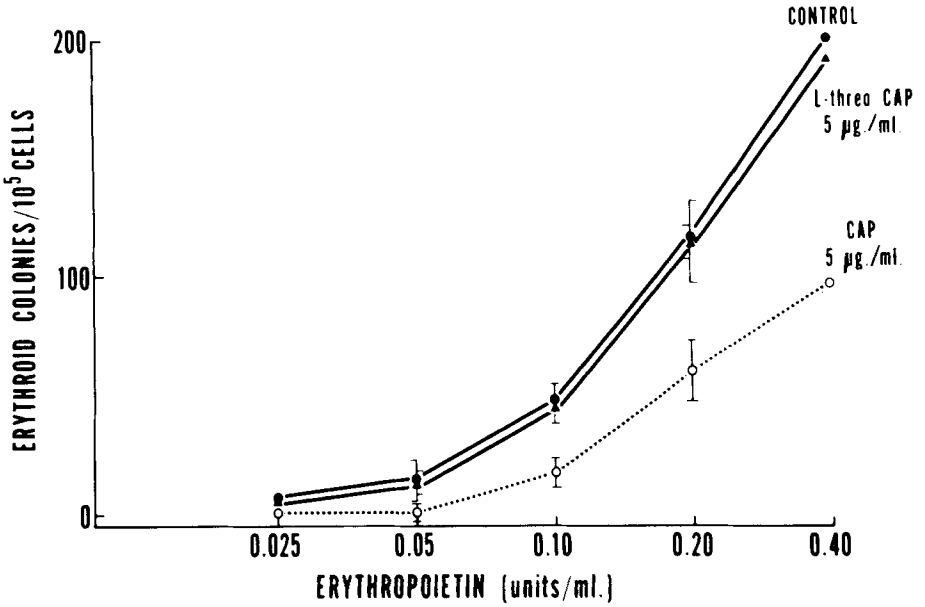

Fig. 4. Effect of a fixed concentration (5 μg/ml) of CAP and its L-threo isomer on human CFU-E growth in relation to ESF. No protection was observed with increasing concentrations of ESF in the cultures.

To test for reversibility of the CAP effect, mouse marrow cells were preincubated with or without drug for 4 hrs in medium. The cells were then washed and plated for CFU-E growth both in the presence and absence of CAP. The results are shown in Figure 3. Although preincubation in medium alone resulted in 25% loss of erythroid colonies below control values, the loss after preincubation with CAP was not significantly different, demonstrating the requirement for CAP's continued presence in order for inhibition to be observed. It is also evident that CAP inhibited the growth of preincubated cells to the same extent as control cells, indicating that prior incubation with the drug did not reduce the number of CAP-sensitive CFU-E.

Relation of Inhibition to ESF Concentration

The effect of increasing ESF concentrations on human CFU-E growth in the presence of a fixed level of CAP is shown in Figures 4 and 5. Approximately 50% inhibition of growth was seen with 5 μg CAP/ml at all ESF levels (Fig. 4). The L-threo isomer had no inhibitory effect.

Virtually complete inhibition of erythroid colony growth was observed at CAP and TAP concentrations of 10 μg/ml, regardless of the ESF levels (Fig. 5). A similar pattern of inhibition was noted in cultures of mouse marrow (Fig. 6). In comparing the results in mouse (Fig. 6) to those in human marrow (Fig. 4 and 5), it can be seen again that comparable CAP concentrations had a greater inhibitory effect on human CFU-E.

Inhibition of CFU-C Growth by CAP and the Protective Effect of CSF

The effect of increasing concentrations of CAP on mouse CFU-C growth (at each of

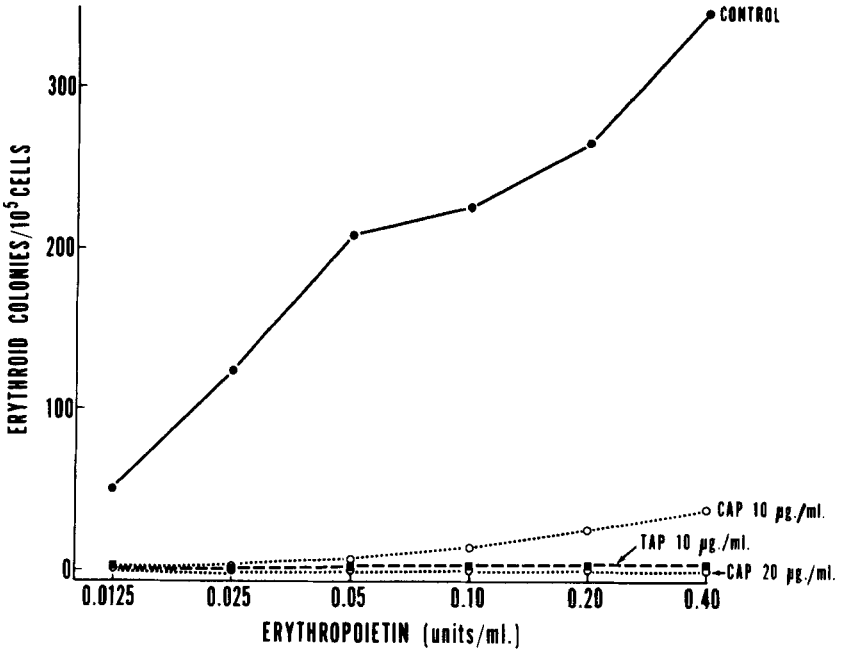

Fig. 5. Effect of CAP (10 μg and 20 μg/ml) and TAP (10 μg/ml) on human CFU-E growth in relation to ESF. No protection was observed with increasing concentration of ESF in the cultures.

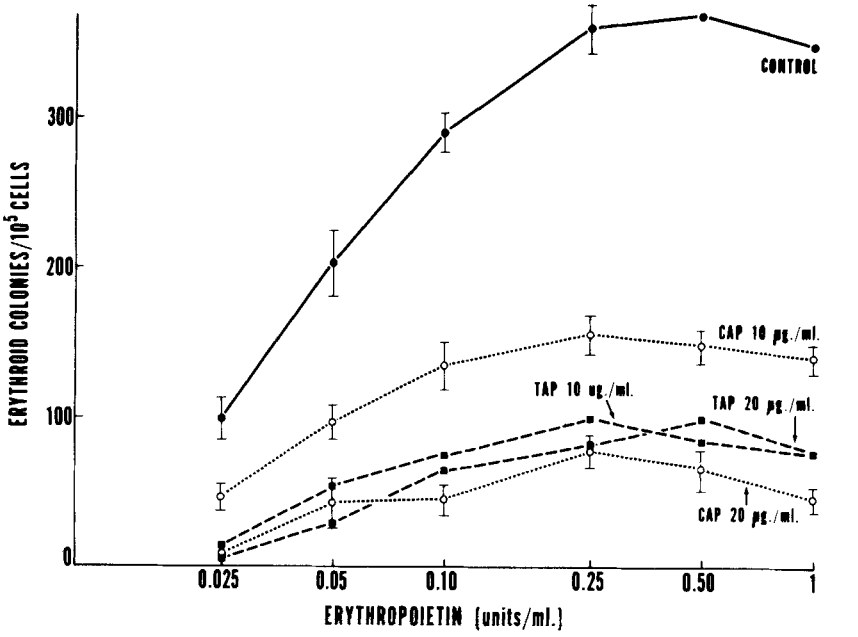

Fig. 6. Effect of CAP (10 and 20 μg/ml) and TAP (10 and 20 μg/ml) on mouse CFU-E growth in relation to ESF concentration. No protection was observed with ESF

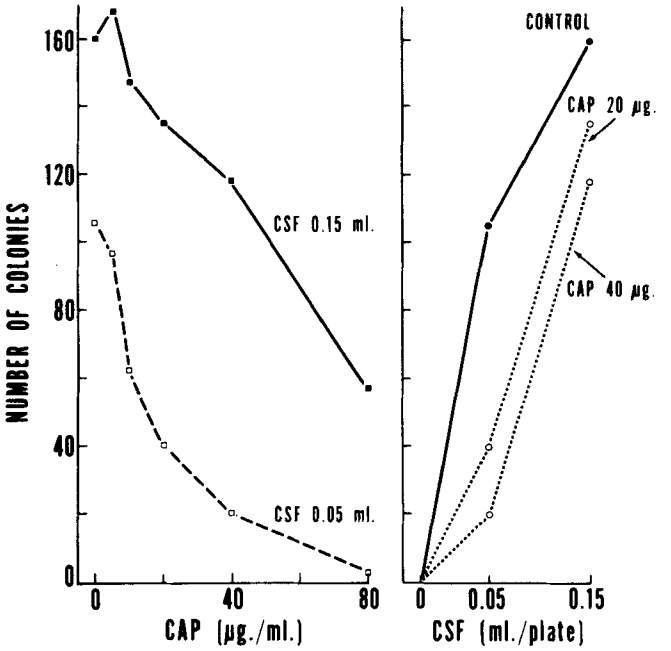

Fig. 7. Effect of increasing concentrations of CAP on mouse CFU-C growth at 2 CSF levels. On the left is a plot relating number of colonies to CAP concentrations at 2 CSF levels. On the right is a plot relating number of colonies to CSF levels at 2 CAP concentrations (20 and 40 μg/ml). Note the reversal of the CAP effect by increasing concentrations of CSF.

two CSF levels) is shown in Figure 7. In the presence of 0.05 ml/ml of CSF, CAP at concentrations of 20 and 40 μg/ml inhibited colony formation by 60 and 80% respectively. In contrast, at a CSF level of 0.15 ml/ml the inhibition of colony growth was 16 and 27%, respectively.

The protective effect of CSF is more readily seen when the number of colonies is plotted against CSF concentration with and without CAP (Fig. 7, right). Both the percent inhibition as well as the total number of colonies lost are significantly lower at the CSF level of 0.15 ml ($P < 0.01$). A similar experiment with human marrow is shown in Figure 8. Comparable CAP concentrations caused less inhibition of human CFU-C than that observed with mouse marrow, but the protective effect of CSF was again clearly seen. When the effects of CAP on CFU-C (Fig. 7 and 8) and CFU-E (Fig. 1 and 2) growth are compared it can be seen that CFU-E are more sensitive to the drug, with almost complete inhibition of growth occurring at 10 μg CAP/ml.

DISCUSSION

The present experiments clearly demonstrate the in vitro inhibition of mouse and human erythroid colony growth by concentrations of CAP and TAP frequently achieved

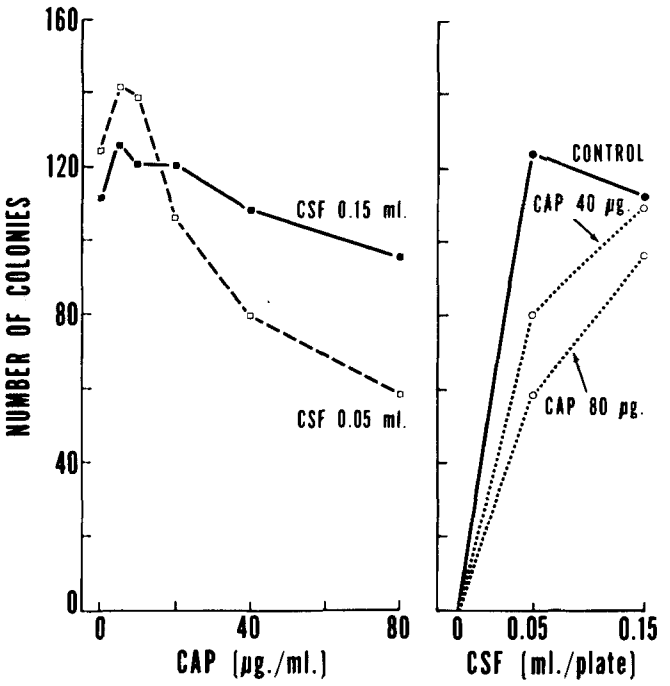

Fig. 8. Effect of increasing concentrations of CAP on human CFU-C growth at 2 CSF levels. On the left is a plot relating number of colonies to CAP concentrations at 2 CSF levels. On the right is a plot relating number of colonies to the CSF levels at 2 CAP concentrations (40 and 80 μg/ml). Note reversal of the CAP effect by incrasing concentrations of CSF.

therapeutically in man. The inhibitory effect of CAP is concentration dependent and appears to be stereospecific. Short term preincubation (4 hrs) of bone marrow cells with CAP does not result in significant inhibition of CFU-E growth, indicating reversibility of the CAP effect within this limited time of cell-drug exposure.

Since in vitro bone marrow culture systems are susceptible to various nonspecific injuries, a certain degree of specificity must be demonstrated when one is studying drug effect. The specificity of the CAP effect observed in these studies is demonstrated by the lack of inhibition by the L-stereoisomer. Furthermore we have shown (not illustrated) that neither Dilantin (20 μg/ml) nor propylthiouracil (20 μg/ml) had any inhibitory effect on CFU-E growth.

Both human and mouse marrow CFU-E appear to be more sensitive than CFU-C to CAP. Thus, complete inhibition of human CFU-E growth was observed at a CAP concentration of 10 μg/ml while a concentration of over 40 μg/ml was required to significantly inhibit CFU-C growth. The possibility that the difference in the sensitivity of CFU-E and CFU-C to CAP is a function of the different assay conditions cannot be excluded. Evidence has been provided that inhibition of cellular growth by CAP is a consequence of mitochondrial injury (8, 9, 13). Whether mitochondria of erythroid progenitors are, for some reason, more sensitive to CAP than those of granulocytic progenitors, has not been determined.

The protection of mouse CFU-C from the inhibitory effect of CAP by high concentrations of CSF, has previously been reported (9). A similar protective effect of CSF on human CFU-C growth is clearly shown in the present study. The mechanism for this protective effect of CSF is not understood. As has been pointed out (9) CAP neither binds to nor inactivates CSF. In contrast, we observed no protection of CFU-E growth by ESF; the degree of inhibition by CAP was relatively constant at all ESF levels. This difference may reflect differences in the mechanism of action of CSF and ESF at the cellular level, but little can be said in this regard until we know more about the function of CSF.

The significance of in vitro observations as related to what occurs in vivo must always be interpreted with caution (9). The pattern of in vitro inhibition of CFU-C and CFU-E growth by CAP observed in these studies is consistant with what we know occurs in vivo. Thus, it is known that suppression of erythropoiesis from large doses of CAP occurs with much more regularity than suppression of granulopoiesis. The results of our studies suggest that the lack of protection by ESF may account for the comparative vulnerability of erythroid cells to CAP in vivo.

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