

# Autocrine production of extracellular catalase prevents apoptosis of the human CEM T-cell line in serum-free medium

(oxygen radicals)

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Communicated by Konrad Bloch, February 16, 1993 (received for review November 23, 1992)

**ABSTRACT** CCRF-CEM is a human T-cell line originally isolated from a child with acute lymphoblastic leukemia. At cell densities  $> 2 \times 10^5$  cells per ml, CEM cells grow in serum-free medium, but at lower cell densities the cultures rapidly undergo apoptosis, or programmed cell death. The viability of low-density CEM cells could be preserved by supplementing the serum-free medium with "conditioned" medium from high-density CEM cultures, but a variety of known growth factors and lymphokines were ineffective. Fractionation of conditioned medium by sequential chromatography on DEAE-cellulose, propyl agarose, chromatofocusing, and hydrophobic-interaction HPLC resulted in the isolation of a 60-kDa protein capable of sustaining CEM growth in the absence of serum. The active protein was identified as human catalase based on its amino acid sequence and composition and was subsequently shown to exhibit catalase activity and to be replaceable by human erythrocyte catalase or bovine liver catalase. Comparison of the level of intracellular catalase activity with the amount released into the culture medium demonstrated that the latter accounted for  $< 3\%$  of the total catalase activity present in the cell culture. These findings show that, despite its low amount, the catalase released by CEM cells, and perhaps by T cells in general, provides a critical first line of defense against hydrogen peroxide ( $H_2O_2$ ) present in the extracellular milieu.

Survival in aerobic conditions has required that organisms develop elaborate antioxidant defense systems to cope with potentially toxic reactive oxygen species (ROS) (1). For example, hydrogen peroxide ( $H_2O_2$ ) is generated from numerous endogenous and exogenous sources including mitochondrial respiration, UV radiation, peroxigenic bacteria, and, in the immune system, from the combined actions of the NADPH oxidase and superoxide dismutase systems of phagocytes (1, 2). It has recently become apparent that ROS can have divergent effects on mammalian cell growth. In some cases low doses of  $H_2O_2$  induced cells to undergo apoptosis, or programmed cell death (3), whereas in other cases  $H_2O_2$  was found to promote cell proliferation (4–7). Such findings suggest that ROS may function as intracellular second messengers (8) and that growth stimulation may occur when cells are protected against excessive ROS toxicity (7).

Both prokaryotes and eukaryotes produce catalase, an enzymatic antioxidant that efficiently breaks down  $H_2O_2$  into  $H_2O$  and  $O_2$ . However, the extensive involvement of the glutathione peroxidase system for intracellular  $H_2O_2$  detoxification in cells of higher organisms (9), coupled with the tenet that catalase activity is restricted to peroxisomes (7), raises the question as to what role extraperoxisomal catalase plays in antioxidant defense (10). We report here that CCRF-CEM, an acute T-lymphocytic leukemia cell line (11), dis-

plays density-dependent growth characteristics in response to cell-derived extracellular catalase and rapidly undergoes apoptosis when cultured below a critical cell density. These results are discussed in the context of a possible relevance of extracellular antioxidant production to normal T-cell function and the etiology of leukemogenesis.

## MATERIALS AND METHODS

**Cell Lines and Culture Conditions.** The CCRF-CEM (11) and MOLT-4 (12) T-cell acute lymphoblastic leukemia cell lines were obtained from the American Type Culture Collection. A3.01, a derivative of CCRF-CEM (13) was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. The T-cell lines were maintained in a described (14) serum-free medium (BHITS) consisting of RPMI 1640 medium supplemented with 0.1% bovine serum albumin (Sigma A6003), 10 mM Hepes (pH 7.2), bovine pancreatic insulin [Intergen 4501 (Purchase, NY)] and human apotransferrin (Intergen 4450) each at 5  $\mu$ g/ml, and sodium selenite at 5 ng/ml (Sigma). Cells were cultured at 37°C in a humidified incubator containing 6%  $CO_2/94\%$  air. Cultures were routinely passed 1:5 every 4–5 days to maintain cell densities of  $0.4\text{--}2 \times 10^6$  cells per ml.

For large-scale production of conditioned medium (CM), the A3.01 cell line was generally used, and the culture conditions and medium were modified as follows. Four-liter cultures of A3.01 cells grown in the serum-free medium were added to 16 liters of RPMI 1640 medium containing 10 mM Hepes and insulin and transferrin each at 1.5  $\mu$ g/ml. The cells were then cultured statically in 150-cm<sup>2</sup> flasks for 6 days, at which time the flasks were stood on end, and incubation was continued. After 24 hr the CM was removed by aspiration.

**CEM Cell Bioassay.** CEM cells from 5-day-old serum-free cultures were used in the bioassay. The cells were washed twice with medium, resuspended in the same medium to a cell density of  $1 \times 10^5$  cells per ml, and added in 0.1-ml aliquots to round-bottomed 96-well culture plates containing duplicate 0.1-ml samples to be tested for growth-promoting activity. After 24 (or in some cases, 48) hr, cultures were treated with 0.5  $\mu$ Ci per well of [<sup>3</sup>H]thymidine (New England Nuclear, 1 Ci = 37 GBq) for 4–6 hr and harvested onto a glass filter with a PHD sample harvester (Cambridge Technology, Cambridge, MA); the incorporated radioactivity was then measured by liquid scintillation counting.

**Viability Assays.** Assay of CEM cell viability was based on the ability of the cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (15). CEM cells grown in 1% fetal bovine serum/RPMI 1640 medium were pelleted, washed once with serum-free medium, and cultured

in round-bottom 96-well plates at a cell concentration of  $2 \times 10^5$  cells per ml in 0.2 ml of either serum-free medium or serum-free medium supplemented with 40% CM. At hourly intervals thereafter, 20  $\mu$ l of an aqueous MTT stock solution (5 mg/ml) was added to triplicate wells of each culture condition. After 3-hr incubation, 0.1 ml of medium was removed from each well, and 0.1 ml of 0.5% HCl/isopropanol was added to lyse the cells. After an additional 10-min incubation at 37°C, the absorbance at 570 nm was read by using an Anthos model 2001 ELISA plate reader.

**Purification of Lymphoid Catalase.** A3.01 cells were generally used as a source of CM (A3.01-CM) because these cells seemed to survive better in high-density, serum-free cultures than CEM cells. Typically, 20 liters of A3.01-CM were collected by aspiration, passed through a 5- $\mu$ m filter to remove residual cells, and concentrated to  $\approx 150$  ml by using an Amicon YM-30 filter. The crude concentrate was dialyzed overnight against two 4-liter changes of 25 mM Hepes, pH 7.0, and applied at a flow rate of 2 ml/min to a DEAE-cellulose (Whatman) column (80-ml bed volume) previously equilibrated with 25 mM Hepes, pH 7.0. The column was washed with the equilibration buffer until the OD<sub>280</sub> reading returned to baseline. A 320-ml linear gradient up to 0.5 M NaCl in 25 mM Hepes, pH 7.0, was then initiated, and column fractions were assayed directly for activity with the CEM bioassay. The activity eluted within the first 70 ml, resolving it from  $\approx 98\%$  of the starting protein and, in particular, from the bovine serum albumin in the serum-free medium. Ammonium sulfate was added directly to the pooled, active material from the DEAE-cellulose column to yield a final concentration of 1.4 M, and the material was loaded at a flow rate of 2 ml/min onto a propyl-agarose (Sigma) column (50-ml bed volume) previously equilibrated with buffer A [0.1 M sodium phosphate buffer, pH 7.2/1.4 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>]. Unbound material was eluted by washing the column with two bed volumes of buffer A, and bound proteins were eluted with a 250-ml linear gradient up to 100% of buffer B (0.1 M sodium phosphate, pH 7.2/12.5% isopropanol). Column fractions containing the activity were pooled and filter-dialyzed into 25 mM triethanolamine buffer, pH 8.3, containing 0.3% octyl- $\beta$ -D-glucopyranoside with an Amicon Centriprep 10 concentrator. The recovered material, which represented  $\approx 2\%$  of the post-DEAE protein, was applied to a Mono P 5/20 column (Pharmacia) equilibrated in the same buffer and chromatofocused by using polybuffer 96/74, pH 5.0, containing 0.3% octyl- $\beta$ -D-glucopyranoside at a flow rate of 1 ml/min. Column effluent was monitored at 280 nm, and 1-ml fractions were collected and assayed directly for activity. The activity eluted over a pH range of 6.3–6.8, coinciding with three minor peaks of protein that accounted for 8% of the protein carried over from the propyl-agarose column. Ammonium sulfate was added to active fractions to yield a 1 M final concentration; the fractions were then individually injected onto a Synchropac propyl hydrophobic-interaction chromatography (HIC) HPLC column (250  $\times$  4.8 mm) equilibrated with 70% buffer A [0.1 M sodium phosphate buffer, pH 7.2/2.0 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>] and 30% buffer B (0.1 M sodium phosphate buffer, pH 7.2/12.5% isopropanol). A 25-min linear gradient up to 100% of buffer B was initiated immediately upon sample injection. Column effluent was monitored at 230 nm, and 1-ml fractions were collected and assayed directly for activity. HIC HPLC of the two fractions corresponding to the middle of the activity peak from the chromatofocusing column generated a single, well-resolved, symmetrical peak of protein that coincided precisely with the peak of activity.

**Analytical Methods.** Reversed-phase HPLC of the post-HIC HPLC material was done using a Synchropac C-1 reversed-phase column (50  $\times$  4.6 mm) developed with a linear gradient starting from 95% buffer A (water/0.1% CF<sub>3</sub>COOH)

and 5% buffer B (isopropanol/0.1% CF<sub>3</sub>COOH) and increased over 30 min to 100% of buffer B. Protein elution was monitored at 230 nm. Essentially one protein peak eluted from the reversed-phase HPLC column, indicating that the protein obtained after HIC HPLC was purified to homogeneity. This result was confirmed by analysis of the post-HIC and post-reversed-phase HPLC materials by electrophoresis on 0.1% NaDodSO<sub>4</sub>/7–20% polyacrylamide gels (16). After overnight staining with Coomassie blue, a single band of 60 kDa was observed under both reducing and nonreducing conditions, thus confirming that the four chromatography steps yielded a single, highly purified protein capable of sustaining CEM growth in serum-free medium.

**Assay of Catalase Activity.** Catalase activity was quantified by measuring the rate of H<sub>2</sub>O<sub>2</sub> catabolism as indicated by a decrease in absorbance at 240 nm (17).

**Cyanogen Bromide Cleavage.** Cyanogen bromide (CNBr) cleavage was done on 200 pmol of purified protein (18). The resulting fragments were separated by Tricine/NaDodSO<sub>4</sub>/PAGE (16.5% T, 3% C) (19), electroblotted onto a poly(vinylidene difluoride) protein-sequencing membrane, and stained with Coomassie blue.

**Apoptosis Assay.** DNA fragmentation was assayed by using a procedure adapted from Hogquist *et al.* (20). Briefly,  $2 \times 10^6$  cells were pelleted and resuspended in 500  $\mu$ l of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4/10 mM EDTA/0.2% Triton X-100) for 10 min, followed by centrifugation (10,000 rpm for 10 min). Proteinase K was added to the supernatant (100  $\mu$ g/ml), which was then incubated at 50°C for 6 hr, followed by the addition of RNase A and further incubation at 37°C for 2 hr. Fragmented DNA was extracted twice with phenol, extracted once with chloroform/isoamyl alcohol, 24:1, and precipitated overnight at –20°C with 50% isopropanol/glycogen at 5  $\mu$ g/ml. The DNA fragments were electrophoresed through a 1.25% agarose gel and stained with ethidium bromide.

**Amino Acid Analysis and Peptide Sequencing.** Amino acid analysis and sequencing were done at the University of California at Los Angeles Protein Microsequencing Facility (Los Angeles).

**Materials.** Recombinant human interleukins -3, -4, -5, -6, and -8 were provided by the Biological Response Modifiers Program (National Cancer Institute, Bethesda, MD). Recombinant human interleukin 2 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. Recombinant human interleukin 7 was purchased from Genzyme. Human erythrocyte catalase was purchased from Calbiochem, and bovine liver catalases were obtained from Worthington and Sigma.

## RESULTS

**Autocrine Growth of CCRF-CEM.** We previously reported that CEM cells could be grown in a serum-free medium consisting of RPMI 1640 supplemented with bovine serum albumin, insulin, transferrin, and selenium (14). However, in subsequent studies it was noted that the ability of CEM cells to proliferate in serum-free medium was cell-density-dependent (Fig. 1). CEM cells proliferated when inoculated at cell densities  $> 2 \times 10^5$  cells per ml but did not proliferate at lower cell densities. By contrast, MOLT-4, a T-cell lineage distinct from CCRF-CEM, grew in the serum-free medium at every cell density tested (Fig. 1).

Density-dependent growth has, in some cases, been attributed to the secretion of one or more essential cell-derived proteins (21, 22). Fig. 2 shows that the addition of CM from high-density ( $2\text{--}3 \times 10^6$  cells per ml) CEM cultures (CEM-CM) sustained CEM cell proliferation at the normally non-permissive cell density of  $5 \times 10^4$  cells per ml. By contrast,

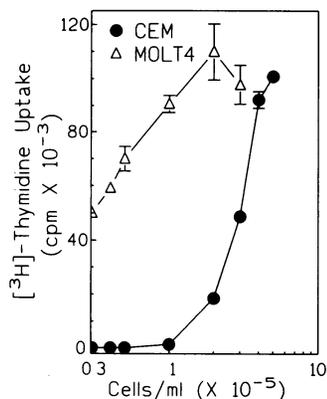


FIG. 1. Density dependence of CEM proliferation. CEM and MOLT-4 cells were cultured in serum-free media at the indicated cell densities. After 72 hr, cultures were treated for 4 hr with [<sup>3</sup>H]thymidine.

CEM cells cultured at  $5 \times 10^4$  cells per ml in the absence of CM or serum supplements underwent a rapid loss of viability (Fig. 3A) with accompanying morphological changes (Fig. 3B) and DNA degradation (Fig. 3C) characteristic of apoptosis (23). Collectively, the data shown in Figs. 1–3 are consistent with CEM cells having a requirement for one or more cell-derived factors to survive in the absence of serum supplements.

A variety of recombinant human cytokines were tested for their ability to sustain CEM proliferation in serum-free medium; recombinant human interleukins 2 through 8 were ineffective, as were a variety of other known growth factors (data not shown).

**Purification of 60-kDa Hydrophobic Protein (HP60).** The inability of CEM cells to proliferate in serum-free medium when plated at low cell densities facilitated development of a bioassay that was used to assay column fractions for growth-promoting activity. As described in *Materials and Methods*, growth-promoting activities were routinely quantified by measurements of [<sup>3</sup>H]thymidine uptake, but visual inspection of the cultured cells (Fig. 3B) also provided a rapid and sensitive assessment of activity during the purification process. Purification of the required factor was accomplished starting with 20 liters of A3.01-CM, which contained  $\approx 4$  g of starting protein. The purification protocol developed involved four chromatographic steps (Fig. 4) and yielded  $\approx 35$   $\mu$ g of a HP60 having a pI of 6.3–6.8.

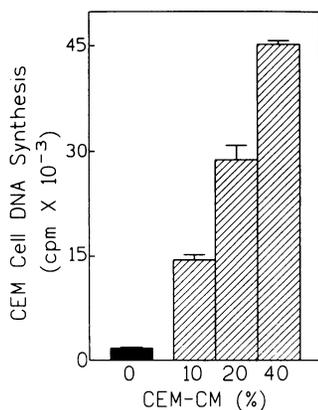


FIG. 2. Factor dependency of CEM. CEM cells were cultured at a density of  $5 \times 10^4$  cells per ml in serum-free medium containing the indicated concentrations of CEM-CM. After 24 hr the cultures were treated for 4 hr with [<sup>3</sup>H]thymidine.

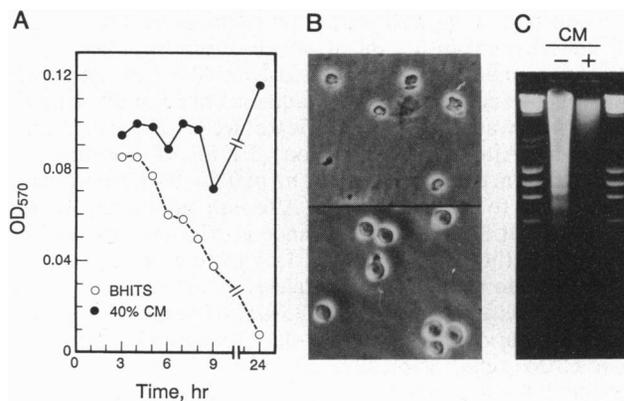


FIG. 3. CEM viability and morphology in serum-free medium. (A) CEM cells were cultured at a density of  $5 \times 10^4$  cells per ml in either serum-free medium (BHITS) or serum-free medium containing 40% CEM-CM. Immediately afterward and at hourly intervals thereafter, culture aliquots were removed, MTT was added, and cell viability was assayed 4 hr later. (B) CEM cells were cultured at a density of  $5 \times 10^4$  cells per ml for 15 hr in serum-free medium either lacking (Upper) or containing (Lower) 40% CEM-CM. (C) CEM cells were cultured at a density of  $5 \times 10^4$  cells per ml in serum-free medium either lacking (–) or containing (+) 40% CEM-CM. After 18 hr, the cells were harvested and assayed for DNA fragmentation. The four low-molecular-size markers (outer lanes) range from 0.3 to 1.4 kb.

**Identification of HP60 as Catalase.** An initial attempt to obtain the N-terminal amino acid sequence of HP60 suggested that its N terminus was blocked. Accordingly, HP60 was treated with CNBr followed by recovery of a  $\approx 5$ -kDa peptide that was subjected to automated sequencing. The 21-amino acid sequence Leu-Gln-Gly-Arg-Leu-Phe-Ala-Tyr-Pro-[?]-Thr-His-Arg-His-Arg-Leu-Gly-Pro-Asn-Tyr-Leu was obtained and found to correspond to region 350–370 of human catalase (24). Further confirmation of HP60 as human catalase was based on its total amino acid composition (25) and its ability to degrade  $H_2O_2$  (17).

**Biological Activity of Lymphoid Catalase.** Dose–response studies were done by using the CEM cell bioassay to compare the ability of the purified lymphoid catalase (HP60) with commercially obtained human erythrocyte catalase. When titrated on the basis of their catalase activities (units/ml), both lymphoid and erythrocyte catalases were found equally effective in supporting CEM cell growth (Fig. 5). Virtually identical results were obtained with bovine liver catalases of various degrees of purity, obtained from two different commercial sources (data not shown).

Finally, studies were done to compare the amount of catalase activity released by CEM cells into the culture medium with the amount of catalase retained intracellularly. Five milliliters of CEM culture containing  $2 \times 10^6$  cells per ml was concentrated 5-fold by ultrafiltration, and after the addition of Triton X-100 (0.5% final concentration) to release intracellular catalase, the total amount of catalase activity measured was 0.081 units per 5-ml culture. Surprisingly,  $>97\%$  of the activity was associated with the cell pellet (0.078 unit per  $1 \times 10^7$  cells), whereas the level of activity in the culture medium (concentrated 5-fold) was below the limit of detection. This finding indicates that despite a large excess of intracellular catalase, it is the minute amount of extracellular catalase that is necessary to protect CEM cells from the cytotoxic effects of extracellular  $H_2O_2$ .

## DISCUSSION

The requirement for oxygen radical scavengers and/or reducing agents in both serum-containing and serum-free me-

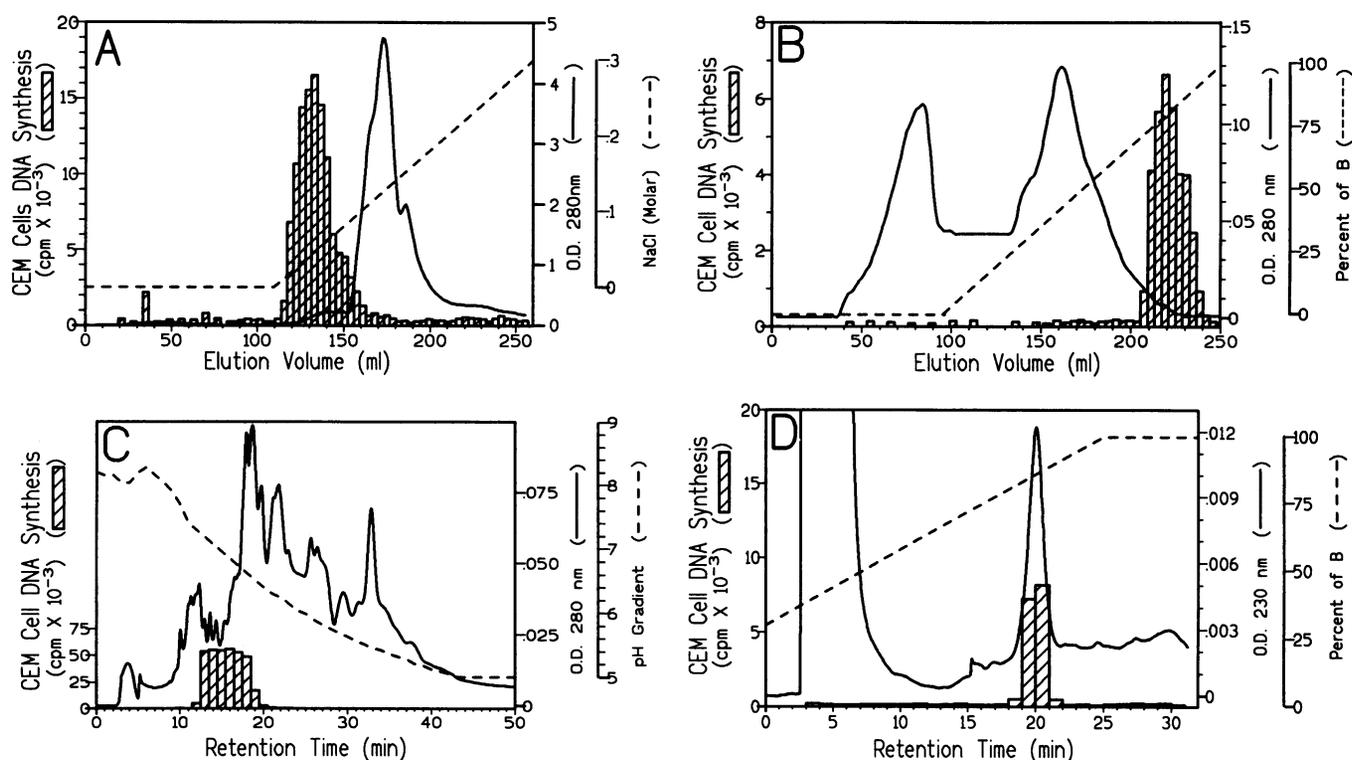


FIG. 4. Purification of HP60. (A) Concentrated A3.01-CM was applied to a DEAE-cellulose column, and bound proteins were eluted with a linear gradient of 0–0.5 M NaCl. (B) Pooled fractions of activity from the DEAE column were applied to a propyl-agarose column, followed by elution with decreased ammonium sulfate to 0 M and a simultaneous increase of isopropanol to 12.5%. (C) Active fractions from the propyl-agarose column were pooled and chromatofocused on a Mono P column with a pH interval between 5.0 and 8.3 by using an HPLC system. (D) Active fractions from the Mono P column were applied in 2-ml aliquots to a Synchropak propyl-HIC HPLC column and eluted with decreased ammonium sulfate to 0 M and increased isopropanol to 12.5%. The peak that eluted between 3 and 9 min contained primarily ampholytes from the chromatofocusing step. Fractions containing HP60 activity are indicated by the hatched bars.

dium is well established. It has previously been reported that the addition of exogenous catalase facilitates the growth of lymphoid cells in serum-free medium (26, 27). Those reports, however, did not provide any evidence that cells of lymphoid origin when cultured at a suitably high density had the potential to condition the growth medium with catalase, or any other antioxidant for that matter. We report here that the density-dependent growth of the CCRF-CEM lymphocytic leukemia cell line in serum-free medium is the result of a requirement for extracellularly released catalase. The apparent inability of large quantities of cell-associated catalase to prevent apoptosis of individual CEM cells in cultures below

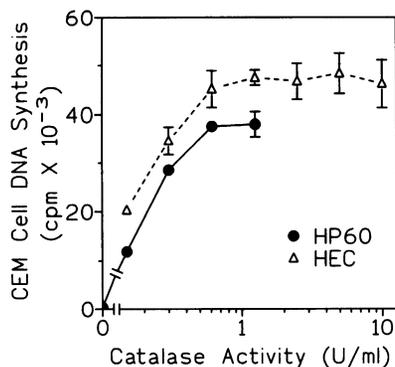


FIG. 5. Titration of lymphoid and erythrocyte catalases in the CEM bioassay. CEM cells cultured at  $5 \times 10^4$  cells per ml in serum-free medium were additionally supplemented with the indicated concentrations of either purified lymphoid catalase (HP60) or human erythrocyte catalase (HEC). After 48 hr, cultures were treated for 5 hr with [<sup>3</sup>H]thymidine to assay DNA synthesis.

a critical cell density suggests a requirement for a relatively minute quantity of extracellular catalase to protect against either H<sub>2</sub>O<sub>2</sub>-mediated plasma membrane damage or by acting on a trace medium component necessary for cell growth. Possible sources of H<sub>2</sub>O<sub>2</sub> in CEM cultures include endogenous production of ROS (2, 4, 5) and small amounts of H<sub>2</sub>O<sub>2</sub> probably present in tissue culture medium (2, 26).

The results described here provide evidence of a leukemic cell line that has an obligate autocrine-like dependence for endogenously produced and extracellularly released catalase. In this regard, it is interesting to note that adult T-cell leukemia-derived factor, an autocrine growth factor, is a homologue of thioredoxin, an enzyme involved in numerous dithiol-dependent reducing processes, and which can catabolize H<sub>2</sub>O<sub>2</sub> (28). Similar to our observations with extracellular catalase, adult T-cell leukemia-derived factor/thioredoxin acts as an autocrine growth factor for Epstein-Barr virus-transformed B-cell lines and facilitates the growth of adult T-cell leukemia cells in low-serum tissue culture (28). Thioredoxin appears to function as an endogenous reducing agent in the process of lymphocyte transformation and activation (28). Hence, autocrine production of extracellular antioxidants, such as catalase and thioredoxin, may play an important role in leukemogenesis.

It has generally been accepted that catalase provides little protection against extracellular H<sub>2</sub>O<sub>2</sub> because the enzyme is predominantly located in peroxisomes (7, 10) and is thought not to be secreted (29). Nevertheless, the present findings with CEM cells when coupled with the reported secretion of thioredoxin by human T-lymphotropic virus I-infected T cells (28) suggest that cells of T helper origin may have the capacity to export functional antioxidant enzymes. Indeed, T helper cells have been reported to be less sensitive to H<sub>2</sub>O<sub>2</sub>-induced

damage than other lymphoid cells, a feature that may be crucial for their ability to survive and function in close proximity to activated macrophages and neutrophils at sites of chronic inflammation (30). In addition, extracellular catalase has been shown to augment a number of T-cell activities, including mixed lymphocyte reactions (31), erythrocyte rosette formation (32), and proliferative responses to phorbol 12-myristate 13-acetate-stimulated macrophages (33). Thus, the observations that some CD4<sup>+</sup> T-cell lines grow in response to extracellular catalase/thioredoxin may have important implications for the functioning of T-helper cells *in vivo*. At or above a critical cell density T helper cells may, through the release of extracellular antioxidants, augment a local immune response by enhancing cell viability and function. Conversely, impaired antioxidant production and/or secretion may be associated with a loss of CD4 T-cell viability or function. It was recently reported that human immunodeficiency virus infection is associated with decreased thioredoxin levels (34). Consistent with that observation, we have recently observed that human immunodeficiency virus gene expression in variants of the CEM cell lineage results in a loss of their ability to grow in serum-free conditions, even at relatively high cell densities (35). This impaired growth in serum-free medium was shown to result from a diminished ability of human immunodeficiency virus-expressing cells to condition the medium with catalase.

We thank Mr. Steve Van Cleave for technical assistance. This research was supported with funds provided by the East Carolina University School of Medicine. Protein sequencing performed at the University of California at Los Angeles Protein Microsequencing Facility was aided by a Biomedical Research Support Shared Instrumentation Grant (1 S10RR05554-01) from the National Institutes of Health.

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