

# Purification of an Inhibitor of Erythroid Progenitor Cell Cycling and Antagonist to Interleukin 3 from Mouse Marrow Cell Supernatants and Its Identification as Cytosolic Superoxide Dismutase

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**Abstract.** We have isolated a protein from media conditioned by a murine marrow-derived cell line (PB6) and from mouse marrow supernatants that antagonizes interleukin 3-dependent proliferation of cells in culture and reversibly inhibits DNA synthesis of erythroid progenitor cells (BFU-E) in vitro. This protein, p16 (monomer  $M_r = 16$  kD on SDS-PAGE), was purified to homogeneity and amino acid sequencing of a polypeptide fragment yielded a sequence identical to that of murine cytosolic Cu,Zn-containing superoxide dis-

mutase (SOD). The identification of p16 as SOD was confirmed by the detection of SOD enzymatic activity in pure p16 fractions, and when a commercial human erythrocytic SOD preparation was tested it showed the same cell inhibitory activities as p16. These observations show that superoxide dismutase is able to affect the cycling and growth factor responses of hematopoietic cells, activities that have not previously been associated with this enzyme.

CELL proliferation and differentiation in general can be affected by many growth factors having stimulatory (8), inhibitory (31), or multiple (28) effects on their targets and showing varying degrees of specificity. Many hematopoietic progenitor cells have been shown to be specifically stimulated or inhibited by different factors (1, 10), and it may be that the control of proliferation of the many cell types in this system involves a balancing of the effects of opposing signals. Within the erythroid lineage the proliferation of late progenitor cells is influenced by erythropoietin (27), while early progenitors (BFU-E) can be stimulated to proliferate by a variety of factors such as interleukin 3 (IL 3), granulocyte-macrophage colony-stimulating factor (11), activin (32), and an endogenous burst promoting activity (12), with the last two having apparently specific effects upon the BFU-E. BFU-E proliferation can also be inhibited, both nonspecifically (e.g., by interferons; 10), and specifically by factors such as inhibin (32).

The demonstration that protein from marrow supernatants of C57BL/6 mice (B6-MS; 2) and from media conditioned by cells of the PB6 continuous murine marrow-derived cell line (PB6-CM; 9) can reversibly inhibit DNA-synthesis of BFU-E in vitro led to attempts to characterize the nature of the inhibitory effect (3) and purify the factors involved (9). Recently we have developed a culture model of the BFU-E that serves as a cell proliferation assay to screen purified fractions for the ability to oppose the effects of IL 3 on dependent cells (4). In this study we describe the purification

of a protein from PB6-CM and B6-MS which is able to both antagonize the effects of IL 3 on dependent cells in culture and reversibly inhibit cell cycling of BFU-E. By amino acid sequencing we have been able to identify this protein as cytosolic copper, zinc superoxide dismutase (SOD<sup>1</sup> · EC 1.15.1.1), an enzyme involved in the protection of tissues from the toxic effects of the superoxide radical (13). This unexpected identification was confirmed by tests for SOD activity in inhibitory protein preparations and by the demonstration that commercially available SOD possesses similar activity in both cell inhibitory assay systems.

## Materials and Methods

### Animals, Cells, and Reagents

The isolation of the PB6 (Pan B6) cell line has been described (30); it is an immortalized marrow-derived line of an undetermined and apparently nonerythroid lineage. Cultures used for the production of conditioned medium for purifications (PB6-CM) were grown in 0.5–2 liters of serum-free  $\alpha$ -minimal essential medium ( $\alpha$ -MEM; Gibco Laboratories, Toronto, Canada) seeded at a density of  $5 \times 10^5$  cells/ml, and allowed to grow for 3–4 d before the medium supernatant was harvested by centrifugation and filtered before concentration (see below). The DA-1 murine marrow cell line is equivalent to an immortalized BFU-E and was obtained from Dr. L. Guilbert (University of Alberta, Edmonton, Canada). Cells were maintained in Iscove's modified Dulbecco's medium (IMDM; Gibco Laboratories) plus

1. *Abbreviations used in this paper:* HSOD, human erythrocytic superoxide dismutase; NRP, negative regulatory protein; SOD, superoxide dismutase.

100 U/ml penicillin, 50  $\mu$ g/ml streptomycin, 10% FCS, and 20% WEHI-3 cell-conditioned medium, and during proliferation assays the WEHI-3 CM was replaced with IL 3 (ICN Biomedical Canada Ltd., Montreal, Canada; dosages expressed in units quoted from the source). Mature C57BL/6U mice were used for harvesting of marrow cells and supernatants according to previously described methods (2). All chemicals and reagents used for chromatography and electrophoresis were of HPLC or ultrapure grade; human erythrocytic SOD was purchased from Sigma Chemical Co., St. Louis, MO.

### Ion Exchange Chromatography of Supernatants

PB6-CM (initial volume  $\sim$ 20 liters) and B6-MS (initial volume  $\sim$ 20 ml) were concentrated via Amicon Hollowfiber, Centriprep, and Centricon concentrators (molecular mass cutoff = 10kD) to a final volume of <1 ml. The buffer was changed to 20 mM Tris, 2% betaine, 20 mM 2-mercaptoethanol, and samples were heated to 55°C for 10 min, cooled to room temperature, and centrifuged before loading onto a Mono-Q HR 5/5 column (Pharmacia Inc., Piscataway, NJ) in an FPLC setup. The column was developed against start buffer plus 1 M NaCl at a flow rate of 1 ml/min according to the gradients indicated in the figures, and outflow samples (1 ml) were collected, pooled as indicated, and concentrated. Samples assayed for inhibitory activity had the buffer changed to culture medium appropriate to the assay and were sterilized by filtration (Millex GV 0.22  $\mu$ m). Protein amounts in purified fractions were determined by amino acid analysis or estimated from band intensity on SDS-PAGE gels relative to standards.

### Electrophoresis and Electroelution of Proteins

Isolation of protein from complex fractions for bioassays was done by electroelution from Laemmli 15% acrylamide SDS-PAGE gels using the Bio-Rad mini electrophoresis system and electroelution module (Bio-Rad Laboratories, Richmond, CA), electroeluted protein samples were put through several concentration and resuspension cycles to remove SDS and change the buffer to culture medium before being assayed for inhibitory activity. Gels used to visualize the low molecular weight region of crude and purified fractions and in the preparation of protein and polypeptide fragments for sequencing contained 20% acrylamide and were prepared and run according to the method of Giulian and Graham (14), with reducing conditions. The molecular mass markers used were prestained SDS-PAGE standards (17–130 kD; Bio-Rad Laboratories) and the polypeptide molecular mass calibration kit (<17 kD; Pharmacia). Gels were silver stained to visualize proteins, dried with Bio-Gel Wrap (Bioscience, Inc., Carmel, NY) and photographed via transmitted light.

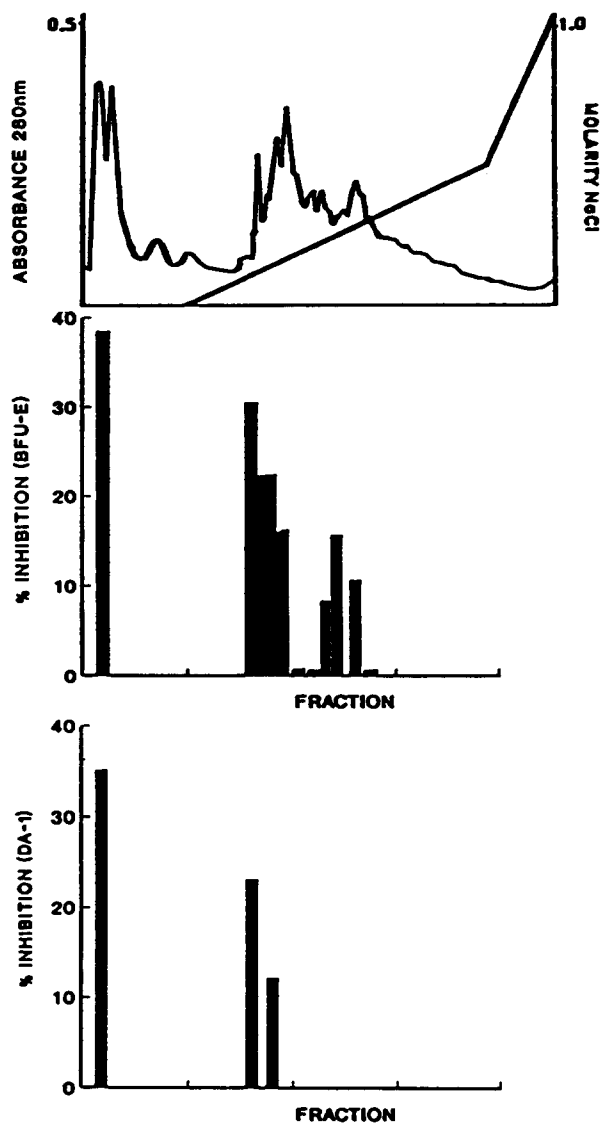
### Peptide Mapping and Amino Acid Sequence Analysis

Peptide mapping was done by running protein preparations on SDS-PAGE polypeptide gels, excising the desired protein size region, and performing in situ cyanogen bromide cleavage (24) using a modified protocol (15) in which the gel slices were soaked in 70% formic acid plus 50 mg/ml CNBr for 2 h at room temperature, then equilibrated in stacking gel buffer (14). The digested slices were soaked in hot sample buffer (14) for 10 min and the polypeptide fragments were resolved on a second gel identical to the first. These conditions consistently gave >70% cleavage of protein in the gel slices.

Samples of intact protein and cleavage products used for amino acid sequence analysis were recovered by electroblotting from SDS-PAGE gels onto Immobilon PVDF membrane (Millipore-Continental Water Systems, Bedford, MA), blotted protein was visualized by Coomassie blue staining (19) and excised on the membrane support for direct amino-terminal amino acid sequence analysis in a gas phase sequencer (model 470A; Applied Biosystems, Inc., Foster City, CA) with online PTH amino acid analyzer.

### Cell Proliferation Assay for IL 3 Antagonism

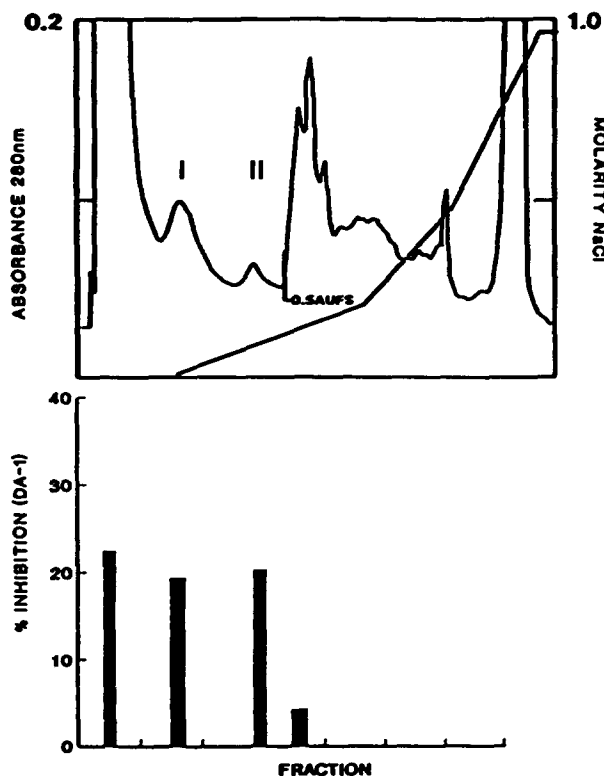
The details of this assay have been described (4). It involves testing protein samples for the ability to inhibit the proliferation of DA-1 cells in the presence of a growth-permissive dose of IL 3. This inhibitory effect is expressed as the % inhibition (DA-1), equal to the difference between mean living cell numbers at 48 h in treated culture wells and untreated controls expressed as a percentage of control. The apparent toxicity of a sample is calculated as the difference in the mean proportion of dead cells between tested and control groups expressed as a percentage of control. Significance of inhibition and toxicity relative to controls were determined via comparisons of raw cell count data with Student's *t* test.



**Figure 1.** Inhibitory activity of B6-MS fractions isolated via Mono-Q ion exchange chromatography at pH 7.2. 8 mg of protein was loaded onto the column developed according to the gradient shown. Aliquots (1–3  $\mu$ g) of fractions were assayed for inhibitory activity and the results are shown in the histograms below the tracing (the width of each bar indicates the region on the tracing from which the samples tested were pooled, all fractions are shown to have some activity to distinguish regions where no material was tested). Only the flow-through and first early eluting fractions that were active in the BFU-E assay were tested in the DA-1 assay. SDS-PAGE analysis of the flow-through fraction is shown in Fig. 3.

### BFU-E Cell Cycling Inhibition Assay

The details of this assay have been described (2). It is used to detect rapid short-term inhibitors of DNA synthesis by their ability to reduce the lethality to BFU-E of brief exposure to the S-phase specific cytotoxin hydroxyurea (HU). Aliquots of marrow cells treated with the test sample are divided into two groups and one is exposed to HU, then both (+HU and –HU) are washed, cultured, and scored after 7 d for number of erythroid bursts (4–6 plasma cultures per group). The inhibitory effect of a tested sample (% inhibition BFU-E) is calculated as the difference in the percentage of BFU-E killed (the difference in mean burst counts between +HU and –HU groups expressed as a percentage of the –HU mean) between the test group and an untreated control. If there is no significant decrease in –HU burst counts



**Figure 2.** Inhibitory effects of early eluting PB6-CM Mono Q fractions. 8 mg of soluble protein was loaded onto the column developed against the NaCl gradient indicated at pH 7.5 (note the absorbance scale changes from 0.2–0.5 U full scale). Results from DA-1 cell proliferation assay for aliquots (0.2–5  $\mu$ g) of pooled early eluting fractions are shown below the tracing, and inhibition was significant for the flow-through and first two early eluting peak fractions (I and II, shown in SDS-PAGE gels in Fig. 3).

between test and control then the % inhibition (BFU-E) represents the proportion of BFU-E that were removed from DNA synthesis during HU treatment by the effects of the test sample. Significance of inhibition was determined by comparing the numbers of +HU bursts for tested and control groups (*t* test), with an increase versus control indicating inhibitory activity.

### Assay of Superoxide Dismutase Activity

SOD activity in purified fractions was measured using the negative assay of Martin et al. (18), which detects SOD activity through interference with the autoxidation of hematoxylin. The reactions were carried out in 2-ml disposable cuvettes at 25°C with an initial hematoxylin concentration of 200  $\mu$ M added to reaction buffer (50 mM potassium phosphate, 0.1 mM EDTA at pH 7) containing the sample to be tested, and the rate of hematoxylin autoxidation was monitored as an increase in absorbance at 560 nm in a spectrophotometer (model DU-8B; Beckman Instruments, Inc., Palo Alto, CA). The SOD activity of a fraction was measured as the decrease in rate of change in absorbance it produced expressed as a percentage of the buffer alone control (% inhibition). Comparisons of SOD activity in various fractions were also made using a gel activity assay (5) on samples run in native PAGE gels. The human erythrocytic SOD preparation tested for comparison had an activity of 3,130 U/mg in the standard xanthine oxidase-cytochrome C assay for SOD activity (20).

## Results

### Fractionation of Supernatants by Ion Exchange Chromatography

The results of inhibition assays of fractions obtained from

B6-MS after Mono Q fractionation (at pH 7.2) are summarized in Fig. 1. BFU-E inhibitory activity comparable to that of the starting material was consistently recovered in flow-through and early gradient eluting fractions (Fig. 1, center), and samples from this range were also inhibitory when tested in the DA-1 cell proliferation assay (bottom). SDS-PAGE analysis of these fractions (not shown) detected strong protein bands in several size ranges, and the major proteins were identified by amino acid sequencing to be serum transferrin, aspartate amino transferase,  $\alpha$  and  $\beta$  globin, none of which had inhibitory activity when tested alone (data not shown).

PB6-CM Mono Q fractions showed a similar distribution of inhibitory activity to those obtained from B6-MS, and our purification efforts were concentrated on this material since it is qualitatively the less complex of the two. Attempts to isolate active components from the Mono Q early eluting fractions via successive application of different chromatographic methods met with some success, however, it was found that altering the elution gradient and running pH during Mono Q separation was the most effective way to isolate inhibitory PB6-CM fractions containing small numbers of different proteins as determined by SDS-PAGE. In the Mono Q purification shown in Fig. 2 (run at pH 7.5) inhibitory activity in the DA-1 cell proliferation assay was localized to the flow-through and early eluting peak fractions, one of which (Fig. 2, peak II) consistently showed a single major protein band at  $M_r = 16$  kD (referred to hereafter as p16) on SDS-PAGE (Fig. 3).

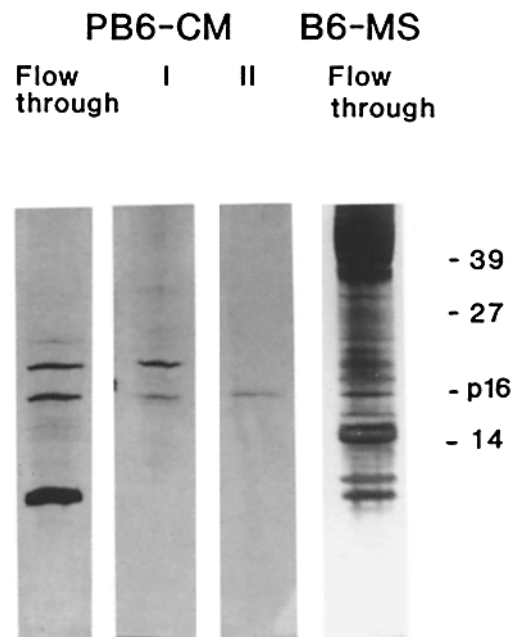
### Inhibitory Activity of p16 Protein

The IL 3 antagonistic and toxic effects of test samples can be distinguished in the DA-1 cell proliferation assay if there are sample concentrations where cell growth is reduced without an accompanying increase in death. Such an analysis is shown in Fig. 4 for p16 from PB6-CM, which had an inhibitory effect at concentrations where there was no apparent toxicity. p16 was able to oppose the effects on DA-1 cells of a growth-permissive dose of IL 3 (1:5,000 dilution from stock equivalent to 1 CFU<sub>50</sub> U/ml) down to a concentration of 60 ng/ml (the high apparent toxicity for larger doses of p16 may be due to p16 actually causing cells to be starved for IL 3). p16 is also inhibitory in the BFU-E assay (Table I) down to a concentration of 45 ng/ml.

As the gel photograph in Fig. 3 shows, the p16 protein band is present in other Mono Q fractions from PB6-CM and B6-MS which show inhibitory activity (Figs. 1 and 2). The identity of p16 protein present in these fractions was established via peptide mapping of the protein bands, and when protein from the p16 region (15–25 kD) was isolated by electroelution it showed inhibitory activity comparable to that of the total fractions (Table I). Protein from the only other size region showing matching bands in PB6-CM and B6-MS (70–90 kD, containing mostly serum albumin and transferrin) did not show inhibitory activity (Table I).

### Sequence Analysis and Identification of p16

Intact p16 did not yield a sequence when analyzed in the sequencer and the results were consistent with a protein having a blocked NH<sub>2</sub> terminal. When the major 12-kD fragment obtained after CNBr cleavage of p16 was analyzed the fol-



**Figure 3.** SDS-PAGE gels of lower molecular weight region for samples corresponding to the flow-through and first two early eluting Mono Q protein peaks from PB6-CM (Fig. 2) and the Mono Q flow-through fraction of B6-MS (Fig. 1). Mobilities of p16 protein and size standards are shown to the right.

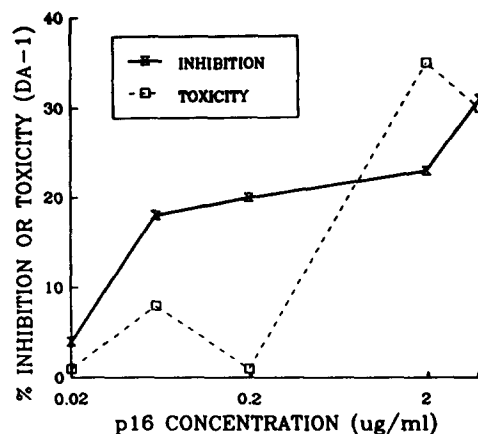
lowing sequence was obtained (with a 93% repetitive yield) for the first 20 amino acids:

KAVXVLKGDGPVQGTIHFEQ.  
MAMKAVCVLKGDPVQGTIHFEQLA

is the amino acid sequence deduced from the first 25 codons of the cloned cDNA for mouse cytosolic CuZn-superoxide dismutase (SOD; 7), which has an unmodified chain molecular mass of 15,943 D and was found as a match to the p16 sequence during a computerized search of the NBRF protein sequence data base. The unknown amino acid at position 4 (X) in the p16 fragment sequence corresponds to cysteine (C) in SOD, and cysteine is not detected during sequencing unless it is first modified to a stable derivative, which was not done in this instance. The residue preceding the NH<sub>2</sub> terminal of the p16 fragment is a methionine (M) in SOD, which is consistent with the fact that CNBr cleaves proteins at M-X bonds, and the expected size of the major CNBr fragment from SOD of 115 amino acids (~11.9 kD) matches that obtained from p16.

#### SOD Activity of p16

The results of SOD activity testing for the commercially prepared human enzyme, p16 protein, and PB6-CM are shown in Table II. PB6-CM and p16 purified from it clearly show SOD activity, and a p16 concentration of ~200 ng/ml (total reaction volume = 2 ml) is sufficient to inhibit the autoxidation of 200 μM hematoxylin by 95%. While it may be that enzymes from different sources will not show the same activity in the assay, a rough estimate of p16 SOD activity can be made by comparing its activity to that of human erythrocytic SOD, for which five standard SOD activity units (20) inhibit hematoxylin autoxidation by 77% (Table II). This compari-



**Figure 4.** Dose vs. inhibitory effect and apparent toxicity in the DA-1 cell proliferation assay for p16 protein purified from PB6-CM (see Materials and Methods for calculations). Undiluted initial p16 concentration was 4 μg/ml.

son gives an activity of ~70 ng p16 per standard SOD activity unit (20), which is greater than the activity of the human SOD preparation (300 ng protein per unit), but comparable to the activity seen for highly purified SOD (12 ng/U; 26). Matching the levels of inhibition seen for PB6-CM with equivalently active amounts of p16 gives an average estimate of p16 concentration of 1 ng/ml, which is in agreement with the estimates of p16 in PB6-CM obtained from silver-stained SDS-PAGE (data not shown).

#### Inhibitory Activity of Superoxide Dismutase

Human erythrocytic SOD (HSOD) was tested for inhibitory activity in the DA-1 cell proliferation assay, with the results shown in Table III. Like p16/SOD tested in the same assay (Fig. 4), HSOD showed strong inhibition and apparent toxicity at high concentration and an intermediate range of concentrations which produced significant inhibition vs. IL 3 with low apparent toxicity.

**Table I. Inhibitory Effects of Purified Fractions**

Fraction tested	Concentration μg/ml	Percent inhibition
DA-1 proliferation assay		
B6-MS Mono Q		
Flow-through	2.5	15*
Flow-through 15-25 kD	0.1	20*
Early eluting	1.0	29*
Early eluting 15-25 kD	0.1	18*
Early eluting 70-90 kD	1.0	9
PB6-CM Mono Q		
Early eluting I	1.0	17*
Early eluting I 15-25 kD	0.2	24*
BFU-E suicide assay		
p16 from PB6-CM	0.1	45*
	0.045	13*
	0.015	1

\* *P* < 0.05 (see Materials and Methods) percentages are averages from two test groups.

**Table II. Superoxide Dismutase Activities of p16, PB6-CM, and Controls\***

Sample tested		Rate of hematoxylin autoxidation <i>A<sub>560</sub></i> U/min × 10 <sup>-3</sup> (SE)	Percent inhibition
Control		44.8 (0.7)	0
Human SOD	5 U	10.3 (0.2)	77.0
p16	600 ng	1.8 (0.4)	96.1
	400 ng	2.1 (0.2)	95.3
	300 ng	11.9 (2.3)	73.4
	200 ng	20.4 (3.9)	54.5
	150 ng	40.0 (3.4)	10.7
PB6-CM‡	300×	3.0 (0.6)	93.4
	167×	16.4 (1.3)	63.4
	60×	29.9 (1.4)	33.3

\* Total reaction volume was 2 ml; see Materials and Methods for calculations.  
 ‡ Concentrations given are relative to freshly harvested PB6-CM; 60× concentrated PB6-CM is effective in both cell inhibition assays.

The original demonstration that PB6-CM was an antagonist to IL 3 involved an experiment similar to the DA-1 cell proliferation assay wherein the cultures were carried for several days and given intermittent "feedings" of IL 3 (4). The successive additions of IL 3 were able to overcome the initial effects of the inhibitor, allowing the treated cultures to "catch up" to the control, a further demonstration that the inhibitor was not merely killing cells. The results of such an experiment done with HSOD are shown in Fig. 5 and the results parallel those seen for PB6-CM under similar conditions (4).

The inhibitory effect of HSOD on BFU-E is shown in Fig. 6, and again it is clear that HSOD has an inhibitory effect in this assay (the protein concentration was equivalent to 300 ng/U of SOD activity). The results for p16/SOD from Table I are plotted for comparison, and the two SOD preparations show similar dose-response profiles in this assay.

We have also made direct comparisons of the amounts of SOD activity present in various fractions using a gel activity assay (5), which confirm that there is sufficient SOD present in PB6-CM (~0.08 U/ml for unconcentrated CM) and B6-MS (~1.3 U/ml) and the fractions derived from them to account for the inhibitory activities observed. This analysis did not detect the presence of any SOD species other than the cytosolic Cu,Zn form in any of the preparations used in this study.

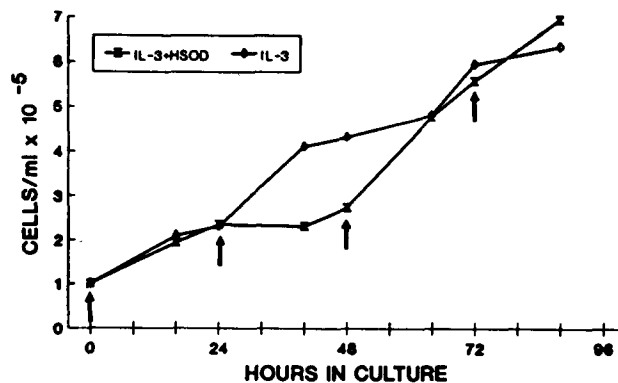
## Discussion

In this study we have shown that a protein, p16, is present

**Table III. Results of DA-1 Proliferation Assay for Inhibitory Activity of Human Erythrocytic SOD**

Concentration U/ml	Percent inhibition	Toxicity
200	32*	47
100	14*	5
50	18*	7
10	3	0

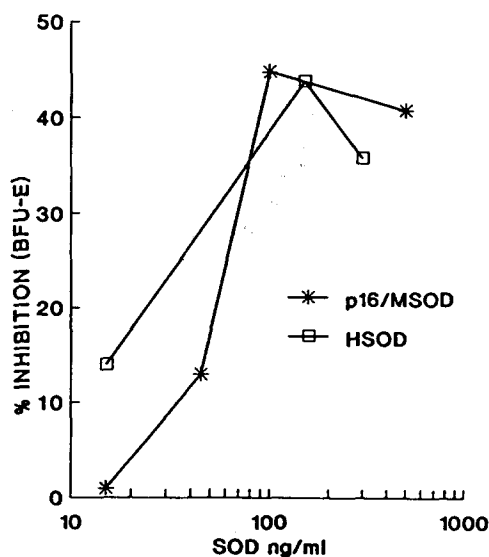
\*  $P < 0.05$  see Materials and Methods for calculations.



**Figure 5.** The inhibition of DA-1 cell proliferation by human CuZn SOD (HSOD) is reversible by the addition of IL 3. Arrows indicate times at which all cultures had IL 3 (0.5 U/ml) added, and the test group was also given HSOD (50 U/ml) at the first two feedings. Cell counts are averages from two groups.

in the marrow supernatant of C57BL/6 mice (B6-MS), in serum-free medium conditioned by the growth of the PB6 cell line (PB6-CM), and in fractions isolated from either via ion exchange chromatography or electroelution that are able to reversibly inhibit BFU-E cycling and oppose the stimulatory effects of IL 3 on DA-1 cell proliferation. We have identified the major protein present in p16 preparations as murine CuZn SOD on the basis of its amino acid sequence and enzymatic activity and we have also shown that a commercial preparation of human erythrocytic SOD displays inhibitory characteristics similar to p16/SOD, PB6-CM, and B6-MS. These facts make it reasonable to propose that SOD present in PB6-CM and B6-MS is responsible for their observed inhibitory effects.

It is unclear what the relationship is between p16/SOD and preparations (negative regulatory protein or NRP; 9) that have been partially purified from media supernatants of PB6



**Figure 6.** Inhibitory effects of different concentrations of human SOD and p16/SOD on BFU-E as measured in the BFU-E suicide assay (data for p16 from Table I). Inhibition by human SOD is significant for each point ( $P < 0.05$ ).

cells grown in the presence of 10% FCS and that show similar activity to p16/SOD in the BFU-E inhibition assay. SOD exists as a homodimer of  $M_r = 32$  kD and the inhibitory component of NRP is reported to have an  $M_r = 79$  kD on gel filtration, and while the partially purified NRP was not tested for SOD activity, SOD was certainly present in the cell culture supernatants used for NRP purification, both murine SOD from the PB6 cells and bovine SOD present as a normal constituent of FCS. We found no evidence for the presence of any inhibitor other than SOD in PB6-CM, B6-MS, or any of the fractions isolated from them that were examined in this study. Assays of protein isolated from the 70–90-kD region of inhibitory fractions gave no indication of inhibitory material in this size range (Table I), and direct enzyme assays showed that there is sufficient SOD activity present in PB6-CM, B6-MS, and the fractions isolated from them to account for their inhibitory effects on cells. We also found that only the cytosolic Cu,Zn form of SOD was present in our crude material, with no other isozymes (see below) detected.

Superoxide dismutase exists in mammals in three different forms: a mitochondrial manganese-containing form (Mn-SOD; 13), and two copper-zinc forms, one cytosolic (CuZn-SOD, 13) and one extracellular (EC-SOD, 17). These distinct enzymes share a common activity and presumably also the important role of shielding living tissues from the toxic effects of the superoxide radical by catalyzing the reaction  $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ . SOD activity has been reported to affect cell differentiation (6, 23), tumorigenesis (22), and tumor promotion (16), and reactive oxygen intermediates have been shown to affect hematopoietic progenitor cell renewal in culture (21). Within the erythroid lineage, the addition of CuZn SOD to the culture media of Friend erythroleukemia cells induces differentiation (6), however, to our knowledge SOD has not been implicated in the control of normal progenitor proliferation nor in the response of cells to growth factors, thus the demonstration here that SOD can inhibit the cycling of BFU-E and oppose the effects of IL 3 on responsive cells in vitro is novel.

The present line of investigation arose from the observation that the majority of marrow BFU-E in C57BL/6 mice are normally in a noncycling state (29), but a large proportion enter DNA synthesis when they are removed from the influence of B6-MS in vitro (2). The addition of B6-MS is sufficient to inhibit DNA synthesis in both B6-derived BFU-E and those derived from another strain (B6S) in which a high proportion of BFU-E are normally in cycle, while B6S-MS has no inhibitory effect on BFU-E (2). It has been proposed that the factor(s) giving rise to these in vitro effects are responsible for the differences in BFU-E cycling state seen in the two strains, and the possible role of SOD in this phenomenon is currently under investigation. The inhibitory effects of B6-MS are specific for BFU-E, with stem cells elsewhere within the erythroid and other myeloid lineages being unaffected (2), and we have evidence that like B6-MS, SOD does not affect the cycling of other progenitors (i.e., CFU-E, CFU-GM, CFU-S) at concentration levels where it is effective on BFU-E (Pluthero, F. G., M. Shreeve, D. Eskinazi, and A. Axelrad, manuscript submitted for publication).

PB6-CM is able to inhibit the proliferation of other cell lines that require IL 3 (like DA-1 cells), while those that do not are unaffected (4), and we are testing whether the same linking of inhibitory effect to IL 3 dependence will hold true

for SOD. While we have only looked at the effects of one SOD isozyme in this study, if it is the enzymatic activity of the protein that is responsible for the inhibitory activities we have observed, then other isozymes should show similar behavior in our assay systems, and experiments with other mammalian and bacterial SODs indicate this is so (Pluthero, F. G., and A. Axelrad, manuscript in preparation).

The molecular mechanisms by which p16/SOD exerts its effects in the systems so far examined are unknown; one possibility may be that superoxide is involved in the transduction of signals from stimulatory factors such as IL 3, and its removal by SOD can interfere with cell stimulation. However it acts, the demonstration that SOD is capable of inhibiting the cycling of BFU-E in vitro and possibly in vivo is an indication that the control of cell proliferation and differentiation may involve components of the physiological milieu that are not discrete signal factors in the classical sense, but yet can have similar effects through less well-understood means.

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