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Increased Antioxidant Enzyme Activity in Amyloid β Protein-Resistant Cells

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Clones of the rat pheochromocytoma cell line PC12 were selected for their resistance to amyloid β protein (Aβ). These Aβ-resistant cells also survive higher concentrations of exogenously applied peroxides than the parent cells. Aβ triggers intracellular H_2O_2 accumulation in the parent PC12 cells but not in the Aβ-resistant cells. The absence of H_2O_2 accumulation in Aβ-resistant cells is not attributable to differences in Aβ binding to the cell surface. However, the mRNA and protein levels of catalase and glutathione peroxidase, as well as the corresponding enzyme activities, are highly elevated in Aβ-resistant clones. These activities correlate well with the increased resistance of cells to Aβ or peroxides. Finally, cells transfected with catalase and glutathione peroxidase are also more resistant to Aβ toxicity. These results indicate that increased antioxidant enzyme activities in Aβ-resistant cells account for at least part of their resistance to Aβ and substantiate further the role of H_2O_2 in Aβ toxicity.

Key words: Alzheimer's disease; amyloid β toxicity; oxidative stress; catalase; glutathione peroxidase; PC12

Cells living in aerobic conditions are exposed constantly to reactive oxygen species and, as a consequence, use effective protection mechanisms against oxidative damage. This protection comprises nonenzymatic antioxidants such as vitamins E and C and the antioxidant enzymes superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px) (Halliwell and Gutteridge, 1989). Healthy cells maintain an equilibrium between free radical generation and these antioxidant defense processes (Halliwell and Gutteridge, 1989; Ames et al., 1993). When cells are unable to maintain this equilibrium, the level of free radicals increases and peroxidation of macromolecules ensues, causing oxidative damage that results in deleterious effects to the cells (Olanow, 1993).

Oxidative damage is particularly detrimental to nondividing cells like neurons. Oxidative damage to neuronal cells has been demonstrated *in vitro* via free radical-mediated glutamate toxicity (Murphy et al., 1989; Coyle and Puttfarcken, 1993) and *in vivo* via mutations found in the SOD gene that cause familial amyotrophic lateral sclerosis (ALS) (Rosen et al., 1993). Free radicals may play a role in other neuropathological situations such as ischemia–reperfusion, inflammation, and traumatic injury (Yamamoto et al., 1983; Hall and Braughler, 1986; Kitagawa et al., 1990; Jenner et al., 1992). Oxidative damage also has been implicated in degenerative disorders such as Parkinson's disease (Slivka and Cohen, 1985) and Alzheimer's disease (AD) (Behl et al., 1992; Harman, 1993; Behl et al., 1994b; Goodman and Mattson, 1994).

One distinct pathological feature of AD is the accumulation of abnormal protein aggregates in the central nervous system (Glenner, 1988). The aggregates, or plaques, contain amyloid β protein (Aβ), a 40- to 43-amino-acid peptide. Several pieces of evidence support the hypothesis that Aβ is a major cause of the neurodegeneration in AD. First, mutations in the Aβ precursor protein (AβPP) cause the overproduction of Aβ in some cases of familial AD (Chartier-Harlin et al., 1991; Goate et al., 1991; Murrell et al., 1991; Citron et al., 1992; Cai et al., 1993). Second, transgenic mice expressing a human mutant AβPP develop histological brain abnormalities similar to those observed in AD patients (Games et al., 1995). Finally, Aβ is toxic to nerve cells in culture (Yankner et al., 1990; Behl et al., 1992; Pike et al., 1993; Behl et al., 1994a; Goodman and Mattson, 1994; Shearman et al., 1994). A possible mechanism of Aβ toxicity was suggested recently by the observation that Aβ increases levels of H_2O_2 and lipid peroxidation in cultured neurons (Behl et al., 1994b). Furthermore, exogenous addition of antioxidants or catalase protects cells from Aβ toxicity (Behl et al., 1992, 1994b). These observations imply that free radicals play a critical role in Aβ cytotoxicity. Therefore, regulation of cellular mechanisms that remove reactive oxygen species may protect cells from Aβ toxicity.

Clones of the sympathetic precursor-like cell line PC12 (Greene and Tischler, 1976) have been selected for Aβ resistance. They are also less sensitive to H_2O_2 toxicity (Behl et al., 1994b), suggesting a role for H_2O_2 accumulation in Aβ cytotoxicity. To define the biochemical mechanisms that lead to Aβ resistance, the antioxidant defense systems in these cells were examined. We report in this study that the expression and activity of antioxidant enzymes are increased in the Aβ-resistant PC12 cell clones. Furthermore, cell lines genetically engineered to express higher levels of the antioxidant enzymes catalase and GSH-Px are also more resistant to Aβ. These data strongly support the hypothesis that Aβ-induced cell death is mediated by H_2O_2.

MATERIALS AND METHODS

Materials. Peptides $A\beta_{(1-40)}$ and $A\beta_{(25-35)}$ were obtained from Bachem (San Diego, CA) and dissolved in water at 1 mg/ml directly before use. Catalase, H_2O_2, 3-amino-1,2,4-triazole (3-AT), reduced GSH, GSH-Px, GSH reductase, NADPH, superoxide dismutase, xanthine, xanthine oxidase, cytochrome *c*, and all other chemicals were from Sigma (St. Louis,

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MO). The fluorescent dyes were from Molecular Probes (Eugene, OR). The catalase antibody was purchased from Calbiochem (San Diego, CA), the monoclonal Aβ antibody (anti-Aβ 8–17) was purchased from Dako (Glostrup, Denmark), and the Mn-SOD and Cu,Zn-SOD antibodies were generously provided by Dr. K. Kato (Aichi Medical University, Japan). The following cDNA were generously provided: human catalase cDNA by Dr. B Shepard (Wadsworth Center for Laboratories and Research, New York Department of Health, Albany, NY); mouse GSH-Px cDNA and human Mn-SOD cDNA by Dr. D. Hockenbery (Fred Hutchinson Cancer Research Center, Seattle, WA).

Cell culture. Rat pheochromocytoma (PC12) cells are a subclone of a high-passage cell line originally obtained from L. Greene (Greene and Tischler, 1976). PC12 cells were grown on tissue culture dishes (Falcon, Indianapolis, IN) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 5% horse serum. Dialyzed FCS and horse sera were from Gibco (Gaithersburg, MD), and DMEM was made according to the original procedure (Vogt and Dulbecco, 1963). Aβ-resistant cells were washed twice with the culture medium before plating to remove the previously added Aβ. Aβ was added to the resistant clones 24 hr after passaging.

Isolation of resistant clones. PC12 cells were grown in DMEM supplemented with dialyzed sera (10% FCS/5% horse serum) for 4 months in the presence of 20 μM $A\beta_{(25-35)}$. Several subclones were isolated in the presence of 20 μM $A\beta_{(25-35)}$ and were confirmed for their resistance to $A\beta_{(25-35)}$ and $A\beta_{(1-40)}$. The subclones were maintained in the presence of 5 μM $A\beta_{(25-35)}$. The data presented here are from three selected Aβ-resistant clones: AβrCl 1, AβrCl 7, and AβrCl 8.

Cytotoxicity assay. To assess cell viability (cytotoxicity), the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Hansen et al., 1989) was performed in 96-well microtiter dishes containing 100 μl of DMEM with 10% dialyzed FCS and 5% dialyzed horse sera per well. Cells (3×10^3 cells/well) were plated and test reagents were added the day after the plating. On the third day, 10 μl/well of a 5 mg/ml MTT stock in PBS was added, and the incubation was continued for 4 hr before the addition of 100 μl of a solution containing 50% dimethylformamide, 20% SDS, pH 4.8. On the fourth day, absorption values at 570 nm were determined with an automatic microtiter plate reader. In all cases, test substances were added to wells containing medium alone to determine whether they interfered with the assay. The data are expressed as mean percent viable cells compared with respective control cultures.

Transmission electron microscopy. For electron microscopy studies, cells were plated on 24-well tissue culture plates and grown until subconfluency. Cultures were fixed *in situ* in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 30 min at room temperature and post-fixed in 1% osmium tetroxide. To increase contrast, cells were double-fixed in saturated thiocarbohydrazide–osmium; then samples were dehydrated using a graded series (50–100%) of ethanol. Tissue culture dishes were embedded in Epon 812, cured *in vacuo* for 48 hr at 60°C, and sectioned.

Intracellular peroxide formation. The formation of intracellular peroxides was detected using 2′,7′-dichlorofluorescein diacetate (DCFDA), which is a nonfluorescent compound (Bass et al., 1983). After entering the cell, DCFDA is de-esterified to form a compound that can be oxidized by cellular peroxides, resulting in its conversion to the fluorescent dye 2′,7′-dichlorofluorescein (DCF) (Keston and Brandt, 1965; Bass et al., 1983; Cathcart et al., 1983). The cells were pretreated with Aβ for 23 hr, and then 10 μM DCFDA was added for 1 hr. The cells were then washed with phenol-red-free HEPES medium and observed using a fluorescence microscope with fluorescein optics. Different fields totaling >100 cells were photographed, and the fluorescent cells were counted.

Blotting. For Northern blotting, total RNA was isolated from the PC12 parent cell (PC12p) and the Aβ-resistant clones according to Chomczynski and Sacchi (1987). Northern blots were performed according to the standard procedure using 20 μg of total RNA (Kimura et al., 1990).

For Western blotting, cells from subconfluent cultures were harvested, washed twice with PBS, resuspended, and lysed by sonication. The protein concentrations were determined using a kit from Pierce (Rockford, IL). Aliquots (50 μg) were mixed with SDS sample buffer containing 5% β-mercaptoethanol and electrophoresed on a 10% polyacrylamide gel containing SDS (Anderson et al., 1973). Proteins were transferred to Immobilon (Millipore, Milford, MA) and reacted with a dilution of the respective antibody, and they were probed with ^{125}I-labeled protein A conjugate or with a secondary antibody coupled to horseradish peroxidase (Bio-Rad, Richmond, CA) and developed using ECL (Amersham, Bucks, UK). Densitometer readings of Northern and Western blot autoradiographs were performed using an LKB scanning densitometer.

Catalase assay. The catalase activity of cell lysates was determined according to Aebi (1974). Decrease in H_2O_2 level was monitored at 240 nm in a reaction mixture containing 50 mM phosphate buffer, pH 7.0, and 10 mM H_2O_2 with 50 μg of cell lysate. A specific inhibitor of catalase, 3-AT, was used to ensure the specificity of the assay (Darr and Fridovich, 1986). In the presence of 5 mM inhibitor, the catalase activity decreased to 5–10% of the original activity.

GSH-Px assay. The GSH-Px assay was performed according to Günzler and Flohe (1985) in a reaction mixture containing 50 mM phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM NaN_3, 1 mM GSH, 10 mM H_2O_2, 2.5 U/ml GSH reductase, 150 μM NADPH, and 50 μg of cell lysate. In this coupled assay, consumption of NADPH was monitored at 340 nm. The GSH-Px activity was inhibited by 1.8 mM *N*-ethyl-maleimide (NEM) (Oyama et al., 1994). To ensure that NEM did not affect GSH reductase, oxidized GSH was added to the reaction mixture after activity determination.

Superoxide dismutase assay. The superoxide dismutase activity was measured according to McCord and Fridovich (1969). Cell lysates were added to a reaction mixture containing 50 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 1 mM NaN_3, 1 mM xanthine, 0.4 U/ml xanthine oxidase, and 20 μM cytochrome *c*, and the decrease in cytochrome *c* oxidation at 550 nm was determined. Pure superoxide dismutase (Sigma) was used to generate a standard curve of the SOD activity, and SOD activities of the lysates were determined according to the standard curve. In addition to this assay, SOD activities were determined using an SOD activity gel assay. The staining for SOD enzymatic activity was performed as described previously (Beauchamp and Fridovich, 1971; Lindau-Shepard and Shaffer, 1993).

Cell transfection. Transfection of B12 cells was performed with Lipofectamine from Gibco following the suggested protocol. The transfected cells were selected by their resistance to 0.8 mg/ml G418 in DMEM with 5% bovine serum and 500 nM sodium selenite as supplement (Buckman et al., 1993). Five to ten different subclones of each transfectant (double-transfectant and the cells transfected with the expression vector alone) were isolated.

RESULTS

Aβ-resistant clones are less sensitive to H_2O_2 and *t*-butyl H_2O_2

PC12 cells were grown for 4 months in the presence of 20 μM $A\beta_{(25-35)}$, a concentration in excess of that required to kill the majority of the PC12 cells (Behl et al., 1992). Clones of the surviving cells are all resistant to high levels of $A\beta_{(25-35)}$ and $A\beta_{(1-40)}$. They also became very resistant to H_2O_2 toxicity compared with PC12p clone (Behl et al., 1994b). Because the addition of Aβ causes H_2O_2 accumulation and membrane lipid peroxidation, three Aβ-resistant clones were also tested for their sensitivity to a more potent lipid peroxidizing agent, *t*-butyl hydroperoxide (Bellomo et al., 1982; Masaki et al., 1989). Aβ toxicity was determined by the MTT assay, which

Table 1. Cytotoxic response of parental PC12 and Aβ-resistant cells to peroxides

	Percent cell viability			
	PC12p	AβrCl 1	AβrCl 7	AβrCl 8
[H_2O_2] (μM)				
125	11.8 ± 5.6	97.0 ± 1.3	95.4 ± 7.1	95.4 ± 0.5
250	0	96.2 ± 2.2	90.5 ± 3.5	91.3 ± 1.5
[*t*-butyl H_2O_2] (μM)				
125	17.5 ± 5.0	100 ± 1.7	95.6 ± 6.0	69.4 ± 8.2
250	0	98.0 ± 8.7	86.7 ± 4.6	71.5 ± 1.2

Exponentially dividing cells were dissociated and plated into 96-well microtiter plates on 10% FCS/5% horse serum. Cells were exposed to the indicated concentrations of H_2O_2 or *t*-butyl hydroperoxide (*t*-butyl H_2O_2) for 24 hr. Cell viability, shown in the table as the percentage of the control, was assayed using the MTT assay. The data are means ± SEM for triplicate cultures.

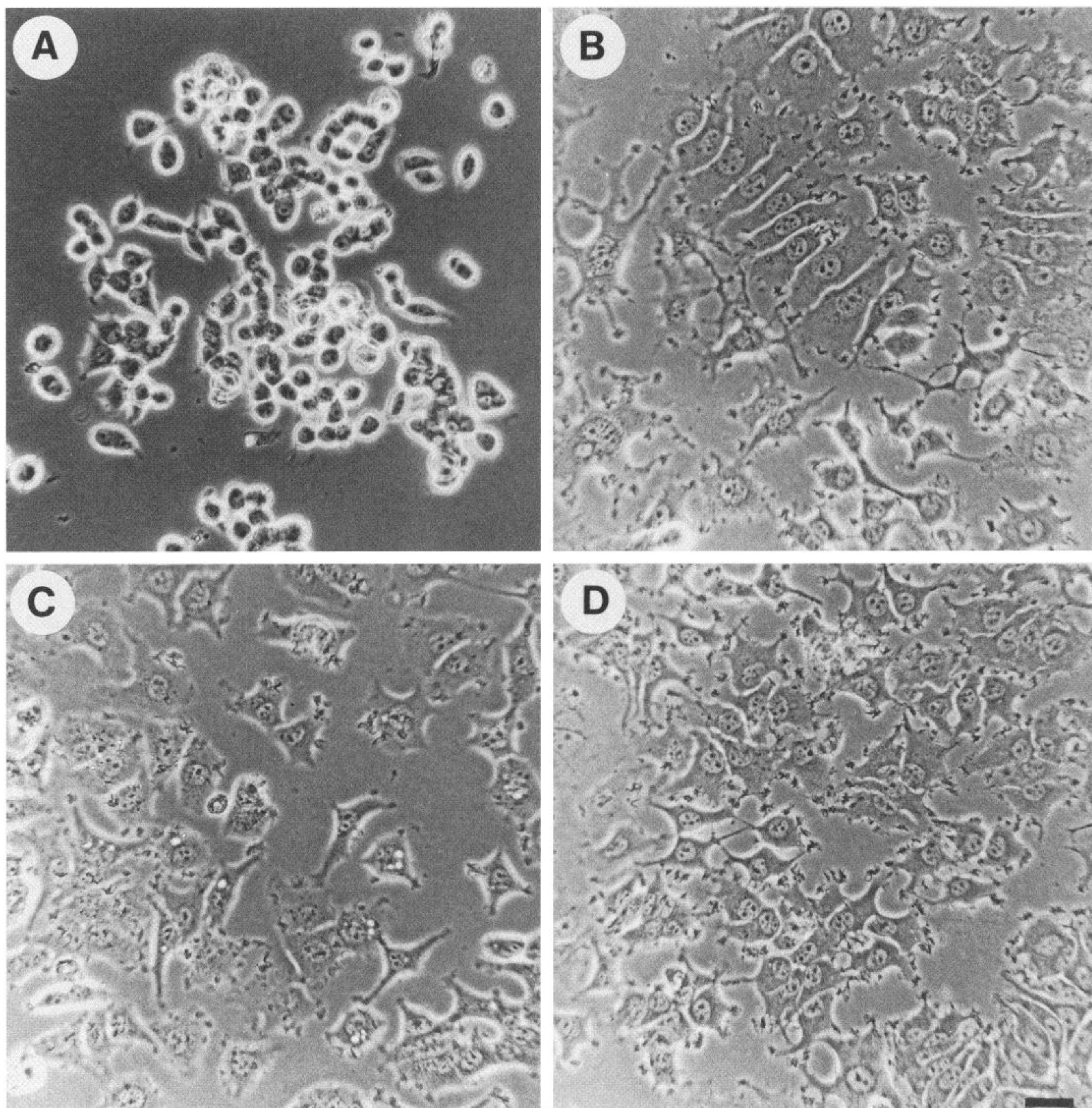

Figure 1. Light microscopy of PC12p and Aβ-resistant cells. Exponentially dividing cells were plated on tissue culture dishes and observed using phase contrast microscopy. *A*, PC12p cells; *B*, AβrCl 1; *C*, AβrCl 7; *D*, AβrCl 8. The resistant cells were isolated initially in the presence of 20 μM $A\beta_{(25-35)}$ and maintained in 5 μM $A\beta_{(25-35)}$. Scale bar, 50 μm.

detects redox changes in cells (Slater et al., 1963). MTT is the earliest identified marker for the cytotoxic effect of Aβ in PC12 cells (Behl et al., 1994b; Shearman et al., 1994). In agreement with the H_2O_2 data, the Aβ-resistant cells are markedly resistant to the toxicity of *t*-butyl hydroperoxide compared with the parent cell line (Table 1).

Morphology of Aβ-resistant clones

The morphology of the Aβ-resistant cells was examined by phase contrast and transmission electron microscopy. The basic cellular morphology of the Aβ-resistant cells is distinct from the parent cells (Fig. 1). The Aβ-resistant cells are more flattened than the parent cells and have small protrusions along the cell membrane. At the ultrastructural level, a great number of vesicles fill the cytoplasm of the resistant cells (Fig. 2, *arrows*). A similar increase in cytosolic vesicles has been observed in endoplasmic reticulum highly activated by the expression of vesicular stomatitis virus glycoprotein in cells carrying a mutation in a GTPase necessary for vesicular transport (Pind et al., 1994). The origin of the

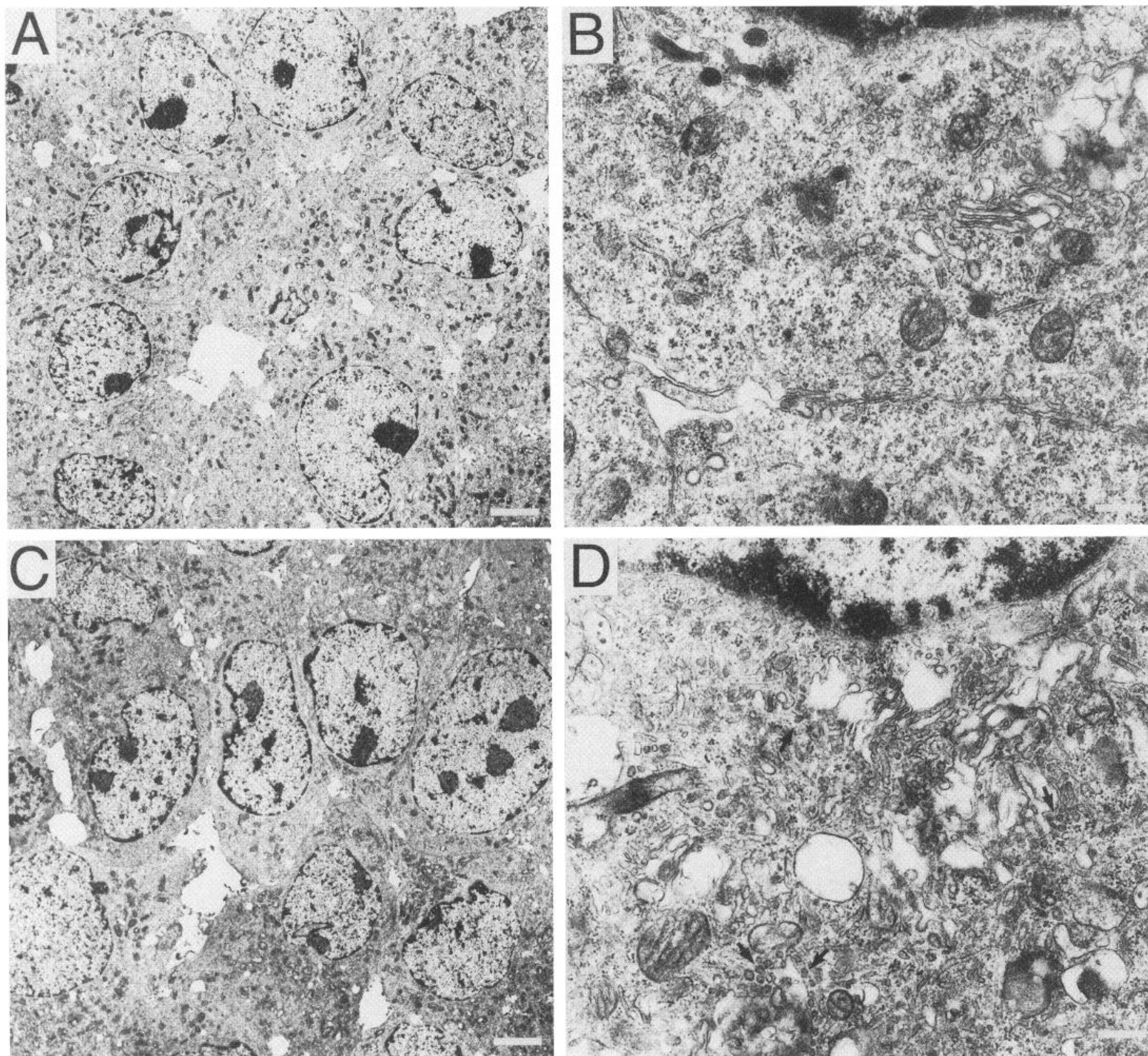

Figure 2. Electron microscopy of PC12p and AβrCl 7 cells. For electron microscopy, cells were plated on 24-well plate tissue culture dishes and grown until subconfluency. The cells were then fixed as described in Materials and Methods and were observed with transmission electron microscopy. *A*, *B*, PC12p cells; *C*, *D*, AβrCl 7 cells. Scale bar in *A* and *B*, 1 μm; scale bar in *C* and *D*, 0.05 μm. *Arrows* in *D* indicate numerous vesicles present in the AβrCl 7 cells.

vesicles is currently under investigation. Vacuoles also are observed more frequently in the resistant cells than in the parent cells. High vacuolization of the cytoplasm in nerve growth factor (NGF)-treated PC12 cells on Aβ addition has been reported (Behl et al., 1994a). Other membranous structures, such as the mitochondria and nuclei, are unchanged (compare Fig. 2*A*,*C*). It follows from these data that Aβ resistance affects the gross morphology of the cells.

Aβ-resistant and parent cells bind similar amounts of Aβ

To determine whether resistance was a consequence of defective Aβ association with the cellular membrane, direct cell-binding studies were performed. Cells were exposed to $A\beta_{(1-40)}$ for 24 h, washed by centrifugation, and lysed, and Aβ was assayed by Western blotting. All cells exposed to $A\beta_{(1-40)}$ bound similar amounts of the peptide (Fig. 3); the association of Aβ with the cells, however, did prevent its dimerization. There were no reproducible differences in peptide amount or size distribution between the cell lines. This conclusion was confirmed using immunofluorescence confocal microscopy (data not shown).

The above data show that the interactions of Aβ with the parent and the Aβ-resistant cells are indistinguishable and that differences in Aβ sensitivity may result from the cascade of

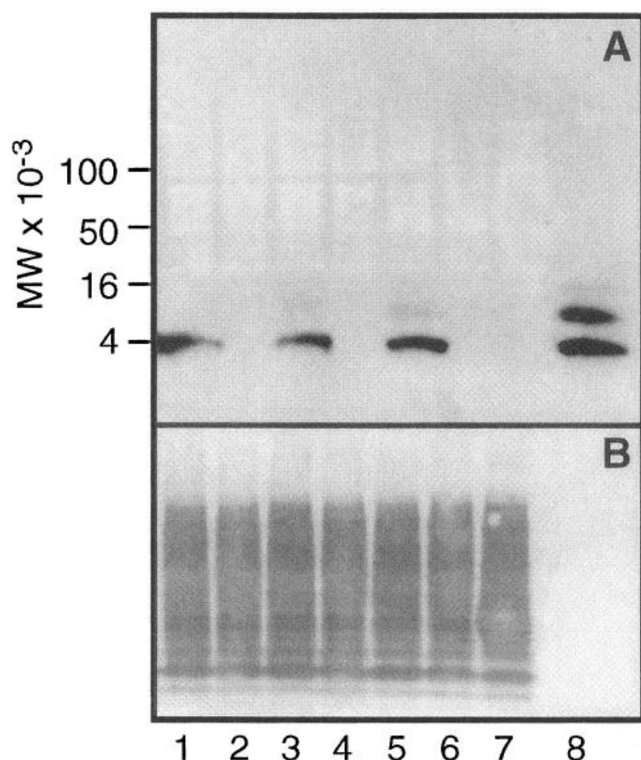

Figure 3. Binding of Aβ to variant cell lines. Exponentially dividing cells (1 × 10^6) were incubated in complete medium with or without 20 μg/ml $A\beta_{(1-40)}$. After 24 hr, the cells were washed three times with serum-free medium, dissolved in sample buffer, and run on 10–20% acrylamide tricine gradient gels. The protein was transferred to Immobilon-P and blotted with an anti-Aβ antibody and developed by the ECL method. The membranes were also stained with Amido Black. *A*, Anti-Aβ. *Lane 1*, AβrCl 8 + Aβ; *lane 2*, AβrCl 8 without Aβ; *lane 3*, PC12p + Aβ; *lane 4*, PC12p without Aβ; *lane 5*, AβrCl 7 + Aβ; *lane 6*, AβrCl 7 without Aβ; *lane 7*, PC12p without Aβ; *lane 8*, 500 ng Aβ alone (no cells). The experiment was repeated three times with no reproducible differences between the clones. *B*, Amido Black staining of the same membrane from *A*.

events after the Aβ–cell interaction. Therefore, the accumulation of H_2O_2 in the parent and resistant cells on exposure to Aβ was examined.

Aβ does not induce H_2O_2 accumulation in Aβ-resistant cells

Aβ causes the accumulation of H_2O_2 in primary and clonal neurons, as detected by the fluorescent dye DCF (Behl et al., 1994b). To determine whether the intracellular H_2O_2 levels of the parent and Aβ-resistant cells were different, the cells were incubated with 20 μM $A\beta_{(25-35)}$ for 24 hr. Cells were then loaded with DCF and examined by fluorescence microscopy. Photographs were taken of each cell clone, and >100 cells were evaluated for the integrity of cell morphology and the DCF fluorescence. Figure 4 shows that the intensity of the DCF fluorescence and the number of fluorescent cells on Aβ treatment were both greater in PC12p than in AβrCl 1. Similar results were obtained with other resistant clones (Table 2). Two possibilities account for the difference in H_2O_2 levels between the parent and resistant cells: (1) H_2O_2 production is decreased in the resistant cells; (2) H_2O_2 degradation is increased in the resistant cells. Because it is virtually impossible to measure *de novo* H_2O_2 production in whole cells, we examined the H_2O_2 degradation enzymes in the parent and resistant cells.

Expression and activities of catalase, GSH-Px, and SOD

Because Aβ initiates H_2O_2 accumulation, catalase and GSH-Px, enzymes that break down peroxides, and SOD, an enzyme that converts superoxide radicals to H_2O_2, were examined. As shown in Figure 5 and Table 3, the expression level of the catalase mRNA is higher in the resistant clones than in the parent cells. Also, up to a fourfold increase in the level of catalase protein could be detected by an anti-catalase antibody in all three resistant clones (data not shown). Similarly, the level of GSH-Px mRNA was increased in the resistant clones compared with the parent cells (Fig. 5, Tablc 3). In contrast, the mRNA expression of superoxide dismutase (Mn-subtype), as well as the Mn- and Cu,Zn-SOD protein expression, was the same in the parent and Aβ-resistant cells (data not shown). Because mRNA and protein levels may not reflect enzyme activity level accurately, the activities of these antioxidant enzymes in the parent and resistant cells were assayed.

Catalase, GSH-Px, and SOD activities of the cell lysates obtained from the parent and the resistant clones were determined by well established spectrophotometric assay systems (McCord and Fridovich, 1969; Aebi, 1974; Günzler and Flohe, 1985). As summarized in Table 4, there is up to 4.8-fold higher catalase activity in the resistant clones than in the parent cells. The GSH-Px activity was even more elevated, up to sevenfold in the resistant clones (Table 4). Higher enzyme activity levels of catalase and GSH-Px (Table 3) than the corresponding mRNA (Table 2) in the resistant cells may reflect enhanced mRNA stability or enhanced translation. In contrast to the catalase and GSH-Px activities, no significant difference in the SOD activity between the parent and the resistant cells was observed. This result was also confirmed in another SOD activity assay using polyacrylamide gels (Beauchamp and Fridovich, 1971) (data not shown).

Transfection of cells with catalase and GSH-Px cDNA

To determine whether increased enzyme levels of catalase and GSH-Px are sufficient to protect cells from Aβ toxicity, B12 cells were doubly-transfected with cDNA for catalase (Korneluk et al., 1984) and GSH-Px (Hockenbery et al., 1990). B12 is a rat central nervous system cell line, and it responds to Aβ similarly to PC12 (Behl et al., 1994b), but it is transfected and cloned more easily than the PC12 cell line (our unpublished observations). The transfected B12 cells were subcloned, and the expression of the two enzymes was confirmed by Northern blots (data not shown) and enzyme assays (Table 4). The double-transfectant (B12CPxN21; Table 4) was more resistant to both H_2O_2 (Fig. 6*A*) and Aβ (Fig. 6*B*) compared with the parent cells and the clones of cells transfected with the selection plasmid alone (B12Neo1). Similar results were obtained with other subclones of the doubly-transfected cell lines (data not shown). The double-transfectants were not as completely resistant to Aβ as the selected Aβ-resistant clones, suggesting that additional factors may be involved in maximizing Aβ resistance. However, because the relative increases in catalase and GSH-Px are lower in the transfected cells than in the Aβ-selected cells, it cannot be ruled out formally that a further increase in antioxidant enzyme activity would be sufficient for complete Aβ resistance.

DISCUSSION

When variant cell lines that are resistant to a toxic agent are compared with their parent cell line, it is frequently possible to infer from their biochemical differences something about the

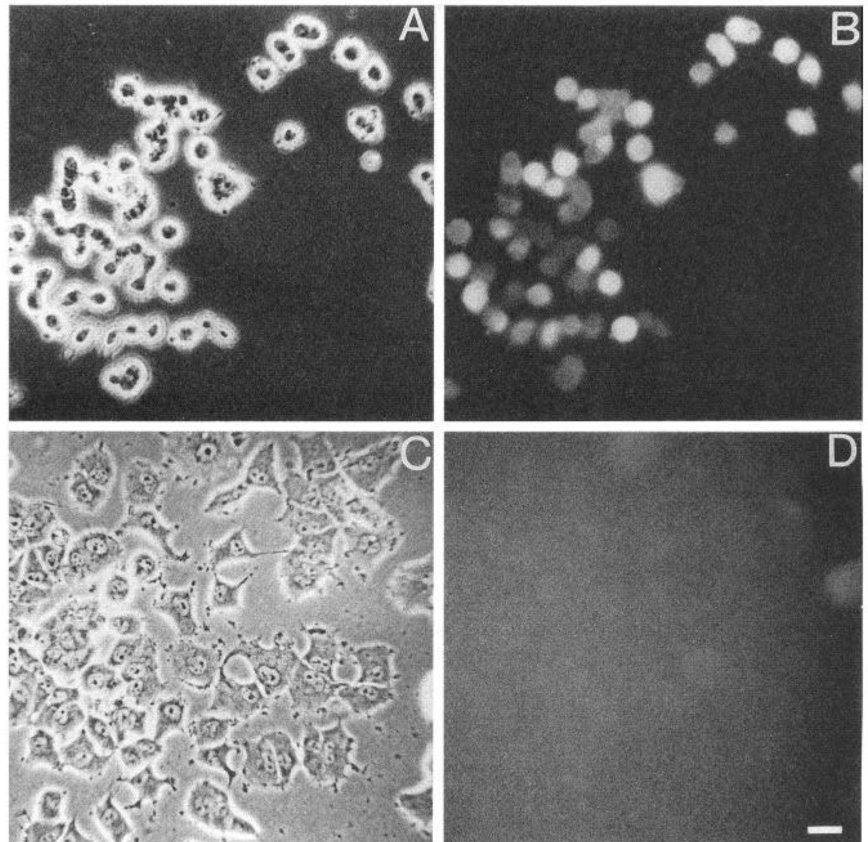

Figure 4. Aβ does not induce peroxide accumulation in Aβ-resistant cells. PC12p cells (*A, B*) and AβrCl 7 cells (*C, D*) were plated on tissue culture dishes, exposed to 20 μM $A\beta_{(1-40)}$ for 23 hr, and loaded with 10 μM DCFDA for 1 hr. The cells were washed and viewed with phase contrast microscopy (*A, C*) and with a fluorescence microscope using fluorescein optics (*B, D*). Scale bar, 50 μm. For quantitative analysis, see Table 2.

mechanism of toxicity. For example, cell lines that are resistant to methotrexate have elevated levels of dihydrofolate reductase, suggesting that methotrexate inhibits this enzyme *in vivo*, resulting in a selection of cells expressing more of the enzyme (Stark and Wahl, 1984). Clones of PC12 cells selected for their resistance to Aβ toxicity also became resistant to the toxic action of H_2O_2 (Behl et al., 1994b). This observation suggests that H_2O_2 and Aβ share a common step in inducing cell death. The above data extend these initial observations to show that Aβ resistance is also associated with resistance to the membrane-peroxidizing agent *t*-butyl hydroperoxide, dramatic changes in cell morphology, and large increases in the expression and activity of GSH-Px and catalase. In contrast, the other major enzyme in free radical metabolism, SOD, is not changed, and neither is the ability of Aβ to bind to

Table 2. Intracellular peroxides after Aβ treatment

	Percent fluorescent cells			
	PC12p	AβrCl 1	AβrCl 7	AβrCl 8
Exp. 1	56.5	0	11.3	2.0
Exp. 2	43.8	5.2	6.4	0
Exp. 3	55.6	3.3	2.1	2.3

Cells were treated as described in the legend to Figure 4. The results of three independent experiments for each cell clone are presented as the percentage of fluorescing cells in a given microscopic field. In some cultures of the resistant cells, the fluorescence level was very low and longer photographic exposure was required to detect the fluorescence in these cells. More than 100 cells were scored for each point.

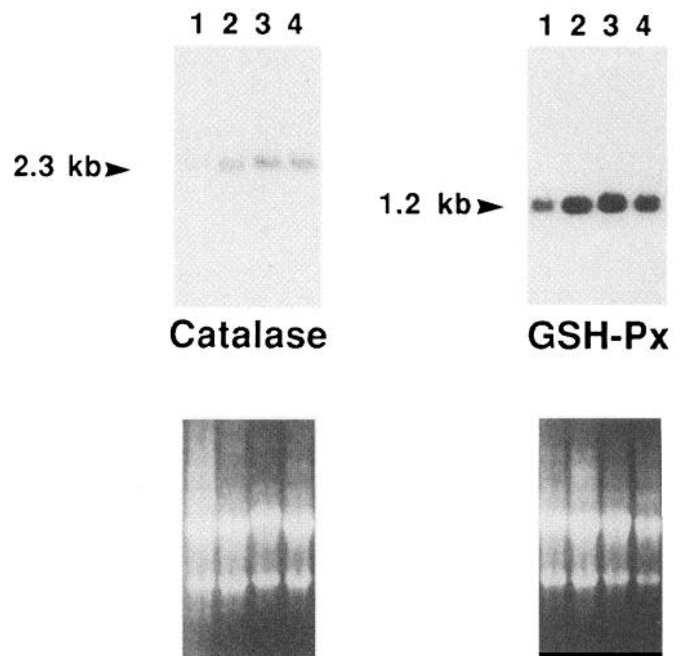

Figure 5. Northern blot analysis. Total RNA (20 μg) from PC12p (*1*), AβrCl 1 (*2*), AβrCl 7 (*3*), and AβrCl 8 (*4*) were hybridized to a cDNA probe of human catalase or rat GSH-Px. The ethidium bromide-stained agarose gels used for the Northern blots are depicted below the autoradiographs. The results of scanning the autoradiographs are shown in Table 3.

Table 3. Increased expression of catalase and GSH-Px mRNA

	Catalase	GSH-Px
AβrCl 1	2.3 ± .09	2.1 ± .09
AβrCl 7	3.7 ± .33	2.3 ± .11
AβrCl 8	3.0 ± .04	1.5 ± .10

Northern blots were performed as described in Materials and Methods. Densitometer readings of autoradiographs were done using an LKB scanning densitometer. The data are presented as fold increase compared with PC12 parental cells, and they are the mean ± SEM from three experiments.

cells. Because GSH-Px and catalase are the major H_2O_2-degrading enzymes in the cell, their induction is in agreement with the observation that H_2O_2 accumulation in the Aβ-resistant clones is greatly reduced compared with the parent cell line (Fig. 3, Table 2). Together, these data constitute a strong argument that H_2O_2 accumulation is involved in Aβ toxicity.

The importance of the antioxidant enzymes catalase and GSH-Px in the resistance of PC12 cells to Aβ is also shown by the double-transfectant studies. Cells transfected with cDNA for catalase and GSH-Px become more resistant to Aβ toxicity, even though the increases in enzyme activity levels in the transfectant are relatively low (twofold for catalase and threefold for GSH-Px). These changes may be sufficient for the removal of H_2O_2 because GSH-Px has a rate constant severalfold higher than catalase and is the primary factor in H_2O_2 degradation in some cells (Flohe et al., 1972; Jones et al., 1981; Chaudiere et al., 1984; Cohen and Duke, 1984) The GSH-Px enzyme level may also explain the similar sensitivities of the parent PC12 and B12 cells to Aβ toxicity even though they have different catalase levels (Table 4).

Increases in catalase and/or GSH-Px activity have been associated previously with oxidative stress. For example, treatment of PC12 cells with NGF leads to an elevation of both catalase and GSH-Px, resulting in protection from the toxicity of H_2O_2 and 6-hydroxydopamine, two generators of hydroxy radicals (Tiffany-Castiglioni and Perez-Polo, 1981; Jackson et al., 1994). The autocrine production of extracellular catalase prevents apoptotic cell death in a human T cell line in serum-free medium (Sandstrom

Table 4. Comparison of catalase, GSH-Px, and SOD enzyme activities

	Catalase		GSH-Px		SOD	
	Units[a]	Increase[b]	Units[c]	Increase[b]	Units[d]	Increase[b]
PC12p	7.56 ± 0.7	—	16.7 ± 8.3	—	23.5 ± 5.1	—
AβrCl 1	30.3 ± 5.3	4.0	118 ± 6.4	7.1	24.4 ± 5.4	—
AβrCl 7	36.6 ± 2.3	4.8	82.3 ± 5.9	4.9	22.8 ± 3.9	—
AβrCl 8	18.3 ± 5.3	2.4	104 ± 10	6.2	21.6 ± 2.9	—
B12Neo1	30.6 ± 1.6	—	18.2 ± 0.6	—	N.D.	
B12CPxN21	55.5 ± 3.2	1.8	54.4 ± 2.6	3.0	N.D.	

Enzyme assays were performed as described in Materials and Methods. The number of independent assays was n = 3–5 for the catalase and GSH-Px assays and n = 9 for the SOD assay. Mean ± SEM of n samples are presented.

[a]μmol H_2O_2 consumed/min/mg cell lysate protein.

[b]Fold increase over the control cells. For the resistant clones (AβrCl 1, -7, and -8), the control cell was the PC12p (parental): for the double transfectant (B12CPxN21), the control was B12Neo1 (transfected with the expression vector alone).

[c]Activity units calculated according to Günzler and Flöhe (Günzler and Flöhe, 1985) with the unit of $\times 10^{-3}$ $min^{-1} \cdot mg^{-1}$.

[d]One unit of SOD is defined as the amount of the SOD enzyme that inhibits the rate of cytochrome c reduction (by O_2^- produced by the xanthine/xanthine oxidase system) by 50%/mg protein (McCord and Fridovich, 1969).

N.D., not determined.

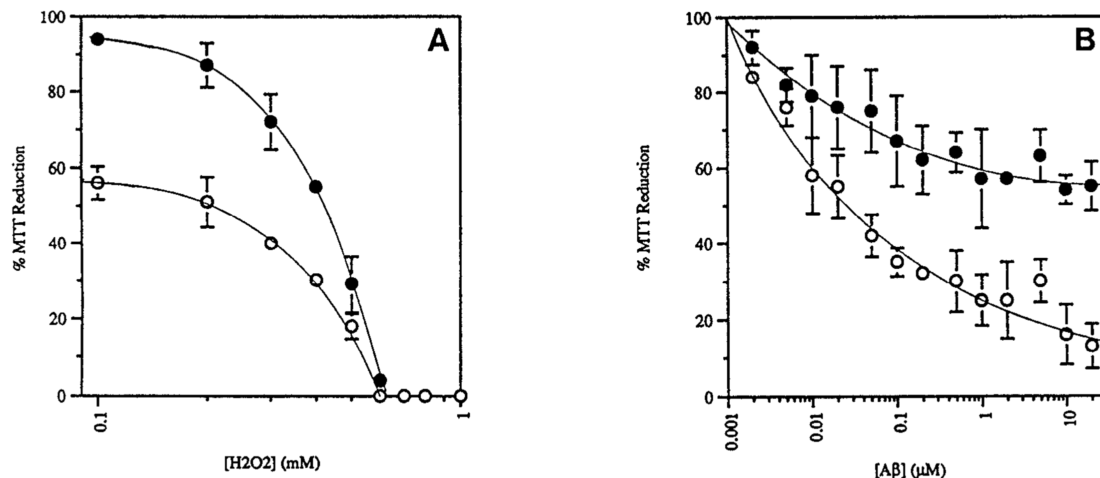

Figure 6. Cytotoxic response of B12 cells transfected with catalase and GSH-Px. Exponentially dividing cells were dissociated and plated into 96-well microtiter plates on 5% FCS. Cells were exposed to the indicated concentrations of either H_2O_2 (*A*) or Aβ (*B*) for 24 hr. Cell viability was assayed using the MTT assay. (○) B12 transfected with the expression vector alone (B12Neo1); (●) B12 transfected with the vectors containing catalase and GSH-Px cDNA (B12CPxN21).

and Buttke, 1993), further supporting the potential protective effect of catalase. Catalase protein could not be detected, however, in the supernatants of the Aβ-resistant cells (our unpublished observations).

The acquisition of Aβ resistance, however, is a more complex phenomenon than simply the induction of the antioxidant enzymes. Morphological observations of several Aβ-resistant cells show shared increases in the number of cytoplasmic vesicles (perhaps pre-Golgi vesicles) and vacuoles, both of which indicate that other crucial changes are associated with Aβ resistance. In contrast, there are no detectable morphological changes in B12 cells transfected with catalase and GSH-Px cDNA (our unpublished observations). In another study, higher catalase or GSH-Px activity was not observed in PC12 cells selected for resistance to increasing concentrations of H_2O_2 (Jackson et al., 1994). We have isolated recently five subclones of Aβ-resistant B12 cells, all of which have morphologies similar to the resistant PC12 clones. Whereas four of them have higher antioxidant enzyme levels than the parent line, one subclone has neither catalase mRNA nor enzyme activity, although it did have increased GSH-Px activity. We are investigating currently the mechanism of Aβ resistance in this subclone.

In addition to the work outlined above and its potential relevance to AD, the importance of antioxidant defense mechanisms in the nervous system is illustrated by a recent finding that some forms of familial ALS are associated with a defect in the superoxide-detoxifying enzyme Cu,Zn-SOD (Rosen et al., 1993). It follows that in addition to increasing the basal levels of nonenzymatic antioxidants such as vitamins E and C, elevation of antioxidant enzymes such as catalase and GSH-Px might be useful in preventing Aβ-induced oxidative damage to neurons and other cells.

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