RESPONSES OF CULTURED ASTROCYTES, C6 GLIOMA AND 1321NI ASTROCYTOMA CELLS TO AMYLOID β -PEPTIDE FRAGMENTS

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□ *The effect of amyloid β -peptide (β AP), which can have both neurotrophic or neurotoxic effects on neurons and has been implicated in the pathogenesis of Alzheimer's disease (AD), was studied on astrocytes using primary cultures and astrocyte cell lines (rat C6 glioma, human 1321NI astrocytoma cells). The cultures were exposed to 0.0005–50 μ g/ml β AP fragments 1–40, 25–35, 31–35, or 40–41 (control) for 24 hr. Some of the fragments were maintained at 37°C for 48 hr to induce aggregation and some of the cell cultures were pretreated with the differentiating agent dBcAMP before the experiments. The astrocyte responses were evaluated for lysosome activity (neutral red assay) and levels of structural proteins, glial fibrillary acidic protein, vimentin, and S-100, which are altered in the dystrophic plaques with associated astrogliosis in AD. The cells frequently responded with biphasic responses, with initial (low-dose) activation-type responses (i.e., increases of indicator compared to controls), before reductions with altered morphology (increased branching of cells) at higher concentrations. However, cell death (with EC_{50} values) was not observed, even at the maximum concentrations of β AP fragments. The findings suggest that the astrocytes have a relatively high resistance against the β AP toxicity.*

Keywords. astrocytes, C6 glioma, 1321 astrocytoma, β -amyloid peptide, activation, toxicity

INTRODUCTION

The primary morphological signs of Alzheimer's disease (AD) are the loss of neurons associated with the presence of neuritic plaques, neurofibrillary tangles, and cerebrovascular amyloidosis. The plaques are composed of a central amyloid core surrounded by aggregates of dystrophic neurites, synaptic boutons with degenerating mitochondria, lysosomes, and activated astrocytes (Terry *et al.*, 1964). The major component of the amyloid is a 4.2 KDa peptide termed β -peptide or A β peptide (Glenner and Wong, 1984; Masters *et al.*, 1985). The amyloid fibrils form a twisted, β -pleated sheet (Glenner and

We thank Donna McHenry for assistance. This work was supported, in part, by the Health and Safety Executive, United Kingdom.

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Wong, 1984). The amyloid β -peptide (β AP) is a derived component from at least three separate amyloid precursor fractions, termed β APP₆₉₅, β APP₇₇₀, which differ by limited numbers of amino acid sequences. The β -amyloid precursors are constitutively processed by at least two enzymatic pathways (Esch *et al.*, 1990; Golde *et al.*, 1992), one of which appears to be within the endosomal-lysosomal pathway, producing intact β AP (Haass *et al.*, 1992).

Approximately half of the β AP is normally buried in the neuronal cell membranes with the other section (N-terminus) projecting extracellularly. The β AP that accumulates as insoluble aggregates (plaques) in the AD brain is a 40–42 amino acid segment of the precursor proteins (Glenner and Wong, 1984). Although the precursor and its genetic control (located on chromosome 21 in man) are highly conserved in many different species (Goldgaber *et al.*, 1987), the reasons for the apparently aberrant accumulation in the AD patient are not yet clear, with both abnormal post-translational modifications and processing of the cell-surface moiety implicated (Robakis *et al.*, 1987). The β AP may be liberated from cells by enzymatic cleavage at the N-terminus (Seubert *et al.*, 1992) and/or other processing mechanisms (Golde *et al.*, 1992). The protein is also present in the cerebrospinal fluid of normal individuals and in the media of neuronal cultures, which might suggest a normal physiological function, possibly associated with the tachykinin receptor (Yankner *et al.*, 1990).

A major area of study in AD pathogenesis, in addition to the processing of β AP, is the involvement of the substance in neurotoxic changes. These have been investigated in a range of studies of cultures of primary neurons and clonal lines. The studies have shown that β AP can be directly neurotoxic (Yankner *et al.*, 1990; Pike *et al.*, 1991; Mattson *et al.*, 1993; Borchelt *et al.*, 1997; Weidemann *et al.*, 1997) and can also render neurons vulnerable to excitotoxic or metabolic insults (Koh *et al.*, 1990; Mattson *et al.*, 1993). Altered calcium homeostasis (Mattson, 1994; Mattson *et al.*, 1992, 1993; Weiss *et al.*, 1994) and generation of reactive free radicals (Behl *et al.*, 1992; Hensley *et al.*, 1994; Muller *et al.*, 1994) are implicated in the mechanism(s) of β AP toxicity. The neurotoxicity may be ameliorated by antioxidants (Behl *et al.*, 1992; Goodman *et al.*, 1994; Guo *et al.*, 1998). However, the toxic process is complicated by the wide variations that occur with different states of aggregation, solubility, and dose of the β AP molecule. Freshly solubilized synthetic β AP (1–40) or newly solubilized β AP 1–42 are nontoxic and may in fact promote neurite outgrowth in hippocampal cultures (Pike *et al.*, 1991). However, with increasing periods of incubation at 37°C (termed aging) the peptide adopts aggregated forms which become progressively toxic, causing rapid cell death after 24 hrs incubation (Pike *et al.*, 1991; Burdick *et al.*, 1992). Furthermore, certain peptide sequences within the β AP 1–42 (e.g., β 25–35) exhibit a rapid aggregation and potent neurotoxicity immediately following solubilization (Yankner *et al.*, 1990). In addition the toxicity of β AP is dose dependent, with low doses

being protective and the high doses toxic (Kaltschmidt *et al.*, 1999). Low-dose pretreatment may also protect against subsequent high dose (Kaltschmidt *et al.*, 1999). A key mechanistic factor shown in these studies was the activation of the transcription factor NF- κ B by free radicals. The activation profile of NF- κ B was inverted U-shaped, with low-dose stimulation followed by inhibition at higher dose, similar to the toxicity curve of β AP (Kaltschmidt *et al.*, 1999). The biphasic form of the β AP responses conforms to the stereotyped hormetic response, as discussed by Calabrese (2001).

In addition to the neuronal toxicity by β AP in AD, several studies have been made on astrocytes. Reactive astrocytes have a major association with the plaques (Duffy *et al.*, 1980; Schechter *et al.*, 1981). The reactive cells, in contrast to normal (nonreactive) astrocytes, may contain significant levels of β AP and its mRNA (Bahmanyar *et al.*, 1987; Card *et al.*, 1988; Siman *et al.*, 1989; Tanzi, 1989). Cultured rat astrocytes undergo reactive morphological changes, with increased stellation, increased GFAP and S-100 immunostaining (Meske *et al.*, 1998), enhanced cytokine and NO production (Hu *et al.*, 1998; Hu and VanEldik, 1999), K^+ and Cl^- channel activities (Jalonen *et al.*, 1997), and glutamate transporter capacity (Abe and Misawa, 2003) when exposed to β AP fragments. Cultured rat astrocytes can also respond to cytotoxic changes and cell death (Brera *et al.*, 2000). However, although both activation and toxicity changes have been reported it is not clear whether they are parts of a graded, biphasic continuum, similar to the responses that occur to a wide range of other toxic compounds (see Pentreath and Slamon, 2000).

The present study was undertaken to further assess the reactive and toxic responses of primary astrocyte cultures and related cell lines to β AP. The aims were to evaluate and compare the responses of β AP fragments 1–40, 25–35, and 31–35 in primary, C6 glioma, and 1321NI human astrocytoma cells using quantitative assays for lysosomes and the cytoskeletal proteins GFAP, vimentin, and S-100, which are sensitive markers of the reactive and toxic responses to a range of toxic substances *in vitro* (see Pentreath, 1999).

MATERIALS AND METHODS

Cell Culture

Primary astrocytes were prepared from 1–3-day-old Sprague-Dawley rats. Cortical tissues were dissected into trypsin-EDTA solution (0.5 g/l trypsin in BME-HEPES with 0.2 g/l tetrasodium EDTA; Gibco, UK). The cells were triturated with sterile pipettes, centrifuged (100g for 10 min), the supernatant removed, and 2.5 ml trypsin inhibitor solution (Sigma; 0.1% w/v in BME-HEPES) plus DNAase (100 μ l of a 1 mg/ml solution; Sigma) was added.

Trituration was repeated followed by centrifugation at 100g for 5 min. The supernatant was poured off and 2 ml growth medium was added; this consisted of DMEM (Dulbecco's modified Eagle's medium 4,500 mg/l glucose; Gibco). This was supplemented with 10% (v/v) heat-activated fetal bovine serum (FBS; Seralab, UK) and 0.5% gentamycin sulphate and 1% nystatin (with respect to total volume; culture media from Gibco, antibiotics and antimycotics from Sigma). The FBS employed was certified to contain less than 6 units/ml bacterial endotoxin. The cultures were used without further purification procedures. Astrocytes prepared by this procedure are more than 90% pure as established by immunostaining for GFAP (see Cookson and Pentreath, 1994).

The C6 rat glial tumour cell line and human 1321NI astrocytoma cell lines were obtained from the European Collection of Animal Cell Cultures, Porton Down, United Kingdom. Both cell lines were subcultured as follows. The cells were initially maintained in 250 cm³ flasks (Nunclon), then washed three times in calcium and magnesium free Hank's Balanced Salt Solution (HBSS; Gibco). Cells were then trypsinized with 1 ml per flask of trypsin-EDTA (1X) solution in HBSS. Following detachment, 9 ml of growth medium (DMEM, 10% FBS, 0.5% gentamycin sulphate) was added to prevent further action of the trypsin-EDTA. A split ratio of 1:3–1:10 was employed and the media was made up to 20 ml in each flask. Subculturing was repeated every 7 days.

Further details of the cell culturing procedures for astrocytes in toxicity testing have been extensively reported (see, e.g., Cookson *et al.*, 1994, 1995; Mead and Pentreath, 1998a; Cookson, 1999).

Toxicity Testing in Multiwell Plates

Two types of test system were employed. The cells were grown either in 96-well microtitre plates (Greiner) or on coverslips in 24-well multidishes from Nunclon. For direct plating (96 well) 100 μ l of a 0.025 mg/ml solution of poly-L-lysine (Sigma) was added to each well, left for 10 min, washed off with sterile (Millipore) water and allowed to dry. Coverslips were dipped in ethanol, flamed, then placed in wells (24-well plate), to which was added 100 μ l of a 0.1 mg/ml solution of poly-L-lysine (Sigma), then washed and dried.

Cells were seeded in each system at a density of 5×10^5 /ml and counted by haemocytometer before addition of the cell suspensions.

Addition of Dibutyrylcyclic AMP (dBcAMP)

Treatment of cultured astrocytes (primary or cell lines) with CAMP is a widely employed procedure that induces differentiation of the cells. Levels of glial fibrillary acidic protein (GFAP) are increased and the cells develop a

more branched form (Fedoroff *et al.*, 1987). Other aspects of cell phenotype are also changed (often upregulated), with several characteristics of reactive astrocytes (Eddleston and Mucke, 1993). In the context of toxicity evaluation the changes provide a valuable adjunct to the untreated cells, because the cells may become more responsive, levels of toxicity markers (e.g., GFAP) are increased, and the increased protective capacities can shed light on mechanisms of toxicity. This is especially relevant for the cell lines (Mead and Pentreath, 1998b).

When the cells reached confluency (2–3 days), some of the cell lines were treated with 0.5 mM N⁶, 2'-0-dibutyryladenine 3':5' cyclic monophosphate (dBcAMP; Sigma) for 48 hrs before toxicity measurements.

Indicators of Toxicity

Neutral Red (NR) Assay

NR selectively targets lysosomes in living cells by binding to anionic and/or phosphate groups of the lysosomal matrix (Borenfreund and Puerner, 1984). It can be extracted from the cells and gives a linear relationship of cell viability (in terms of lysosome activity and numbers). For this a 0.1% w/v solution of NR dye (Sigma) in phosphate buffered saline (PBS) was filtered (0.2 μ m pore size) and 10 μ l aliquots added to the media in the wells. The plates were incubated at 37°C for 3 hr. The media was replaced with 100 μ l of acid alcohol (1% v/v acetic acid in 50% ethanol), which elutes NR from the viable cells. Absorbance was read at 550 nm on a plate reader. The mean absorbance for each concentration of compound was expressed as a percentage of the value obtained for the solvent blank and was plotted against the log of compound concentration.

Measurement of GFAP, Vimentin, and S-100 by ELISA

The cytoskeletal proteins were measured quantitatively by ELISA. The methods have been previously described (O'Callaghan, 1991; Cookson and Pentreath, 1994). Briefly, following incubation in the wells, cells were washed with PBS:ethanol. The cells were fixed in 100 μ l ethanol at –20°C (30 min) and washed (three times) in PBS-CMG. To each well 100 μ l of primary antibody (anti-GFAP, anti-S-100, or monoclonal antivimentin; all Sigma), diluted 1:500 with a solution of PBS-CMG, 1% bovine serum albumin (BSA; Sigma), and 0.1% Triton X-100 (Sigma) were added and the plates incubated for 1–2 days at 4°C. The cells were washed three times with a solution of PBS-CMG/0.1% Triton X-100. To each was added 100 μ l rabbit antimouse IgG conjugated to alkaline phosphatase (Sigma) diluted 1:500 with PBS-CMG, 1% BSA, and 0.1% Triton X-100. For S-100 measurement the secondary antibody was goat antirabbit IgG conjugated to horseradish

peroxidase diluted 1:5000 in PBS-CMG with 1% BSA and 0.1% Triton X-100. Cells were incubated with gentle shaking (2 hr, room temperature) and then washed five times with PBS-CMG/0.1% Triton X-100 and blotted dry. The plates were developed with 100 μ l per well of paranitrophenylphosphate solution (Sigma; 1 mg/ml in 10% diethanolamine buffer with 0.5 mM MgCl_2 , pH 9.8). The reaction was stopped after 10 min by the addition of 100 μ l per well of 0.4 M NaOH. The amount of antigen per well was expressed as a percentage of the absorbance at 410 nm compared to the control (untreated) wells. Appropriate control wells (i.e., without either primary or secondary antibodies, substrate, or cells) were included in duplicate.

Treatment with AP

The cells were treated with β AP fragments 1–40, 25–35, 31–35, or 40–41 (as control), all purchased from Sigma. The treatments were started immediately after the cells had reached confluency or been treated with dBcAMP and were maintained for 24 hr.

The β AP fragment 1–40 was made up either fresh on the day of testing as a 1 mg/ml stock solution in sterile distilled water, or three days before testing in a 37°C incubator to age. Serial dilutions of both (from 50–0.0005 μ g/ml) were made in DMEM containing 10% FBS and 0.5% gentamycin. The β AP fragments 25–35 and 31–35 were not aged and were made up fresh on the day of treatment. Serial dilutions were prepared as described earlier and all fragments were added to the astrocyte cultures at confluency.

Monolayer Morphology

The monolayer cultures were observed at intervals during the exposures with β AP by phase contrast microscopy.

Statistical Analysis

The values for a minimum of three sets of measurements for each β AP fragment and concentration were assessed by a paired student *t*-test.

RESULTS

The primary, C6, and 1321NI cells frequently responded with activation-type responses, with the aged β AP fragment 1–40 and fragment 25–35, recorded as increased in NR uptake, GFAP, vimentin, or S-100 at 24 hr (Table 1). The responses were also frequently nonlinear, with reduction in indicators at higher concentrations; however, cell death with EC_{50}

TABLE 1 Activation in Primary, C6, and 1321NI Astrocytes (% of Control) When Treated with β AP Fragment 1–40)

		C6 cells			1321NI	
		Primary	+dBcAMP	−dBcAMP	+dBcAMP	−dBcAMP
Neutral	Fresh	130(0.0045)	125(0.008)	(NS)	110(NS)	110(NS)
Red	Aged	165(0.000)	152(0.033)	120(0.036)	117(NS)	(NS)
GFAP	Fresh	108(NS)	157(0.0037)	95(NS)	(NS)	(NS)
	Aged	105(NS)	108(NS)	103(NS)	(NS)	(NS)
Vimentin	Fresh	108(NS)	115(NS)	(NS)	110(NS)	120(0.017)
	Aged	103(NS)	(NS)	(NS)	(NS)	(NS)
S-100	Fresh	(NS)	(NS)	(NS)	(NS)	148(0.000)
	Aged	(NS)	(NS)	(NS)	(NS)	(NS)

p-values in brackets; NS = not significant at the 5% level using the student's *t*-test.

Activation expressed as a percentage of the control for neutral red, GFAP, vimentin, and S-100 when primary, C6, and 1321NI astrocytes were treated with fresh and aged β AP fragment 1–40 for 24 hr.

values was rarely observed even at the maximum concentrations employed (50 μ g/ml).

β AP Fragment 1–40

The primary astrocytes were activated by low concentrations of aged β AP (i.e., 0.0005–0.005 μ g/ml), whereas fresh β AP caused activation at 50 μ g/ml when assessed by NR uptake (Table 1). C6 cells also showed significant increases in NR uptake after dBcAMP treatment and the aged β AP fragment, whereas smaller responses occurred with fresh β AP and/or no dBcAMP treatment (Figure 1). The activation maxima were followed by reductions at higher concentrations (Figure 1). The 1321NI cells, on the other hand, showed only modest (not significant) biphasic changes with aged or fresh β AP 1–40, either with or without dBcAMP pretreatment (Table 1).

When evaluated with measurement of GFAP levels, small but nonsignificant nonlinear responses were recorded for the primary cells (Figure 2), but some relatively large increments, without inhibition at the highest concentrations employed, were recorded with the C6 cells and the fresh β AP (Figure 3). The 1321NI cells were generally unresponsive (Table 1). In relation to this several properties of GFAP in the 1321NI cell line (for example, responses to dBcAMP) can differ significantly from the primary and C6 cells (see Cookson, 1999).

Vimentin and S-100 levels also underwent modest, generally nonsignificant, changes in the different cell types with or without dBcAMP treatments (Table 1). For example, in the 1321NI cells the increases (maximum 20%) were not followed by cell death even with 50 μ g/ml aged β AP (Figure 4).

FIGURE 1 Effects of β AP fragments 1–40 on C6 glioma cells, either with or without dBcAMP treatment. Cells were exposed to the fresh or aged fragments for 24 hr and responses measured with neutral red. Each point is the mean of three replicates.

Examination of the monolayers by phase contrast showed progressive morphological changes in the primary cells and cell lines with increasing concentrations of β AP, which appeared to correspond with the quantitative measurements. These were altered fibrillar arrangements with changes in cell shape to more branched morphology, with increased gaps between the cells. However, despite the alterations of morphology there was no evidence

FIGURE 2 GFAP levels in primary astrocyte cultures following 24 hr exposure to fresh or aged β AP 1–40. Each point is the mean of seven replicates.

of cell detachments or cell death, even at the highest concentrations of β AP employed.

β AP Fragments 25–35 and 31–35

Similar patterns of responses, with some modest biphasic alterations in indicator levels in the different cell types, were caused by the different

FIGURE 3 GFAP levels in C6 glioma cells following 24 hr treatment with fresh or aged β AP 1–40. Some of the cultures were treated with dBcAMP. Each point is the mean of three replicates.

fragments. The activation data are summarized in Table 2. However, many of the changes were not significant, and cell death did not take place even at the highest concentrations ($50 \mu\text{g/ml}$) of β AP fragments employed. Examples of the responses of the levels of vimentin and S-100 in the C6 cells are shown in Figures 5 and 6.

Phase contrast microscopy showed altered morphology with increased branching of the cell processes after exposure to $50 \mu\text{g/ml}$ β AP 25–35. The

TABLE 2 Activation in Primary, C6, and 1321N1 Astrocytes (% of Control) When Treated with β AP Fragment 25–35 and 31–35

	Primary		C6 cells		1321N1	
	+dBcAMP	–dBcAMP	+dBcAMP	–dBcAMP	+dBcAMP	–dBcAMP
Neutral red						
(25–35)	184(0.001)	80(0.032)	122(0.008)	103(NS)	163(0.002)	140(0.005)
(31–35)	(NS)	(NS)	132(0.2)	(NS)	(NS)	(NS)
GFAP						
(25–35)	155(0.002)	117(NS)	(NS)	107(NS)	(NS)	(NS)
(31–35)	169(0.000)	110(NS)	(NS)	(NS)	(NS)	(NS)
Vimentin						
(25–35)	(NS)	123(NS)	(NS)	(NS)	110(NS)	118(NS)
(31–35)	(NS)	125(0.016)	108(NS)	(NS)	105(NS)	115(NS)
S-100						
(25–35)	(NS)	(NS)	112(NS)	(NS)	170(0.004)	(NS)
(31–35)	(NS)	(NS)	127(0.001)	116(NS)	(NS)	104(NS)

p-values in brackets; NS = not significant at the 5% level using the student's *t*-test.

Activation expressed as a percentage of the control for neutral red, GFAP, vimentin, and S-100 when primary, C6, and 1321N1 astrocytes were treated with fresh and aged β AP fragment 1–40 for 24 hr.

changes were apparent after 2 hr exposure in each cell line, and progressed until 24 hrs when there appeared to be significant disruption of the cells, with gaps forming in the previously confluent cell layer. However, there did not appear to be any cell death (i.e., detachment). With β AP fragment 31–35 the changes were less marked, with no alterations in the 1321NI cells at 24 hr at a concentration of 50 μ g/ml.

DISCUSSION

A range of toxicity studies of astrocytes have been made using cultures of primary, subcultured, or permanent cell lines. These have been extremely valuable in toxicity evaluation and understanding the mechanisms of action of a number of neurotoxic substances (see Pentreath and Slamon, 2000, for review). The common experimental procedure in the studies has been to expose the cultures to a range of concentrations of the toxic substances and assess the responses by different cytological and biochemical markers (e.g., Tiffany-Castiglioni *et al.*, 1989; Cookson and Pentreath, 1994, 1996; Cookson *et al.*, 1994, 1995; Cookson, 1999; see also Pentreath, 1999). The data concerning several features of toxicity, for example EC₅₀ values and associated rankings of toxicants, are generally closely comparable to the *in vivo* studies, but additional novel mechanistic information on gliotoxicity has been obtained (see Pentreath, 1999). In particular it has been established that many of the toxic substances produce biphasic responses with the different endpoints, with the suprathreshold concentrations causing increases

FIGURE 4 Vimentin levels in 1321N1 astrocytoma cells, either with or without dBcAMP treatment, following 24 hr exposure to fresh or aged β AP 1–40. Each point is the mean of seven replicates.

(i.e., activation) in endpoint values followed by reductions at higher concentrations. The suprathreshold concentration increases were frequently in the range 110–160% control values (see Figure 7). Mounting evidence shows that these responses are associated with the phenomenon of astrocyte activation (astrogliosis) and involve, in part, increased protective capacities against toxicity caused by oxidative damage (Pentreath and Slamon, 2000). The reductions at higher concentrations are associated with increasing toxic damage, which for many neurotoxicants cannot be compensated for and leads to cell

FIGURE 5 Vimentin levels in rat C6 glioma cells, either with or without dBcAMP treatment, following 24 hr treatment with β AP fragments 25–35 or 31–35. Each point is the mean of seven replicates.

death. The response patterns in most of these situations corresponds to the nonlinear, hormetic responses which appear common to multiple biological systems (see Calabrese and Baldwin, 2001).

The present findings are consistent with a fairly frequent biphasic toxicity dose-response for β AP and its 11-amino acid toxic fragment β AP 25–35 in cultures of primary astrocytes, C6 glioma, and 1321NI astrocytoma cells. Most of the treatments induced activation-type responses, with the aged β AP

FIGURE 6 S-100 levels in C6 glioma cells, with or without dBcAMP treatment, following 24 hr treatment with β AP fragments 25–35 or 31–35. Each point is the mean of seven replicates.

fragment 1–40 causing greater activation (increase in NR uptake) than fresh β AP fragment 1–40. However, none of the β AP fragments caused the marked toxic changes with EC_{50} values followed by cell death comparable to those described for a number of other chemical toxicants (see, e.g., Cookson and Pentreath, 1994; Cookson *et al.*, 1994). A similar lack of overt toxicity has been reported by Brera *et al.* (2000), using the MTT assay as an indicator

FIGURE 7 Responses of cultured primary astrocytes and astrocyte cell lines to toxic substances. The curve depicts the generalized response derived from the study of a large number of different toxicants, assessed by different endpoints (including structural proteins, lysosomal activity, and energy metabolism) over a range of concentrations (see Pentreath and Slamon, 2000). The shape, dose range, time course, and amplitude of the activation phase and the subsequent reductions vary with test substance, indicator/endpoint, and cell type. The different amyloid beta fragments studied in the present work frequently produced responses within the activation phase but did not cause cell death, even at high concentrations (i.e., reach 0% on the downward phase). There is growing evidence that the responses to the lower doses of toxicants, measured by different endpoints, reflect different types of insult/stress, which in turn initiate activation (i.e., upregulation) of different components of the cells' protective systems (Pentreath and Slamon, 2000).

of mitochondrial activity, who found that neither β AP 25–35 nor β AP 1–40 caused more than 50% cell death in the astrocyte population after 24 hr. Evidence was presented that the limited toxicity was due to peroxide generation by β AP, but that the cells' high antioxidant capacities were sufficient to counteract the damage.

This contrasts with previous studies on neuronal cultures; for example, Pike *et al.* (1991) found that β AP 1–42 in the aggregated or “aged” form caused marked cell death of cultures of hippocampal neurons after 24 hr at concentrations of 10–50 μ g/ml. Similarly, Yankner *et al.* (1990) have shown that β AP fragment 25–35 has a direct toxic effect on neurons in rat hippocampal cultures. However, the findings are consistent with the well-established high levels of protective systems, especially against oxidative/radical mediated damage, in astrocytes compared to neurons (see review by Pentreath

and Slamon, 2000). Further experiments using extended culture periods may shed light on this.

Astrocyte activation with enhanced staining for GFAP and invasion of the neuritic plaques is a hallmark of AD (Duffy *et al.*, 1980; Schechter *et al.*, 1981). The calcium-binding protein S-100, which is normally concentrated in glial cells (reviewed by Kligman and Hilt, 1988), is also elevated in the AD patient. It is concentrated in the neuritic plaques of dystrophic dendrites (Kosik *et al.*, 1986) and may be released from astrocytes to promote neurite extension (Kligman and Hilt, 1988).

Increased GFAP and S-100 immunostaining in astrocytes exposed to toxic β AP fractions, but without significant cell death, has been observed by Meske *et al.* (1998). Although the staining showed altered morphology with condensation (i.e., apparent increases in fluorescence) of the proteins, it did not provide quantitative data on the changes.

Vimentin is another cytoskeletal component, chiefly of immature, undifferentiated glia, which is coexpressed with GFAP in reactive astrocytes following damage to nervous tissue (Calvo *et al.*, 1991). Treatment with dB-cAMP induces activation-associated increases in the cytoskeletal components along with many other structural and metabolic constituents of astrocytes (Eddleston and Mucke, 1993) and, in accord with this, greater activation by the β AP fragments when measured by NR uptake, GFAP, vimentin, and S-100 expression. Several studies have demonstrated that β AP fragments induce other components of the reactive response in astrocytes, including upregulation of IL-1 β , NO synthase (Hu *et al.*, 1998, 1999), K⁺ and Cl⁻ channel activities (Jalonen *et al.*, 1997), neprilysin (a putative β AP degrading enzyme; Apelt *et al.*, 2003), and glutamate transport (Abe and Misawa, 2003). Thus, taken together, the present work and those of others demonstrate that the significant responses of astrocytes to the toxic β AP fractions are the activation-type changes, with relatively limited toxicities. They also strongly imply that β AP may be an etiologic agent in the glial changes that accompany AD.

The significance of the astrocyte activation in response to the β AP is not yet clear. Although the activation repertoire embraces a change in cell phenotype with alterations (normally upregulation) of a large number of enzymes (see Eddleston and Mucke, 1993), substantial evidence is available for a number of toxic compounds showing that part of the changes confer increased protection against oxidative/radical mediated cell damage (reviewed by Pentreath and Slamon, 2000). In relation to this the toxic forms of β AP can initiate oxidative damage in neurons, which in turn causes activation of NF- κ B, which is responsible for initiating the cellular antioxidant defense program (Kaltschmidt *et al.*, 1997, 1999). High, chronic exposure to increased levels of β AP may exceed the capacities for repair and lead to neuronal apoptosis and death (Kaltschmidt *et al.*, 1997, 1999; see also Calabrese, 2001). The

likely protective roles of astrocytes in β AP toxicity are important areas for further study.

REFERENCES

- Abe K, Misawa M. Amyloid β protein enhances clearance of extracellular L-glutamate by cultured rat cortical astrocytes. *Neurosci Res.* 2003;45:25–31.
- Apelt J, Ach K, Schliebs R. Aging-related down-regulation of neprilysin, a putative β -amyloid-degrading enzyme, in transgenic Tg2576 Alzheimer-like mouse brain is accompanied by an astroglial upregulation in the vicinity of β -amyloid plaques. *Neurosci Lett.* 2003;339:183–186.
- Bahmanyar S, Higgins GA, Goldgaber D, Lewis DA, Morrison JH, Wilson MC, Shankar KC, Gajdusek C. Localization of amyloid β protein mRNA in brains from patients with AD. *Science.* 1987;237:77–80.
- Behl C, Davis J, Cole GM, Schubert D. Vitamin E protects nerve cells from amyloid β protein toxicity. *Biochem Biophys Res Comm.* 1992;186:944–950.
- Borchelt DR, Ratovitski T, van Larc J, Lee MD, Gonzales V, Jenkins NA, Copeland NG, Price DL, Sisodia SS. Accelerated amyloid deposition in the brains of transgenic mice expressing mutant presenilin 1 and amyloid precursor proteins. *Neuron.* 1997;19:939–945.
- Borenfreund E, Puerner JA. A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/MR-90). *J Tiss Cult Meth.* 1984;9:7–9.
- Brera B, Serrano A, Ceballos ML. β -amyloid peptides are cytotoxic to astrocytes in culture: A role for oxidative stress. *Neurobiol Dis.* 2000;7:395–405.
- Burdick D, Soreghan B, Kwon M, Kosmoski J, Knauer M, Henschen A, Yates J, Cotman CW, Glabe C. Assembly and aggregation properties of synthetic Alzheimer's A4/ β -amyloid peptide analogs. *J Biol Chem.* 1992;267:546–554.
- Calabrese EJ. Amyloid β -peptide: Biphasic dose responses. *Crit Rev Toxicol.* 2001;31:605–606.
- Calabrese EJ, Baldwin LA. Hormesis: A generalizable and unifying hypothesis. *Crit Rev Toxicol.* 2001;31:353–424.
- Calvo JL, Carbonell AL, Boya J. Co-expression of GFAP and vimentin in reactive astrocytes following brain injury in rats. *Brain Res.* 1991;566:333–336.
- Card JP, Meade RP, Davis LG. Immunocytochemical localization of the precursor protein for β -amyloid in the rat CNS. *Neuron.* 1988;1:835–846.
- Cookson MR. Functional biochemical indicators. In: Pentreath VW, ed. *Neurotoxicology in Vitro*. London: Taylor and Francis; 1999:149–175.
- Cookson MR, McClean R, Williams SP, Davenport-Jones J, Egan C, O'Hare S, Atterwill CK, Pentreath VW. Use of astrocytes for in vitro neurotoxicity testing. *Toxicol in vitro.* 1994;8:817–819.
- Cookson MR, Mead C, Austwick SM, Pentreath VW. Use of the MTT assay for estimating toxicity in primary astrocyte and C6 glioma cultures. *Toxicol in vitro.* 1995;9:39–48.
- Cookson MR, Pentreath VW. Alterations in the glial fibrillary acidic protein content of primary astrocyte cultures for evaluation of glial toxicity. *Toxicol in vitro.* 1994;8:351–359.
- Cookson MR, Pentreath VW. Protective roles of glutathione in the toxicity of mercury and cadmium to C6 glioma cells. *Toxicol in vitro.* 1996;10:257–264.
- Duffy PE, Rapport M, Graf L. GFAP and Alzheimer-type senile dementia. *Neurol.* 1980;30:778–782.
- Eddleston M, Mucke L. Molecular profile of reactive astrocytes: Implication for their role in neurologic disease. *Neurosci.* 1993;54:15–36.
- Esch FS, Keim PS, Beattie EC, Blacher RW, Culwell AR, Oltersdorf T, McClure D, Ward PJ. Cleavage of amyloid β peptide during constitutive processing of its processor. *Science.* 1990;248:1122–1124.
- Fedoroff S, Ahmed I, Opas h, Kalnins VI. Organization of microfilaments that form in the presence of dibutyl cyclic AMP in cultures and which are similar to reactive astrocytes in vivo. *J Neurosci.* 1987;22:255–266.
- Glenner GG, Wong CW. Alzheimer's disease and Down's syndrome: Sharing of a unique cerebrovascular amyloid fibril protein. *Biochem Biophys Res Comm.* 1984;122:1131–1135.
- Golde TE, Estus S, Younkin LH, Selkoe DJ, Younkin SG. Processing of the amyloid protein precursor to potentially amyloidogenic derivatives. *Science.* 1992;255:728–730.

- Goldgaber D, Lerman MI, McBride OW, Saffiotti U, Gajdusek DC. Characterization and chromosomal location of a cDNA encoding brain amyloid of Alzheimer's disease. *Science*. 1987;235:877–880.
- Goodman Y, Steiner MR, Steiner SM, Mattson MP. Nordihydroguaiaretic acid protects hippocampal neurons against amyloid β -peptide toxicity and attenuates free radical and calcium accumulation. *Brain Res*. 1994;654:171–176.
- Guo Q, Robinson N, Mattson MP. Secreted β -amyloid precursor protein counteracts the proapoptotic action of mutant presenilin-1 by activation of NF- κ B and stabilization of calcium homeostasis. *J Biol Chem*. 1998;273:1234–1235.
- Haass C, Schlossmecher MG, Hung AY, Vigo-Pelery C, Mellon A, Ostaszewski BL, Lieberburg I, Koo EH, Scheuk D, Teplow DB, Selkoe DJ. Amyloid β -peptide is produced by cultured cells during normal metabolism. *Nature*. 1992;359:322–325.
- Hensley K, Carney JM, Mattson MP, Aksenova M, Harris M, Wu JF, Floyd RA, Butterfield DA. A model for β -amyloid aggregation and neurotoxicity based on free radical generation by the peptide: Relevance to Alzheimer disease. *Proc Natl Acad Sci USA*. 1994;91:3270–3274.
- Hu J, VanEldik LJ. Glia-derived proteins activate cultured astrocytes and enhance beta amyloid glial activation. *Brain Res*. 1999;842:46–54.
- Hu J, Akama KT, Krafft GA, Chromy BA, Van Eldik LJ. Amyloid- β peptide activates cultured astrocytes: morphological alterations, cytokine induction and nitric oxide release. *Brain Res*. 1998;785:195–206.
- Jalonen TO, Charniga CJ, Wielt DB. β -amyloid peptide-induced morphological changes coincide with increased K^+ and Cl^- channel activity in rat cortical astrocytes. *Brain Res*. 1997;746:85–97.
- Kaltschmidt B, Uherek M, Volk B, Baeuerle PA, Kaltschmidt. Transcription factor NF- κ B is activated in primary neurons by amyloid β peptides and in neurons surrounding early plaques from patients with Alzheimer disease. *Proc Natl Acad Sci USA*. 1997;94:2642–2657.
- Kaltschmidt B, Uherek M, Wellman H, Volk B, Kaltschmidt C. Inhibition of NF- κ B potentiates amyloid β -mediated neuronal apoptosis. *Proc Natl Acad Sci USA*. 1999;96:9409–9414.
- Kligman D, Hilt DC. The S-100 protein family. *TIBS*. 1988;13:437–443.
- Koh JY, Yang LL, Cotman CW. β -amyloid protein increases the vulnerability of cultured cortical neurons to excitotoxic damage. *Brain Res*. 1990;533:315–320.
- Kosik KS, Joachim CL, Selkoe DJ. Microtubule-associated protein tau is a major antigenic component of paired helical filaments in AD. *Proc Natl Acad Sci USA*. 1986;83:4044–4048.
- Masters CL, Simms G, Weinmain NA, Multhaup G, McDonald BL, Beyreuther K. Amyloid plaque core in AD and Down Syndrome. *Proc Natl Acad Sci USA*. 1985;82:4245–4249.
- Mattson MP. Calcium and neuronal injury in Alzheimer's disease. Contributions of β -amyloid precursor protein mistreatment, free radicals and metabolic compromise. *Ann NY Acad Sci*. 1994;747:50–76.
- Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I, Rydel RE. β -amyloid peptides destabilize calcium homeostasis and render human cortical neurones vulnerable to excitotoxicity. *J Neurosci*. 1992;12:376–389.
- Mattson MP, Tomaselli KJ, Rydel RE. Calcium de-stabilizing and neurodegenerative effects of aggregated β -amyloid are attenuated by basic FGF. *Brain Res*. 1993;621:35–49.
- Mead C, Pentreath VW. Evaluation of toxicity indicators in rat primary astrocytes, C6 glioma and human 1321N1 astrocytoma cells: Can gliotoxicity be distinguished from cytotoxicity. *Arch Toxicol*. 1998a;72:372–380.
- Mead C, Pentreath VW. Hypertrophy and increased glial fibrillary acidic protein are coupled to increased protection against cytotoxicity in glioma cell lines. *Toxicol in vitro*. 1998b;12:141–152.
- Meske V, Hamker U, Albert F, Ohm TG. The effects of β /A4-amyloid and its fragments on calcium homeostasis, glial fibrillary acidic protein and S100 β staining, morphology and survival of cultured hippocampal astrocytes. *Neurosci*. 1998;85:1151–1160.
- Muller U, Cristina N, Li ZW, Wolfer DP, Lipp HP, Rulicke T, Brandner S, Aguzzi A, Weissmann C. 1994. Behavioral and anatomical deficits in mice homozygous for a modified beta-amyloid precursor protein gene. *Cell*. 1994;79:755–765.
- O'Callaghan JP. Assessment of neurotoxicology: Use of GFAP as a biomarker. *Biochem Env Sci*. 1991;4:197–206.
- Pentreath VW. *Neurotoxicology In Vitro*. London: Taylor and Francis; 1999.
- Pentreath VW, Slamon ND. Astrocyte phenotype and prevention against oxidative damage in neurotoxicity. *Human Exptl Toxicol*. 2000;19:641–649.

- Pike CJ, Walencewicz AJ, Glabe CG, Cotman CW. In vitro aging of amyloid β protein causes peptide aggregation and neurotoxicity. *Brain Res.* 1991;563:311–314.
- Robakis NK, Ramakrish N, Wolfe G, Wisniewski HM. 1987. Molecular cloning and characterization of a cDNA encoding the cerebrovascular and the neuritic plaque amyloid. *Proc Nat Acad Sci USA.* 1987;84:4190–4194.
- Schecter R, Yen SC, Terry RD. Fibrous astrocytes in senile dementia of the Alzheimer type. *Am J Neuropathol Exp Neurol.* 1981;30:95–101.
- Seubert P, Vigo-Pelfrey C, Esch F, Lee M, Dovey H, Davis D, Sinha S, Schlossmacher M, Whaley J, Swindlehurst C, McCormack R, Wolfert R, Selkoe D, Lieberburg I, Scheuk D. Isolation and quantification of soluble Alzheimer's β -peptide from biological fluids. *Nature.* 1992;359:325–327.
- Siman R, Card JP, Nelson RB, Davis LG. Expression of β -amyloid precursor protein in reactive astrocytes following neuronal damage. *Neuron.* 1989;3:275–285.
- Tanzi RE. Molecular genetics of Alzheimer's disease and the amyloid beta peptide precursor gene. *Ann Med.* 1989;21:91–94.
- Terry RD, Gonatas NK, Weiss M. Ultrastructural studies in Alzheimer's presenile dementia. *Am J Pathol.* 1964;44:269–297.
- Tiffany-Castiglioni E, Sierra EM, Wu J-N, Rowles TK. Lead toxicity in neuroglia. *Neurotoxicol.* 1989;10:417–444.
- Weidemann A, Paliga K, Durrwang U, Czech C, Evin G, Masters CL, Beyreuther K. Formation of stable complexes between two Alzheimer's disease gene products presenilin-2 and beta-amyloid precursor protein. *Nat Med.* 1997;3:328–332.
- Weiss JH, Pike CJ, Cotman CW. Calcium channel blockers attenuate β -amyloid peptide toxicity to cortical neurons in culture. *J Neurochem.* 1994;62:147–158.
- Yankner JA, Duffy LK, Kirschner DA. Neurotrophic and neurotoxic effects of amyloid β protein reversal by tachykinin neuropeptides. *Science.* 1990;250:279–282.