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# Beta Amyloid Toxicity Does Not Require RAGE Protein

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**It has been suggested that a receptor for advanced glycation end products (RAGE) is the nerve cell receptor for amyloid $\beta$ protein (A$\beta$). To determine if this is indeed the case, two neural cell lines as well as rat cortical neurons were examined for the presence of the mRNA for RAGE by PCR and northern blot analysis. Although lung was strongly positive, in no case was RAGE mRNA detected in the cultured neural cells. Glycated albumin is a major ligand for RAGE and the cell surface RAGE protein is trypsin sensitive. In agreement with the mRNA data, trypsin treatment did not alter A$\beta$ toxicity, nor did glycated albumin modify the A$\beta$ response. It follows that RAGE is not the neural receptor for A$\beta$.** 

A significant body of human genetic, mouse transgenic, and biochemical evidence shows that amyloid $\beta$ protein (A$\beta$) is involved in the pathogenesis of Alzheimer's disease (AD) (for review, see Yankner et al., (1)). Since A$\beta$ is directly toxic to neuronal cells, several alternatives have been suggested for the initial interaction between A$\beta$ and the cell. These include interactions with the SEC receptor (2) and with the tachykinin receptors (3, 4), a nonspecific insertion into the membrane (5), the formation of ion pores (6), and the internalization of peptide via pinocytosis (7). More recently it has been claimed that a protein called RAGE (receptor for advanced glycation end products) is responsible for A$\beta$ neurotoxicity (8). However, since a variety of chemically distinct amyloid peptides are neurotoxic via indistinguishable pathways (5), and since these synthetic peptides are not glycated, it is not clear how RAGE could recognize them. If there exists a unique receptor which mediates A$\beta$ neurotoxicity, it mostly likely recognizes the amphiphilic $\beta$ sheets that are common to all amyloid peptides. To examine the potential role of RAGE in A$\beta$ nerve cell toxicity, PCR and northern blot analysis were used to identify RAGE mRNA in A$\beta$ sensitive clonal and primary nerve cell cultures. Competition experiments for RAGE binding by glycated albumin were also carried out. It is shown that none of these A$\beta$ sensitive nerve cell culture systems express RAGE mRNA, nor does glycated albumin interfere with A$\beta$ toxicity. Therefore RAGE is not the neuronal receptor for A$\beta$.

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Abbreviations: RAGE, receptor for advanced glycation end products; A$\beta$, amyloid beta protein; DMEM, Dulbecco's modified Eagles' medium; AD, Alzheimer's Disease.

## MATERIALS AND METHODS

*Cells.* The B12 cell line was derived from a rat brain tumor and is very sensitive to A$\beta$. B12 cells responds to A$\beta$ with a rapid decrease in the ability to reduce MTT and by limited cell lysis(9). PC12 cells have a similar response to A$\beta$ (10, 11). Both cell lines respond equally well to A$\beta_{1\text{-}40}$, A$\beta_{1\text{-}42}$, and A$\beta_{25\text{-}35}$; they are nonresponsive to A$\beta_{40\text{-}1}$ and scrambled A$\beta_{25\text{-}35}$. B12 cells were maintained in Dulbecco's modified Eagles' medium with 10% fetal bovine serum and PC12 cells in DMEM plus 5% horse and 10% fetal calf serum. Primary rat cortical neuron cultures were prepared from E17 embryos and maintained in $N_2$ synthetic medium as described by (12). They were essentially devoid of GFAP positive astrocytes.

*Cytotoxicity.* Cytotoxicity assays were carried out as described (9). Briefly, clonal cells were plated in 96 well plates at $2\times10^3$/well in DMEM plus 5% dialyzed fetal calf serum. Eighteen hours later the test reagents were added, and after 24hrs the cytotoxic effect of A$\beta$ was monitored by the MTT assay (9, 11). Cell viability in primary cultures was monitored either by the release of LDH or by visual counting.

*RT-PCR and Northern blot analysis.* Total RNA was prepared from cultured cells and embryonic tissues by the guanidinium thiocyanate method (13). Northern blot analysis was done according to standard procedures using 20 $\mu$g of total RNA per lane and the specified cDNA probes (14). The following PCR primers were synthesized based upon the rat cDNA RAGE sequence nomenclature (Lundh, E. R., Morser, J., McClary, J. and Nagashima, M. unpublished, accession number L33413): Upstream primer I, residues 20-40 (5′end); upstream primer IV, residues 760-780; downstream primer III, residues 520-540; downstream primer II residues 1400-1420 (3′ end). eIF-2 primers were between bases 59-79 and 569-589 (15). 10 $\mu$g of total RNA were used for reverse transcription with these primers using first-strand reaction mix beads from Pharmacia. PCR analysis was carried out in 10 mM Tris. HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 10$\mu$g/ml gelatin, and 200 $\mu$M of each deoxynucleotide triphosphate for 35 cycles. Each cycle is composed of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by a 10-min extension period at 72°C. PCR products were analyzed by 2% agarose



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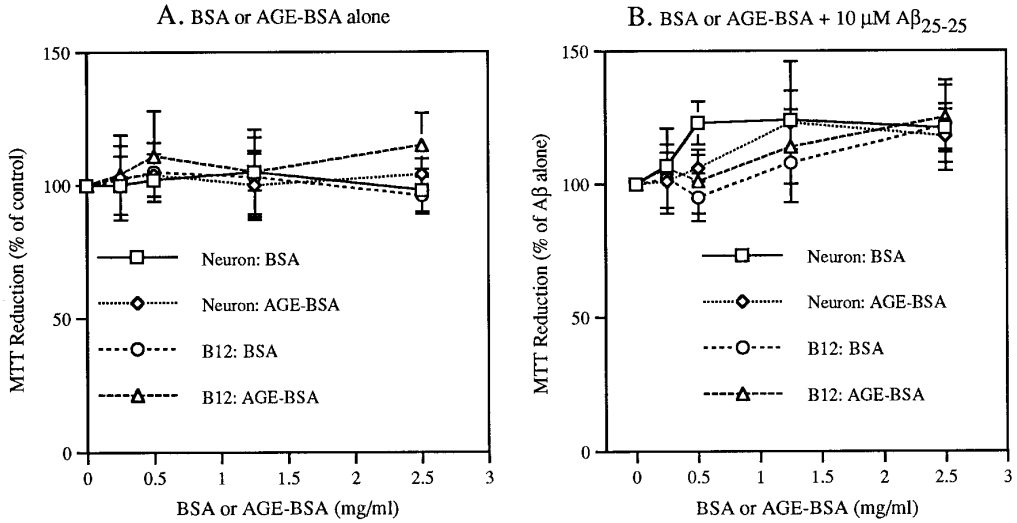


**FIG. 1.** The effect of AGE-BSA on cell viability and the possible competition between AGE-BSA and A$\beta$. (A). The effect of control BSA and AGE-BSA on the viability of rat primary cortical neurons and B12 cells. Cells were treated with various concentrations of AGE-BSA or control BSA for 16 hr at 37° C. The viability of the cells was then determined by the MTT assay. (B). The possible competition between AGE-BSA and A$\beta_{25\text{-}35}$. Various concentrations of AGE-BSA or control BSA were added to rat primary cortical neurons or B12 cells, followed by the immediate addition of 10 $\mu$M A$\beta_{25\text{-}35}$. Cell viability was then assayed with the MTT assay after 16 hr of incubation at 37° C. MTT reduction in the presence of 10$\mu$M A$\beta_{25\text{-}35}$ was taken as 100%. Similar results were obtained with A$\beta_{1\text{-}40}$. All data are the means ± S.D. of three determinations.

gel electrophoresis. DNA sequencing was done by the Scripps Research Institute DNA sequencing facility.

*Glycosylation and receptor competition assays.* Bovine serum albumin (BSA, fraction V, Sigma) was incubated with glucose to form the advanced glycosylation end products (AGE-BSA) according to Schmidt et al., (16). Briefly, BSA (25 mg/ml) was incubated with 0.5 M glucose at 37°C for 6 weeks in calcium and magnesium-free phosphate buffered saline containing protease inhibitors (PMSF, 1mM; leupeptin, 0.5 $\mu$g/ml; pepstain, 0.1$\mu$g/ml; aprotonin, 2 $\mu$g/ml). Control proteins were exposed to the same conditions except glucose was omitted, and these proteins are designated as control BSA. The incubation mixtures were sterilized by filtration. The glycosylated proteins appear yellowish and had a 9-fold increase in fluorescence (excitation wavelength, 370 nm; emission wavelength, 440 nm). The effect of AGE-BSA on cell viability and the possible competition between AGE-BSA and A$\beta$ were studied with the MTT assay.

*Effect of A$\beta$ on MTT reduction by trypsinized B12 cells.* Cell surface RAGE protein is sensitive to trypsin treatment (8). To study whether RAGE is involved in mediating the effect of A$\beta$ on cellular MTT reduction, B12 cells ($5 \times 10^6$ cells in 2 ml medium) were treated with 1 mg/ml trypsin for 30 min at 37°C. Trypsin was inactivated by an excess of serum-containing media. Trypsinized cells were resuspended in serum-containing media and treated with 10 $\mu$M A$\beta_{25\text{-}35}$ for 10 min at 37°C. The cells were then washed three times to remove unbound A$\beta$ so that no free A$\beta$ will be available to bind newly synthesized RAGE. After three hours of incubation at 37°C, the MTT assays were performed. Maximal inhibition of cellular MTT reduction by A$\beta_{25\text{-}35}$ is achieved after 10 min incubation of the cells with A$\beta_{25\text{-}35}$ if the MTT assay is performed three hours later. The continued presence of A$\beta$ is not necessary (Liu and Schubert, unpublished results).

## RESULTS

It has been suggested that A$\beta$ interacts with a specific receptor on the surface of nerve cells to generate a cyto-

**TABLE 1**

Effect of Trypsin Treatment on A$\beta$-Induced Inhibition of Cellular MTT Reduction

| | | MTT reduction ($A_{570\text{-}630}$nm/2 × $10^4$ cells/3 hr) | |
|---|---|---|---|
| Cell type | A$\beta_{25\text{-}35}$ | Control cells | Trypsin-treated cells |
| B12 cells | 0 $\mu$M | 0.405 ± 0.014 (100%) | 0.376 ± 0.087 (100%) |
| | 10 $\mu$M | 0.193 ± 0.007 (48%) | 0.226 ± 0.034 (60%) |
| PC12 cells | 0 $\mu$M | 0.421 ± 0.019 (100%) | 0.379 ± 0.019 (100%) |
| | 10 $\mu$M | 0.262 ± 0.021 (62%) | 0.223 ± 0.025 (59%) |

B12 cells ($5 \times 10^6$ cells in 2 ml medium) were treated with 1 mg/ml trypsin for 30 min at 37°C. Trypsin was inactivated by serum-containing media. Trypsinized cells were resuspended in serum-containing media and treated with 10 $\mu$M A$\beta_{25\text{-}35}$ for 10 min at 37°C. The cells were then washed three items to remove unbound A$\beta$ so that no free A$\beta$ will be available to bind newly synthesized RAGE. MTT assays were then performed after three hours of incubation at 37°C.

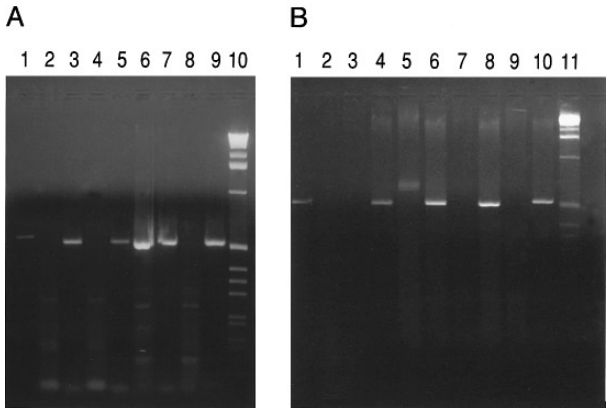


**FIG. 2.** PCR analysis of RAGE in cultured cells. Total RNA was isolated and analyzed by PCR with the indicated primers as described in Materials and Methods. (A). RAGE primers III to I, and eIF primers. Lanes: 1. Reverse transcriptase kit control (550bp); 2. B12 RAGE; 3. B12 eIF; 4. PC12 RAGE; 5. PC12 eIF; 6. Lung, RAGE; 7. Lung, eIF; 8. Heart, RAGE; 9. Heart, eIF; 10. molecular weight markers (λ phage, Hind III). (B). RAGE primers II to IV, and III to I, eIF primers. Lanes: 1. kit control; 2. B12, II-IV; 3. PC12, II-IV; 4. PC12 eIF; 5. Lung II-IV; 6. Lung eIF; 7. Primary, II-IV; 8. Lung III-I; 9. Primary III-I; 10. Primary eIF; 11. Molecular weight markers.

toxic response (8). This receptor is a 50kd protein called RAGE, which was originally isolated in lung as a receptor for glycated proteins (16, 17). Since RAGE interacts strongly with glycated albumin, if RAGE is a receptor for A$\beta$ then glycated albumin should either directly activate the biological response elicited by A$\beta$ or at high concentrations block the A$\beta$ response. To determine if RAGE is indeed involved in A$\beta$ toxicity, increasing concentrations of AGE-BSA were added to primary cultures of rat cortical neurons and to two A$\beta$ responsive cell lines, B12 and PC12. Cytotoxicity was assayed by MTT reduction. The inhibition of cellular MTT reduction by A$\beta$ is an early indicator of A$\beta$ toxicity (9-11). Figure 1A shows that increasing concentrations of AGE-BSA alone do not mimic the A$\beta$ response in both rat primary neurons and B12 cells, nor do high concentrations of AGE-BSA inhibit A$\beta$ toxicity (Figure 1B). Similar results were obtained with PC12 cells. RAGE is inactivated by trypsin treatment of cultured cells (8). However, A$\beta$ is still able to inhibit MTT reduction by trypsinized B12 or PC12 cells (Table 1). These results strongly suggest that the RAGE protein is not involved in mediating the toxic effect of A$\beta$ on nerve cells.

A necessary condition for the functional expression of a protein is the presence of its mRNA. Two experiments were done to determine if the A$\beta$ sensitive cell lines and A$\beta$ sensitive cortical neuron cultures express RAGE mRNA. Two sets of PCR primers were synthesized which covered the 5′ and 3′ halves of the rat RAGE cDNA. PCR analysis was done using RNA isolated from B12, PC12, and primary cultures of rat cortical neurons. Lung RNA was used as a positive control. Another positive control to verify the structural integrity of the individual RNA preparations was a set of primers for eIF-2, a low abundance translational initiation factor (15). Figure 2 shows that none of the cells except lung expressed RNA sequences for RAGE. Although the PCR primers cover virtually the entire molecule, we cannot rule out the existence of alternatively spliced variants of RAGE which were not identified in the original cloning of the cDNA (17).

To confirm the PCR result, a 520bp cDNA from the 3′ half of rat RAGE cDNA was isolated by PCR, sequenced to confirm its identity, and used as a probe for northern analysis of mRNA isolated from several A$\beta$

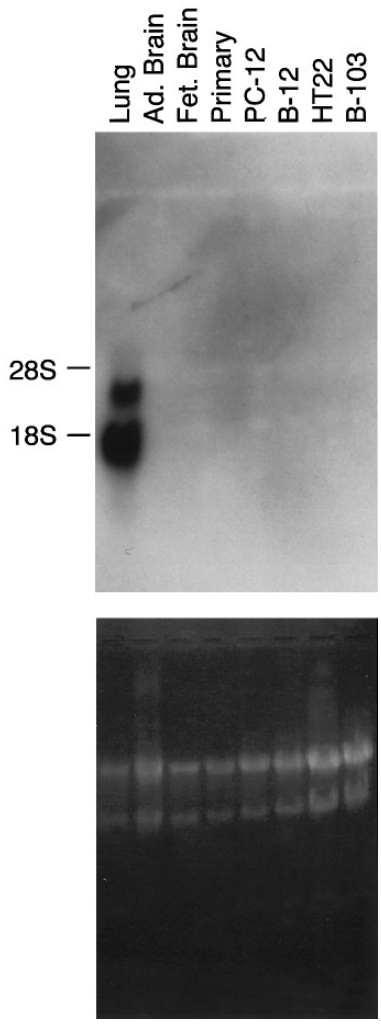


**FIG. 3.** Northern analysis of cell lines, primary cortical cultures, and tissues for RAGE mRNA. 10$\mu$g of RNA from the indicated cells or tissues were electrophoresed in agarose, transferred to nitrocellulose, and probed with a 520bp cDNA probe corresponding to the N-terminal half of the RAGE protein. Primary cells are from E17 mouse cortex. HT22 and B-103 are mouse hippocampal and rat CNS nerve cell lines, respectively. (A). Even overexposed film showed no sign of RAGE mRNA. (B). UV image of gel showing equivalence of RNA loading.

sensitive cell lines, primary neuron cultures, lung, heart, and adult and fetal brain. Figure 3 shows that while lung was strongly positive, none of the other cells expressed detectable levels of RAGE mRNA, even when the autoradiography films were greatly over exposed. These results, in conjunction with the glycated albumin and trypsin data, show that A$\beta$ toxicity to nerve cells is not mediated by the RAGE protein.

## DISCUSSION

The following data show that the RAGE protein is unlikely to be involved in A$\beta$ mediated nerve cell toxicity. 1). Glycated albumin, which binds to RAGE on endothelial cells (16), neither activates the A$\beta$ toxic response nor blocks A$\beta$ induced toxicity in neural cells (Fig. 1). 2). Trypsin treatment of the neural cells does not block A$\beta$-mediated toxicity while it is known to inactivate RAGE (Table 1). 3). mRNA for RAGE is not detectable by PCR analysis (Fig. 2) or by northern blotting (Fig. 3) in A$\beta$-sensitive neural cell lines and primary CNS cells. In addition, the A$\beta$ toxic response is mimicked by a variety of nonglycated peptides which have no sequence homology, but share the common trait of forming amphiphilic beta sheets. It is also inherently unlikely that RAGE can recognize such a diverse collection of peptides, particularly since its primary ligand is glycated protein (8, 16).

Although it is still possible that RAGE is involved in Alzheimer's disease through the activation of microglia, it is highly unlikely that RAGE mediates A$\beta$ nerve cell toxicity as suggested by others (8). The facts that neither A$\beta$ sensitive clonal cell lines nor CNS nerve cells synthesize RAGE argues that either another receptor is present in nerve cells or that A$\beta$ toxicity is not receptor mediated. A$\beta$ is able to interact specifically with tachykinin receptors (3, 4), and SEC receptors (2), but neither of these receptors mediate the amyloid cytotoxic response (18, 19). Since many structurally unrelated amphiphilic peptides, including amylin, calcitonin, lysine-leucine copolymers, and atrial natriuretic peptide all activate a similar toxicity pathway in clonal and primary nerve cells (5, 20, 21), it has been argued that they are all toxic by direct membrane perturbation (5). This hypothesis has not been ruled out, and it is still a viable alternative to receptor mediated A$\beta$ toxicity.

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