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Neurotoxicity of Acetaldehyde-Derived Advanced Glycation End Products for Cultured Cortical Neurons

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Abstract. The Maillard reaction that leads to the formation of advanced glycation end products (AGEs) plays an important role in the pathogenesis of angiopathy in diabetic patients, in aging, and in neurodegenerative processes. We hypothesize that acetaldehyde (AA), one of the main metabolites of alcohol, may be involved in alcohol-induced neurotoxicity in vivo by formation of AA-derived AGEs (AA-AGEs) with brain proteins. Incubation of cortical neurons with AA-AGE produced a dose-dependent increase in neuronal cell-death, and the neurotoxicity of AA-AGE was neutralized by the addition of an anti-AA-AGE-specific antibody, but not by anti-N-ethyllysine (NEL) antibody. The AA-AGE epitope was detected in human brain of alcoholism. We propose that the structural epitope AA-AGE is an important toxic moiety for neuronal cells in alcoholism.

Key Words: Acetaldehyde; Acetaldehyde-derived advanced glycation end products (AA-AGEs); Advanced glycation end products (AGEs); Alcoholism; Neurotoxicity.

INTRODUCTION

Alcohol induces a multitude of effects on the brain that lead to changes in behavior, and brain damage is often seen in alcoholics. Recent reports suggest a role for acetaldehyde (AA), one of the main metabolites of alcohol, in alcohol-induced neurotoxicity in the brain (1, 2). After prolonged consumption of alcohol, if AA concentration increases in brain, it could potentially react with cellular proteins and form covalent AA-protein adducts with several proteins and nucleophilic biomolecules. Despite the prevalence of this severe clinical problem, molecular mechanisms that underlie neuronal degeneration in chronic alcoholism remains unexplained.

The modification, aggregation, and deposition of proteins are prominent parts of many pathological processes and can play a direct role in tissue damage. The pathological role of the nonenzymatic modification of proteins by glucose, a process known as glycation, has become increasingly evident in different diseases. It is now well established that early glycation products undergo progressive modification over time in vivo to the formation of irreversible cross-links, after which these molecules are termed advanced glycation end products (AGEs). AGEs have been implicated in the development of many of the pathological sequelae of diabetes and aging, such as atherosclerosis and renal insufficiency (3–6). Recently, it has become clear that AGEs also have a role in neurodegenerative diseases such as Alzheimer disease (7–11), Parkinson disease (12), Creutzfeldt-Jakob disease (13), and amyotrophic lateral sclerosis (14, 15).

AGEs form by the Maillard process, a nonenzymatic reaction between ketones or aldehydes and the amino groups of proteins that contributes to the aging of proteins and to the pathological complications of diabetes (4–6, 16). In the hyperglycemia produced in diabetes, this process begins with the conversion of reversible Schiff base adducts to more stable, covalently bound Amadori rearrangement products. Over the course of days to weeks, these Amadori products undergo further rearrangement reactions to form the irreversibly bound moieties known as advanced glycation end products (AGEs). In previous reports (17–20), we described the contribution of glucose, α-hydroxyaldehydes (glyceraldehyde and glycolaldehyde), and dicarbonyl compounds (methylglyoxal, glyoxal and 3-deoxyglucosone) to the glycation of proteins, and we developed anti-AGE antibodies that specifically recognize 6 distinct classes of AGE structures (AGE-1, glucose-derived AGEs; AGE-2, glyceraldehyde-derived AGEs; AGE-3, glycolaldehyde-derived AGEs; AGE-4, methylglyoxal-derived AGEs; AGE-5, glyoxal-derived AGEs; and AGE-6, 3-deoxyglucosone-derived AGEs) within the circulating proteins and

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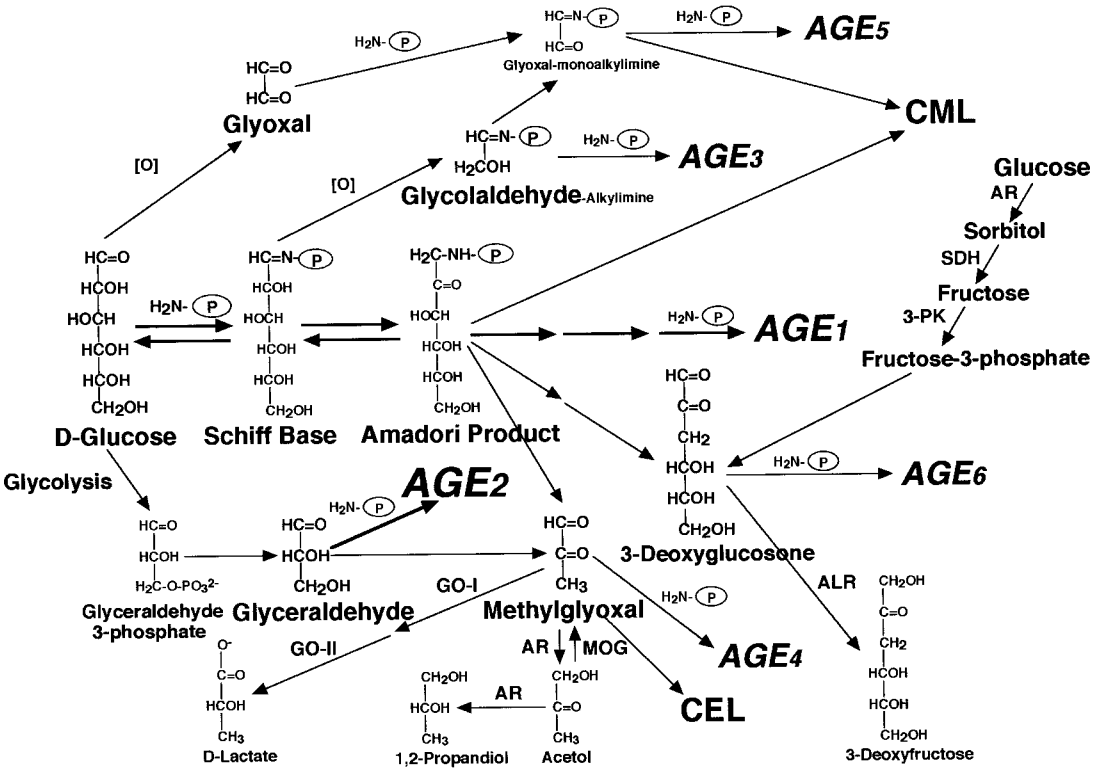

Fig. 1. Alternative routes for the formation of immunochemically distinct AGEs in vivo. Advanced glycation end products (AGEs) arise from the decomposition of Amadori products (AGE-1), the glycolysis intermediate product glyceraldehyde (AGE-2), the Schiff base fragmentation product glycolaldehyde (AGE-3), the triose phosphate and the Amadori product fragmentation product methylglyoxal (AGE-4), the glucose autoxidation product glyoxal (AGE-5), and decomposition product of Amadori products and fructose-3-phosphate to 3-DG (AGE-6). CML, N-(carboxymethyl)lysine; CEL, N-(carboxyethyl)lysine; H_2N-P, free amino residue of protein. Abbreviations: AR, aldose reductase; SDH, sorbitol dehydrogenase; 3-PK, fructose-3-phosphokinase; ALR, aldehyde reductase; GO, glyoxalase; MOG, monooxygenase.

peptides present in serum from diabetic patients on hemodialysis. Based on these data, we proposed a pathway for the formation of distinct AGEs by the Maillard reaction, sugar auto-oxidation, and sugar metabolic pathways in vivo, as shown in Figure 1.

We have recently shown that an elevation of serum AGE levels was found to be associated with severity of diabetic retinopathy (21, 22). We have also shown that AGEs in the serum of diabetic patients on hemodialysis have diverse biological activities on vascular wall, kidney mesangial, and cortical neuronal cells (23–25).

To better understand the role of AA in the modification of proteins in alcoholism, we developed a new antibody to AA-derived AGEs (AA-AGEs) that could recognize serum proteins modified by AA. Based on our previous studies (17–20), we proposed a pathway for the formation of AA-AGEs by the Maillard reaction in vivo, as shown in Figure 2. The specific anti-AA-AGE antibody has also enabled us to identify immunochemically cross-reactive compounds that exist in vivo. In the present study, we provide the first evidence for the specific neurotoxicity of a particular AA-AGE epitope in primary cultured cortical neuronal cells.

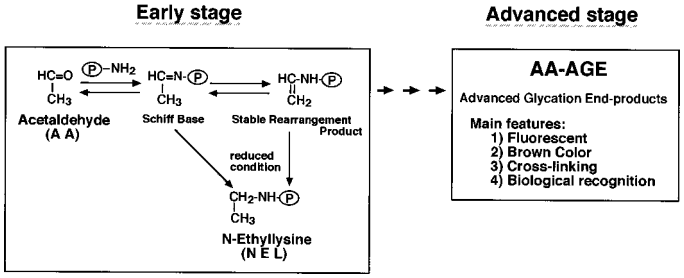

Fig. 2. Possible route of acetaldehyde-derived AGEs (AA-AGE) formation by Maillard reaction.

MATERIALS AND METHODS

Materials

Acetaldehyde, RSA (A-0764), BSA (A-0281), sodium cyanoborohydride, glyoxal, methylglyoxal, cytosine-β-D-arabinofuranoside (AraC), and polyethylenimine were purchased from Sigma-Aldrich Co., Inc. (St. Louis, MO). DL-glyceraldehyde and glycolaldehyde were purchased from Nakalai Tesque (Kyoto, Japan). Freund's complete adjuvant, D-glucose, 3-deoxyglucosone, glyoxylic acid, pyruvic acid, Zeta-Pore filter, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and diethylenetriamine-pentaacetic acid (DTPA) were

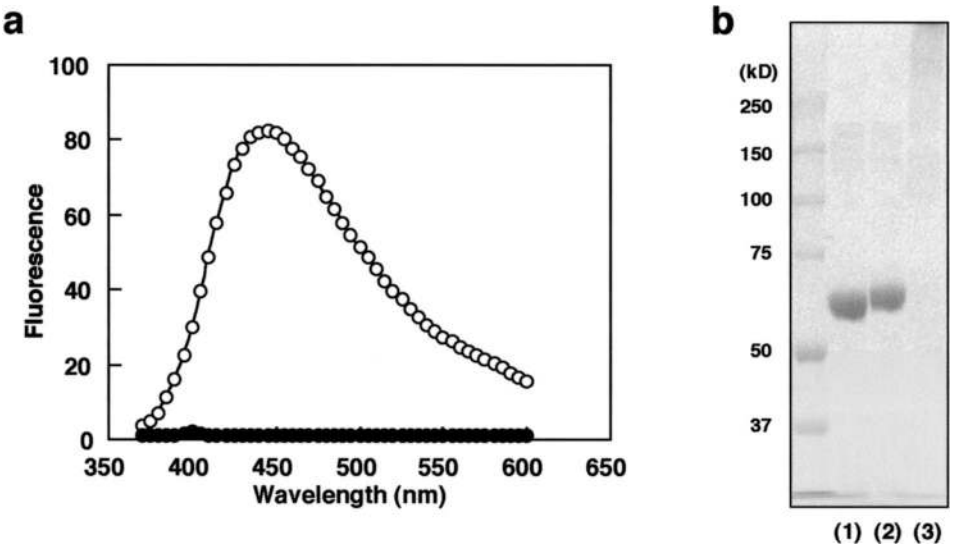

Fig. 3. Characteristics of rabbit serum albumin incubated with AA (AA-AGE-RSA). **a:** Fluorescence emission spectra of 0.5 mg/ml AA-AGE-RSA (○) and control RSA (●) at 360 nm. **b:** SDS-PAGE of AA-AGE-RSA. 2.5 μg of control RSA (lane 1), NEL-RSA (lane 2), and AA-AGE-RSA (lane 3) were loaded on polyacrylamide gel, stained with Coomassie brilliant blue. Size markers (kD) are shown on the left.

purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). PD-10 column and CNBr-activated Sepharose 4B were purchased from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). Dc protein assay reagent was purchased from Bio-Rad Lab. (Richmond, CA). Sheep anti-rabbit IgG alkaline phosphatase conjugated secondary antibody was purchased from Chemicon International (Temecula, CA). Phosphatase substrate kit was purchased from Pierce (Rockford, IL). 5-Bromo-4-chloro-3-indoyl phosphate-nitroblue tetrazolium (BCIP/NBT) was purchased from Kirkegaard & Perry Lab. (Gaithersburg, MD).

Preparation of Various AGE Proteins, N-ethyllysine (NEL), N-carboxymethyllysine (CML), and N-carboxyethyllysine (CEL) Protein

AA-AGE bovine serum albumin (BSA) and AA-AGE-rabbit serum albumin (RSA) were prepared as described previously (17–19). BSA or RSA at 25 mg/ml was incubated under sterile conditions with 0.1 M acetaldehyde and 5 mM diethylenetriamine-pentaacetic acid (DTPA) in 0.2 M phosphate buffer (pH 7.4) at 37°C for 7 days. Low molecular weight reactants and AA were removed using a PD-10 column chromatography and dialysis against PBS. Various AGE-BSAs were prepared as described previously (17–19). Briefly, each protein was incubated under sterile conditions with glucose (AGE-1), glyceraldehyde (AGE-2), glycolaldehyde (AGE-3), methylglyoxal (AGE-4), glyoxal (AGE-5), or 3-deoxyglucosone (AGE-6) and 5 mM diethylenetriamine-pentaacetic acid (DTPA) in 0.2 M phosphate buffer (pH 7.4) at 37°C for 7 days, except in the case of incubation with 3-deoxyglucosone (2 weeks) or glucose (8 weeks). Low-molecular weight reactants and aldehydes were removed using PD-10 column chromatography and dialysis against PBS. N-ethyllysine (NEL)-BSA and NEL-RSA were prepared as described elsewhere (26). Briefly, 50 mg/ml of BSA or RSA was incubated at 37°C for 24 h with 50 mM acetaldehyde and 150 mM sodium cyanoborohydride ($NaCNBH_3$) in 2 ml of 0.2 M phosphate buffer (pH 7.4), followed by PD-10 column chromatography and dialysis against PBS. N-Carboxymethyllysine (CML)-BSA and N-carboxyethyllysine (CEL)-BSA were prepared as described (27, 28). Briefly, 50 mg/ml of BSA was incubated at 37°C for 24 h with 50 mM glyoxylic acid or pyruvic acid and 150 mM $NaCNBH_3$ in 2 ml of 0.2 M phosphate buffer (pH 7.4), followed by PD-10 column chromatography and dialysis against PBS. All preparations were passed through Zeta-Pore filter (Cuno Co., Tokyo, Japan) to remove endotoxin. Protein concentrations were determined with the Dc protein assay reagent using BSA as a standard.

Preparation of Polyclonal Anti-AA-AGE and NEL Antibodies

Four milligrams of AA-AGE-RSA or NEL-RSA was emulsified in 50% Freund's complete adjuvant and injected intradermally into rabbits. This procedure was repeated at weekly intervals for 6 weeks. After a 2-week pause, the rabbits were given a booster injection of 4 mg of each antigen. Animals were bled on the tenth day after this injection and serum was obtained for purification.

Purification of Antibody to AA-AGE from AA-AGE Antiserum by Affinity Chromatography

Antibody specific for AA-AGE was isolated from rabbit antiserum by affinity chromatography. AA-AGE-BSA or NEL-BSA (125 mg of each protein) was coupled to 25 ml of CNBr-activated Sepharose 4B according to the manufacturer's instructions. Twenty-five ml of rabbit anti-AA-AGE-serum was applied to a column (2.5 × 5.5 cm) of Sepharose 4B coupled with AA-AGE-BSA. After extensive washing with PBS, the adsorbed fractions were eluted with 20 mM sodium phosphate

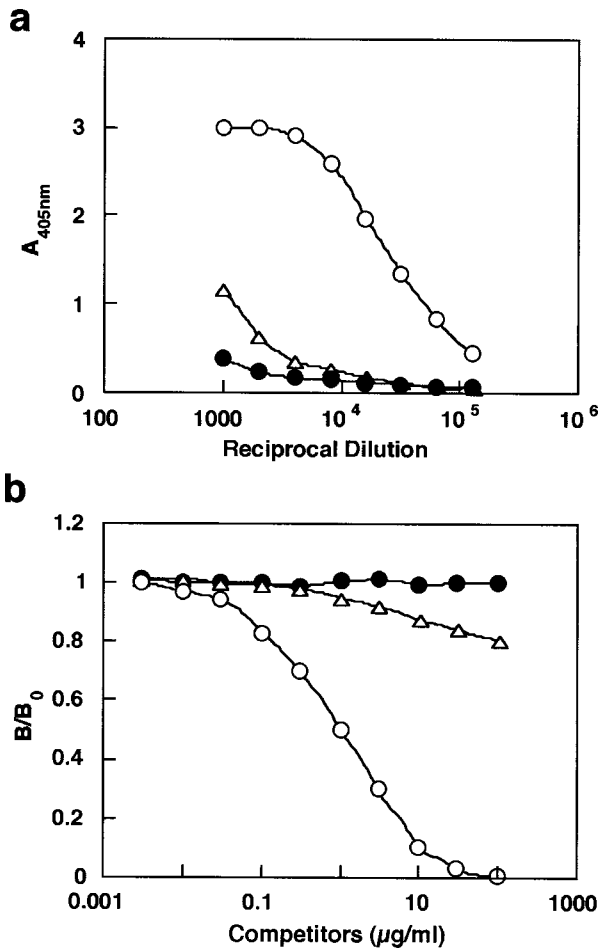

Fig. 4. Immunoreactivity of AA-AGE antiserum. The immunoreactivity of AA-AGE antiserum with AA-AGE-BSA (○), NEL-BSA (△), and control BSA (●) was determined at various concentrations by competitive ELISA. **a:** Noncompetitive ELISA employing bound ligand. **b:** Competitive ELISA employing bound ligand, AA-AGE-BSA, NEL-BSA, or control BSA as competitors.

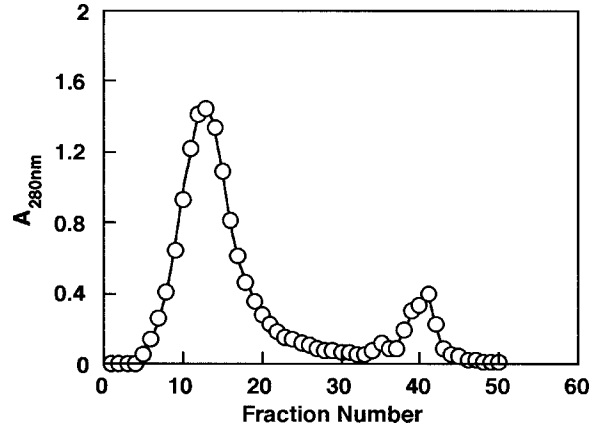

Fig. 5. Separation of AA-AGE antibody from AA-AGE antiserum by NEL-BSA affinity chromatography.

buffer containing 1 M potassium thiocyanate (pH 7.4). The AA-AGE antibody fractions were pooled, concentrated using Centriprep-10, and passed through a PD-10 column equilibrated with PBS. The AA-AGE antibody thus obtained then was loaded onto a column (1.5 × 5.5 cm) of Sepharose 4B coupled with NEL-BSA, which was washed with 30 ml of PBS to obtain the unadsorbed fraction (AA-AGE antibody). The adsorbed fraction (NEL antibody) was eluted with 20 ml of 20 mM sodium phosphate buffer containing 1 M potassium thiocyanate (pH 7.4). Fractions (1.0 ml) were monitored for absorbance at 280 nm. The unadsorbed fractions were pooled, concentrated with Centriprep-10, and passed through a PD-10 column equilibrated with PBS for use in this study.

NEL-specific antibody was isolated from the anti-NEL-serum by NEL-BSA affinity chromatography. Briefly, 25 ml of rabbit anti-NEL-serum was applied to a column (2.5 × 5.5 cm) of Sepharose 4B coupled to NEL-BSA. After extensive washing with PBS, the adsorbed fractions were eluted with 20 mM sodium phosphate buffer containing 1 M potassium thiocyanate (pH 7.4). NEL antibody fractions were pooled, concentrated using Centriprep-10, and passed through a PD-10 column equilibrated with PBS.

Enzyme-Linked Immunosorbent Assay (ELISA)

Ligand inhibition and AA-AGE measurements were performed with a competitive ELISA, as described previously (17–19). Briefly, test samples (50 μl) were added to each well as a competitor for 50 μl of AA-AGE or NEL antibody (1:1,000–1:2,000), followed by incubation for 2 h at room temperature with gentle shaking on a horizontal rotary shaker. Results were expressed as B/B_0, which was calculated as follows: (experimental OD-background OD)/(total OD-background OD). The immunoreactivity of each fraction was read from the calibration curve for AA-AGE-BSA and was expressed as AA-AGE units (U) per ml, with 1 unit corresponding to the amount of antibody-reactive material found in AA-AGE-BSA at a protein concentration of 1 μg/ml.

Immunoblot Analysis

AGE-proteins, serum, and brain samples were subjected to electrophoresis on 7.5% SDS gel or 5–20% gradient SDS gel. The proteins then were transferred electrophoretically to PVDF membranes for 30 min at 2 mA per cm^2. Each membrane was blocked with 3% skim milk and 0.2% BSA in PBS for 1 h at room temperature, reacted for 1 h with immunoaffinity-purified AA-AGE or NEL antibody (1:500–1:1,000 diluted in 3% skim milk and 0.2% BSA in PBS), washed 3 times for 5 min each with PBS-Tween 20 buffer, incubated for 1 h in 3% skim milk and 0.2% BSA in PBS with 1:2,000 diluted anti-rabbit IgG coupled to alkaline phosphatase, washed 5 times for 5 min each with PBS-Tween 20, and finally incubated with 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium.

Primary Rat Cortical Cell Culture

Primary cortical neuronal cell cultures were prepared as described by Ogura et al (29). Briefly, embryos from pregnant Wistar rat were removed on embryonic day 18. The meninges and hippocampal cell layers were removed under a dissecting microscope and the cortical cells incubated with 0.25% trypsin

and 0.2% deoxyribonuclease I (DNase I) at 37°C for 20 min with shaking. The cells were triturated, washed 3 times with Dulbecco's modified Eagle's medium (DMEM), and seeded onto 0.25% polyethylenimine-coated 24-well culture plates (in a volume of 1 ml) at final density of 3.7×10^5 cells/ml. The cells were maintained for 48 h in DMEM supplemented with D-glucose, L-glutamine, sodium hydrogen carbonate, and kanamycin sulfate plus 10% pre-colostrum fetal calf serum in a humidified incubator at 37°C (5% CO_2 atmosphere). Non-neuronal cell growth was inhibited by the addition of 10 μM cytosine-β-D-arabinofuranoside (AraC) at 37°C for 24 h; after which time the cells were washed twice with DMEM. After 24 h in culture, 0.25 to 2.0 mg/ml of control BSA, AA-AGE-BSA, or NEL-BSA was added to medium and the cultures incubated for 72 h. Preparations were tested for endotoxin using the Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan); no endotoxin was detectable.

Neuronal Toxicity Assay

A tetrazolium salt has been used to develop a quantitative colorimetric assay for mammalian cell survival and proliferation. The assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells. This method can therefore be used to measure cytotoxicity, proliferation or activation (30). For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, MTT was dissolved in Ca^{2+} and Mg^{2+} free phosphate buffered saline (CMF-PBS) at 5 mg/ml. Five hundred μl of fresh medium was added after the cells were washed 3 times with CMF-PBS, and then 50 μl of MTT solution was added to the medium in each well of triplicate cultures. The wells then were incubated at 37°C for 2 h. Five hundred μl of acid-isopropanol (0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue formazan crystals. The absorbance was read at a wavelength of 570 nm using a Microplate reader (Model 550, BIO-RAD).

Detection of Apoptotic Cell Death

Neuronal cells cultured on 25-mm coverslips were washed twice with CMF-PBS and fixed with 0.1% glutaraldehyde in CMF-PBS at room temperature for 1 h. After fixation, the coverslips were washed and exposed to 100 μg/ml Hoechst 33258 in CMF-PBS at room temperature for 5 min. After washing, the neurons were observed and photographed in an Olympus IX70 microscope using a U filter (Ex = 330–385 nm/Em = 420 nm).

Statistical Analysis

The data were analyzed by ANOVA/Scheffe test. All data were shown as the mean ± SE. Statistical significance was considered at both the $p < 0.05$ (*) and $p < 0.01$ (**) levels.

Postmortem Brain Samples and Tissue Preparation

Postmortem brains were obtained from the Austro-German Brain Bank at the department of Psychiatry, University of Wuerzburg. The procedure used, acquisition, clinical diagnosis, dissection, storage, and distribution of brain material in the Brain Bank was previously described in detail (31). Postmortem brain tissue samples were obtained from 2 patients with alcohol dependence and 2 controls that were matched with respect to postmortem delay time and were confirmed to be free of both psychiatric disease and other substance abuse according to their records. Diagnosis for patients with alcohol dependence was established using Diagnostic and Statistical Manual of Mental Disorders, third edition (DSM-3) criteria. Whole cell homogenates for immunoblot analysis were prepared from postmortem orbitofrontal cortex.

RESULTS

Characterization of the Immunogen

The formation of AGE by rabbit serum albumin (AA-AGE-RSA) during incubation with AA for 7 days was monitored by AGE-specific fluorescence. Fluorescence was only associated with the AA-AGE-RSA complex, and was not seen in control RSA after 7 days of incubation without AA (Fig. 3a) and NEL-RSA (data not shown). The emission maximum of AA-AGE-RSA was 445 nm, in close agreement with the values for other AGE-RSAs (17–19). SDS-PAGE under reducing conditions revealed that control RSA (incubated without AA) migrated at 67 kD. NEL-RSA (incubated with 50 mM AA and 150 mM $NaCNBH_3$ for 24 h) migrated more slowly than control RSA, which is consistent with the previously reported loss of anionic character that occurs with protein glycation. AA-AGE-RSA (incubated with 0.1 M AA for 7 days) was visible only at the top of the gel, suggesting that extensively cross-linked adducts of RSA had been formed nonenzymatically during incubation (Fig. 3b).

Characterization of AA-AGE Antiserum

We prepared AA-AGE antiserum from rabbit immunized with AA-AGE-RSA (7 days of incubation with AA). This antiserum was applied to an affinity column coupled with AA-AGE-BSA to obtain a purified anti-AA-AGE antibody. Figure 4 shows the cross-reactivity for AA-AGE-BSA, NEL-BSA, and control BSA of the polyclonal antibody obtained from rabbit immunized with AA-AGE-RSA. The antibody reacted with AA-AGE-BSA, whereas no reaction was observed with control BSA that had been incubated without AA. In cross-reactivity studies, this antibody also showed a weak reaction with NEL-BSA. It therefore appeared likely that the polyclonal antiserum not only contained an antibody specific for AA-AGE, but also an antibody for NEL.

Separation of AA-AGE Antibody from Anti-AA-AGE Serum

The AA-AGE antiserum obtained after incubation of AA-AGE-RSA with AA was purified by AA-AGE-BSA affinity chromatography, and then was separated by NEL-BSA affinity chromatography (Fig. 5). We calculated the percentage of antibodies bound to the NEL affinity gel (eluted as the second peak) relative to that of antibody

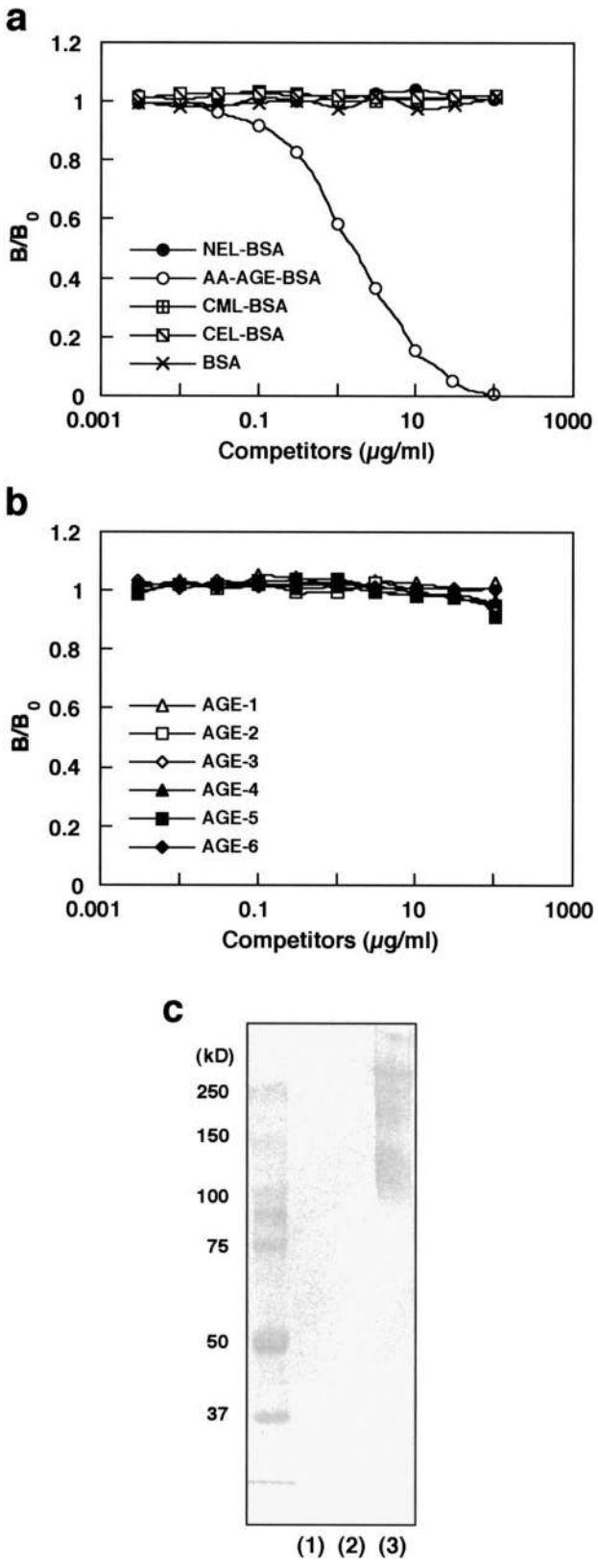

Fig. 6. Immunoreactivity of affinity-purified AA-AGE antibody with various AGE preparations. **a, b:** Characterization of the AA-AGE antibody using a competitive ELISA with several AGE-modified proteins. **c:** Immunoblot analysis of AA-AGE antibody. Aliquots (0.5 μg) of control BSA (lane 1), NEL-BSA (lane 2), and AA-AGE-BSA (lane 3) were loaded on polyacrylamide gel. Formation of AA-AGE in the protein was determined by immunoblot analysis using AA-AGE antibody. Size markers (kD) are shown on the left.

unbound to the affinity gel (eluted as the first peak). Unbound AA-AGE antibody (passed fraction) accounted for 90% of the total obtained. The NEL antiserum obtained after incubation of NEL-RSA with AA and $NaCNBH_3$ was purified by NEL-BSA affinity chromatography (data not shown).

Characterization of AA-AGE Antibody

We characterized the AA-AGE antibody obtained by NEL-BSA affinity chromatography by a competitive ELISA with several AGE-modified proteins. First, we investigated whether AA-AGE antibody purified by NEL-BSA affinity chromatography could react with NEL-BSA, CML-BSA and CEL-BSA. These compounds did not inhibit the binding of AA-AGE antibody to AA-AGE-BSA, even when added in high concentrations (Fig. 6a). To determine which AGEs were recognized by the AA-AGE antibody, BSA was incubated with glucose (AGE-1), glyceraldehyde (AGE-2), glycolaldehyde (AGE-3), methylglyoxal (AGE-4), glyoxal (AGE-5), and 3-deoxyglucosone (AGE-6), and the products tested for reactivity in a competitive ELISA. As shown in Figure 6a and b, only AA-AGE specifically inhibited the binding of anti-AA-AGE antibody. Immunoblot analysis with immunoaffinity-purified AA-AGE antibody confirmed the results obtained by ELISA (Fig. 6c). These findings, taken together, indicate that AA-AGE-BSA had the highest reactivity for AA-AGE antibody. By similar methodology the immunoaffinity-purified NEL antibody was found to cross-react specifically with NEL-BSA, but not with CML-BSA, CEL-BSA, or other types of AGEs (data not shown).

In preliminary studies we have observed AA-AGE and NEL structures were present in human serum by ELISA and immunoblot analysis with the immunoaffinity-purified AA-AGE and NEL antibodies (data not shown).

AA-AGEs Induce Neuronal Cell Death in Cultured Cortical Neurons

Cortical neuronal cells were incubated for 72 h with AA-AGE or NEL conjugated to BSA as a carrier protein. Cell viability then was assessed with MTT reduction. MTT, a tetrazolium salt, has been used to develop a quantitative colorimetric assay for mammalian cell survival and proliferation. Neurotoxic effects were determined for the AA-AGE-BSA, NEL-BSA, and control BSA (Fig. 7). Incubation of cortical neurons with AA-AGE-BSA, but not NEL-BSA or control BSA, induced significant cell

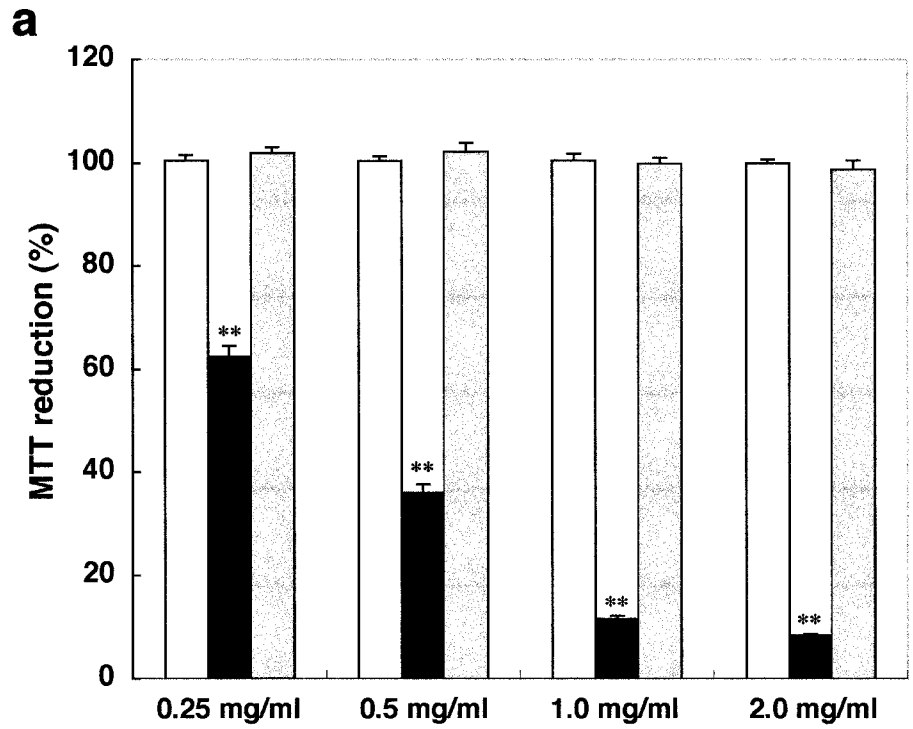

Fig. 7. AA-AGE-induced neuronal cell death in cultured cortical neurons. **a:** Cortical neuronal cells were incubated with control BSA (open bar), AA-AGE-BSA (filled bars), and NEL-BSA (gray bars) in concentrations between 0.25 and 2.0 mg/ml. Cell viability was determined after 72 h by the MTT assay. Data are expressed as mean ± SE (n = 15; five separate experiments, each in triplicate). **b–i:** Neuronal cells were exposed to 0.25, 0.5, 1.0, and 2.0 mg/ml NEL-BSA **(b–e)** or AA-AGE-BSA **(f–i)**. Cultures were photographed using a differential-phase microscope with a ×300 objective.

death (Fig. 7a). AA-AGE caused significant cytotoxicity in a concentration-dependent manner. The effects of AA-AGE-BSA and NEL-BSA on neuronal survival were also assessed by examining cell morphology. Incubation of cortical neurons with 0.25 to 2.0 mg/ml of AA-AGE-BSA for 72 h resulted in cell body shrinkage and in a decrease in the number and length of neurites (Fig. 7f–i), but not NEL-BSA (Fig. 7b–e) or control BSA (data not shown).

AA-AGE also induced apoptotic neuronal cell death associated with nuclear condensation and cell body shrinkage (chromatin condensation in cells stained with the Hoechst 33258) (data not shown).

AA-AGE-Induced Neuronal Cell Death is Neutralized by an Anti-AA-AGE-Specific Antibody

The anti-AA-AGE-specific antibody then was tested for its ability to protect the cells against AA-AGE-induced cell death. The neurotoxicity of AA-AGE-BSA was prevented completely by the addition of anti-AA-AGE-specific antibody, but not by NEL-specific antibody (Fig. 8a), or by other anti-AGE-specific antibodies (AGE-Ab-1 to -6) (data not shown), or nonimmune rabbit IgG (data not shown). With respect to cell morphology, the addition of AA-AGE-BSA led to cell body shrinkage and neurite destruction (Fig. 8b, c). These changes were prevented by the addition of anti-AA-AGE antibody (Fig. 8e), but not by NEL-specific antibody (Fig. 8d) or other anti-AGE-specific antibodies (AGE-Ab-1 to -6) (data not shown).

The apoptotic neuronal cell death associated with nuclear condensation and cell body shrinkage (chromatin condensation in cells stained with the Hoechst 33258) was also completely prevented by the addition of anti-AA-AGE-specific antibody (data not shown).

Immunoblot Analysis of AA-AGE in Brain Tissue from Alcoholism

Immunoblot analysis of whole brain homogenates from postmortem orbitofrontal cortex by using affinity-purified AA-AGE antibody showed the presence of AA-AGE in human brain of alcoholism. The alcoholism brain homogenates, but not control brain, revealed AA-AGE immunoreactivity with an apparent molecular weight of 185 kD (Fig. 9b). The same band shown in human brain homogenates with anti-NEL antibody (data not shown).

DISCUSSION

Alcohol is metabolized to AA, a 2-carbon carbonyl compound that can react with nucleophiles to form covalent addition products. Bucala et al have identified a

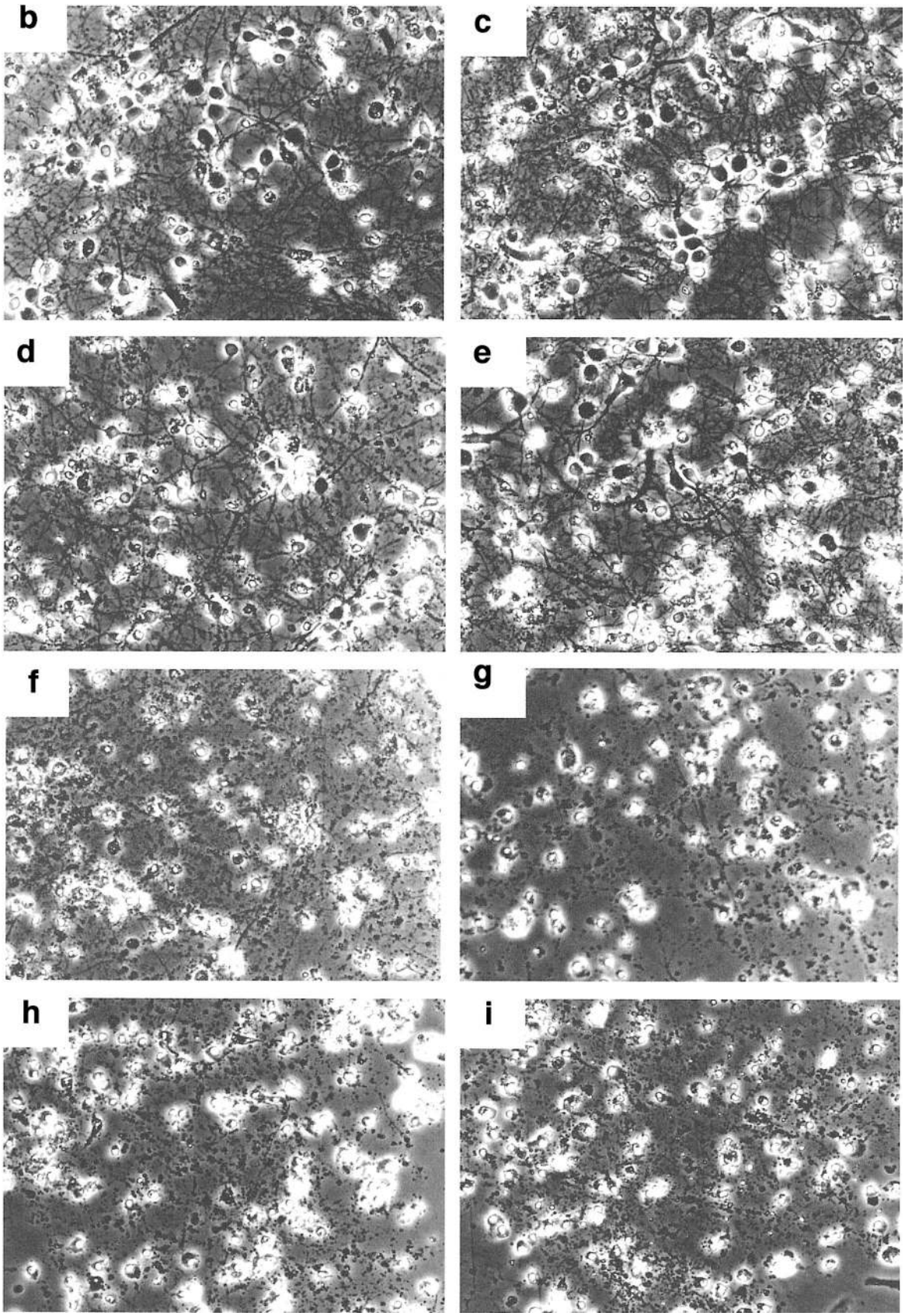

Fig. 7. Continued.

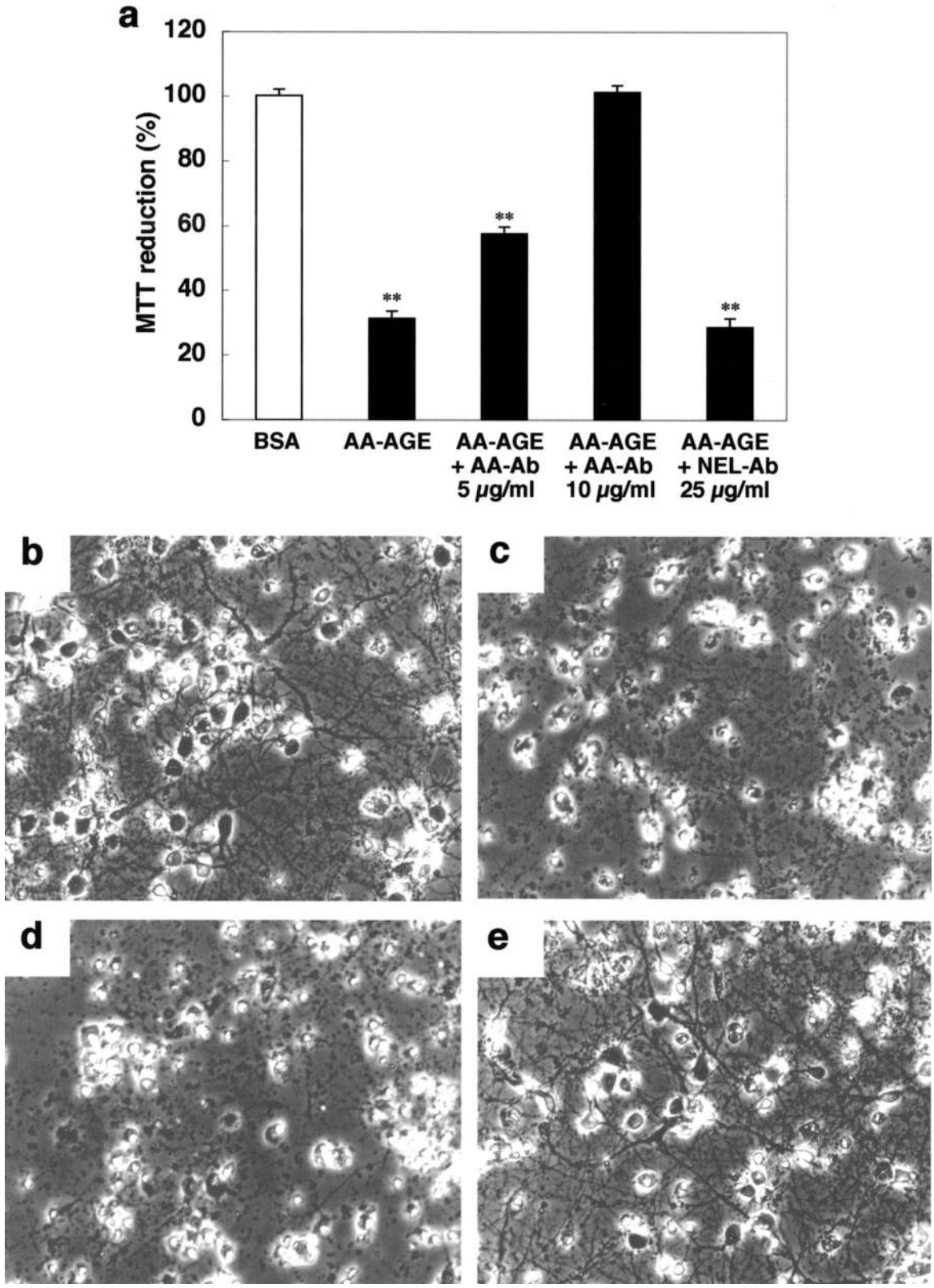

Fig. 8. AA-AGE-induced neuronal cell death is neutralized by the AA-AGE-specific antibody. Cortical neuronal cells were incubated with control BSA and AA-AGE-BSA at a concentration of 0.6 mg/ml. Cell viability was determined after 72 h by the MTT assay. **a:** In neutralization experiments with AA-AGE antibody, AA-AGE-BSA was preincubated with an AA-AGE (5–10 μg/ml) or NEL-specific antibody (25 μg/ml) at room temperature for 1 h before addition to the culture medium. Data are expressed as mean ± SE (n = 12; four separate experiments, each in triplicate). **b–e:** Cells were exposed to 0.6 mg/ml control BSA **(b)**, 0.6 mg/ml AA-AGE-BSA **(c)**, 0.6 mg/ml AA-AGE-BSA plus NEL antibody **(d)**, or 0.6 mg/ml AA-AGE-BSA plus AA-AGE antibody **(e)** for 72 h. Cultures were photographed using a differential-phase microscope with a ×300 objective.

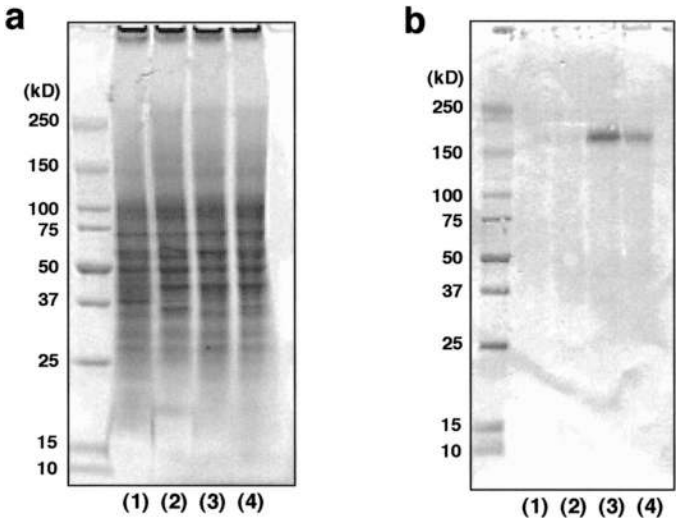

Fig. 9. Immunoblot analysis of brain homogenate from alcoholics with affinity-purified AA-AGE antibody. Fifty μg of human brain homogenate was treated with 1% SDS in the absence of 2-mercaptoethanol and the sample was loaded onto 5%–20% gradient polyacrylamide gel. **a:** Detection of brain protein bands was performed with Coomassie brilliant blue staining. **b:** Formation of AA-AGE was determined by immunoblot analysis using affinity-purified AA-AGE antibody. Aliquots (50 μg) of 2 control brains (lanes 1, 2) and alcoholic brains (lanes 3, 4) were loaded on 5%–20% gradient gel. Size markers (kD) are shown on the left.

biochemical modification produced by the reaction of AA with protein-bound Amadori products (aceraldehyde-amadori product) (32). Amadori products typically arise from the nonenzymatic addition of reducing sugars (such as glucose) to protein amino groups and are the precursors to irreversibly bound, cross-linking moieties called advanced glycation end products (AGEs). AGEs accumulate over time on plasma lipoproteins and vascular wall components, and play an important role in the development of diabetes- and age-related cardiovascular disease.

We recently reported that an elevation of serum AGE levels was found to be associated with severity of diabetic retinopathy (21, 22). We also reported that AGEs in the serum of diabetic patients on hemodialysis have diverse biological activities on vascular wall cells and cortical neurons (23, 25). Therefore, these results suggest a causal role for these types of AGEs in the pathogenesis of diabetic complication and neurodegenerative disease.

Based on our previous studies (17–20), we proposed a pathway for the formation of AA-AGE by the Maillard reaction in vivo, as shown in Figure 2. We hypothesize that AA may be involved in alcohol-induced neurotoxicity in vivo by formation of AA-AGE with human brain. In the present study, we provide new data on the pathway of AGE formation from AA and methods for the immunochemical detection of AA-AGE (Figs. 6, 9). We also provide evidence for a direct toxic effect of AA-AGE, but not the reduced AA-protein adduct (N-ethyllysine: NEL) on rat primary cultured cortical neuronal cells (Figs. 7, 8).

Alcohol is metabolized to AA in the cell, which is potentially deleterious because it can react with cellular proteins and form an AA-protein adduct that can interfere with normal cellular function. NEL is a reduced form of AA-protein adduct and it has previously been detected in the livers of patients with alcoholic liver disease and experimental animals fed alcohol (2, 33). NEL has been hypothesized to mediate alcohol-induced damage in brain. Indeed, recent reports have demonstrated by immunohistochemistry and immunoblot analysis using an anti-NEL antibody the presence of NEL in the brains of experimental animals fed alcohol (34–36). After lifelong ethanol consumption such reactions were found in the white matter, large neurons in the deep layers of the frontal cortex, and in the molecular layer of the cerebellum, particularly in a rat strain that usually shows high concentrations of AA during ethanol oxidation.

Although a role for AA as a possible mediator of ethanol-induced neurotoxicity has also been previously suggested, the formation of AA-AGE in human brain has not been examined. In the present study, we found that incubation of cortical neurons with AA-AGE produced a dose-dependent increase in neuronal cell-death, and the neurotoxicity of AA-AGE was neutralized by the addition of an anti-AA-AGE-specific antibody, but not by anti-NEL antibody (Figs. 7, 8). We also found the AA-AGE and NEL epitopes were detected in brains of alcoholic persons. The alcoholic brain homogenates revealed immunoreactivity with an apparent molecular weight of 185 kD (Fig. 9). Upadhya et al reported that chronic ethanol-treated rat brain immunostained with anti-NEL antibody showed bands of approximate molecular mass 188, 107, 60, and 50 kD (36). Our results indicate that the AA-AGE structure is likely to play an important role in the neuropathological processes associated with alcoholism.

In summary, we provide the first evidence for the toxicity of a specific, alcohol-derived AGE structure, defined as AA-AGE, on rat primary cultured cortical neuronal cells. Studies are presently in progress to elucidate the precise chemical structure of AA-AGE and the molecular mechanism of its cytopathological action.

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