Association of End-Product Adducts with Increased IgE Binding of Roasted Peanuts

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Recently, we have shown that roasted peanuts have a higher level of IgE binding (i.e., potentially more allergenic) than raw peanuts. We hypothesized that this increase in IgE binding of roasted peanuts is due to an increased levels of protein-bound end products or adducts such as advanced glycation end products (AGE), *N*-(carboxymethyl)lysine (CML), malondialdehyde (MDA), and 4-hydroxynonenal (HNE). To support our hypothesis, we produced polyclonal antibodies (IgG) to each of these adducts, determined their levels in raw and roasted peanuts, and examined their ability to bind to IgE from a pooled serum of patients with clinically important peanut allergy. Results showed that AGE, CML, MDA, and HNE adducts were all present in raw and roasted peanuts. Roasted peanuts exhibited a higher level of AGE and MDA adducts than raw peanuts. IgE was partially inhibited in a competitive ELISA by antibodies to AGE but not by antibodies to CML, MDA, or HNE. This indicates that IgE has an affinity for peanut AGE adducts. Roasted peanuts exhibited a higher level of IgE binding, which was correlated with a higher level of AGE adducts. We concluded that there is an association between AGE adducts and increased IgE binding (i.e., allergenicity) of roasted peanuts.

Keywords: *Peanuts (Arachis hypogaea L.); allergenicity; IgE; ELISA; polyclonal antibodies; Maillard reaction adducts; lipid oxidation adducts; AGE; CML; MDA; HNE*

INTRODUCTION

Peanut components such as proteins, sugars, and fatty acids may react, particularly during roasting, to give different kinds of end-product adducts (i.e., products covalently linked to the lysine residues of proteins). These include advanced glycation end products (AGE), N^{ϵ}-(carboxymethyl)lysine (CML), malondialdehyde (MDA), and 4-hydroxynonenal (HNE). AGE and CML adducts are formed as a result of protein−sugar reactions (i.e., Maillard reaction) (*1*, *2*) and/or protein−lipid reactions (*3*). MDA and HNE adducts are produced due to lipid oxidation and cross-linking with proteins (*4*, *5*). These adducts were chosen because of their association with foods, allergy, and diseases. For instance, adducts such as AGE or CML are involved with milk products (*1*, *6*), nut allergy (*7*), milk allergy (*8*, *9*), and numerous diseases such as diabetes (*10*) and aging (*11*). Proteins carrying these adducts have a substantial affinity for transition metals such as iron and copper (*12*−*14*), which may lead to lipid oxidation and consequently the formation of a number of highly reactive aldehyde products such as HNE and/or MDA in foods (*4*, *5*, *15*, *16*).

In peanuts, AGE, CML, MDA, and/or HNE adducts are likely to occur during roasting. This is because when roasted, peanuts generate glycated proteins (containing AGE adducts) due to heat-induced protein−sugar reactions. Heating also enhances lipid oxidation. As a result, HNE and MDA adducts are formed. Also, when cured, peanuts undergo water stress (*17*, *18*). This in turn results in the production of MDA and/or HNE adducts (*19*). All of these adducts are known to render proteins more resistant to proteolytic degradation (*20*−*22*). Such proteins that are stable to digestion tend also to be the major allergens of foods (*23*). Like allergens, adducts are capable of stimulating T-cell and antibody responses (*24*−*28*). For this reason, adducts were thought to have a potential role in food allergy.

It is estimated that 1.6 million Americans, including 1 in 100 preschool-age children, are allergic to peanuts (*29*). While deaths from food allergies are rare, experts think peanut allergies are on the rise. To date, several major peanut allergens including Ara h 1, Ara h 2, and Ara h 3 have been identified (*30*). However, little research is done on the above end-product adducts as to their role in peanut allergenicity. Previously, we have shown that end-product adducts produced by a peanut lectin−glucose model system are capable of binding IgE antibodies (*31*). In addition, we showed that roasted peanuts have a higher level of IgE-binding than raw peanuts (*31*, *32*). We postulated that the increase in IgE-binding of roasted peanuts is due to an increase in end-product adducts. To support this hypothesis, we prepared polyclonal antibodies (IgG) to each adduct (i.e., AGE, CML, MDA, and HNE) and used these antibodies to determine the levels of adducts in raw and roasted peanuts, and the affinity of IgE antibodies for the adducts.

MATERIALS AND METHODS

Apparatus. Xcell II Mini-Cell and Blot Module were purchased from Novex (San Diego, CA). CERES 900C microtiter plate reader was purchased from Bio-Tek Instruments, Inc. (Winooski, VT).

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Reagents and Materials. Precast 4−20% Tris-glycine gels and SDS running buffer were purchased from Novex (San Diego, CA). Prestained low and high molecular standards, Coomassie Brilliant Blue R-250, and alkaline phosphatase conjugate substrate kit were purchased from BioRad Laboratories (Hercules, CA). Immobilon-P transfer membrane was purchased from Millipore Corp. (Bedford, MA). Anti-rabbit IgG alkaline phosphatase conjugate, *ρ*-nitrophenyl phosphate, rabbit anti-human IgE-peroxidase conjugate, *o*-phenylenediamine, bovine serum albumin (BSA), Tween 20, ethylene glycol-bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and phosphate buffer saline (PBS) were purchased from Sigma Co. (St. Louis, MO). HNE was purchased from Cayman Chemical (Ann Arbor, MI). Normal human serum and serum from a pool of three patients with peanut anaphylaxis were obtained from the University of Arkansas, Children's Hospital (Little Rock, AR). Superblock blocking buffer and bicinchoninic acid (BCA)-protein assay kit were purchased from Pierce Chemical Co. (Rockford, IL). Raw and roasted peanut seeds (NC 9) were provided by Dr. Tim Sanders, USDA-ARS, Food Science Department, North Carolina State University, Raleigh, NC.

Preparation of Immunogens for Production of Antibodies to End-Product Adducts. Four immunogens, namely, AGE-, CML-, MDA-, and HNE-BSA conjugates, were prepared, respectively. AGE-BSA was prepared with a modification of the method of Chung and Champagne (*31*). Briefly, BSA (10 mg/mL) was incubated at 37 °C with glucose (300 mM) in 0.3 M phosphate buffer, pH 7.8, 0.04% sodium azide for 90 days and dialyzed. CML-BSA was prepared according to the method of Reddy et al. (*33*). Briefly, BSA (175 mg) was incubated with glycoxylic acid (0.15 M) and $NaBH_3CN$ (0.45 M) in 1 mL of 0.2 M phosphate buffer, pH 7.8, for 24 h at 37 °C and dialyzed. MDA-BSA was prepared according to the method of Palinski et al. (*34*). Prior to conjugation, MDA (0.5 M) was prepared by incubating malonaldehyde bis dimethylacetal (88 *μ*L) with 0.1 N HCL (400 *μ*L) at 37 °C for 10 min, followed by adjusting the pH to 7.4 with 1 N NaOH and bringing the volume to 1 mL with deionized water. MDA-BSA was then prepared by incubating BSA (10 mg) with 0.5 M MDA (1 mL) for 3 h at 37 °C and dialyzing. HNE-BSA was prepared according to the method of Cohn et al. (*22*). Briefly, BSA (2.5 mg/mL) was incubated with 5 mM HNE, 100 mM $NaCNBH_3$ in 0.03 M phosphate buffer, pH 7.8, at 37 °C for 24 h and dialyzed. The resultant conjugates were then dialyzed 4 times against PBS. Concentration of proteins in each of the dialyzed samples was determined, using the BCA protein assay. Conjugates of ovalbumin-end product were prepared in the same way for use as the coating antigens or inhibitors in ELISA.

Production of Antibodies (IgG) Against End Products. Antibodies against AGE or MDA were produced by Covance Research Products Inc. (Richmond, CA). Antibodies against HNE and CML were respectively produced by Bethyl Laboratories (Montgomery, TX) and Strategic Biosolutions (Ramona, CA). Two rabbits were used in each case. Each was injected with the above end product-BSA conjugate. Injection and boosters were performed according to the company's protocol (70−90 days). Doses for initial injection and booster (every two weeks) were 250 and 500 *μ*g, respectively. Bleeds were collected 10 days after each booster and centrifuged. The resultant sera and preimmune sera were analyzed in this laboratory for antibody titer and cross-reactivity.

Determination of Antibody (IgG) Titer and Crossreactivity. This was carried out in an enzyme-linked immunosorbent assay (ELISA). Briefly, a microtiterplate was coated with AGE-, CML-, MDA-, or HNE-ovalbumin conjugate (20, 1.2, 2.5, and 10 *μ*g/mL, respectively) by incubating the ovalbumin conjugate (100 *μ*L; diluted in 0.1 M sodium bicarbonate, pH 9.6) in the well for 90 min at 37 °C. This was followed by washing 4× with PBS/Tween (0.05%), blocking with Superblock solution, and washing again with the buffer. For control, ovalbumin was used. Antibody titer was then determined by incubation of the antiserum or preimmune serum at various dilutions [100 *μ*L; in PBS/Tween:Superblock (1:1), pH 7.4] in the wells of the coated plate at room temperature for 30 min, followed by washing, incubating with a secondary antibody [100 *μ*L; anti-rabbit IgG alkaline phosphatase conjugate (1:12 500)] for 15 min at room temperature, washing, and finally incubating with a substrate solution (100 *μ*L; *ρ*-nitrophenyl phosphate in 10% diethanolamine, pH 9.8, 0.5 mM $MgCl_2$; 1 mg/mL) for 30 min. The absorbance was then read at 405 nm, using the Ceres 900C BioTek microplate reader. Antibody titer was defined as the antiserum dilution that gave an absorbance value of 1.0 in 20 min. Maximum antibody titers were observed at weeks 6, 8, 8, and 10 for antibodies against AGE, CML, HNE, and MDA, respectively.

Cross-reactivity of the antibody with each of the adducts (i.e., ovalbumin conjugates) was determined in the same way as above except that the antiserum (50 *μ*L; at a dilution of 60% of the maximum titer) was incubated with the conjugate (50 *μ*L) for 10 min before being added to the plate. A control (ovalbumin) was also performed. Cross-reactivity was defined as B/B_o, where B is the absorbance value of a sample containing antibodies and a specific adduct, and B_o the absorbance value of a control containing antibodies only. A reduction in the B/B_o value is an indication of cross-reactivity of the antibody toward the adduct.

Preparation of Peanut Protein Extracts. Extracts were prepared from defatted meals of raw and roasted peanuts (*31*) by stirring 0.1 g of meals in 0.7 mL of 0.02 M sodium phosphate, pH 7.4, containing 10 mM EGTA for an hour at 4 °C, followed by centrifugation at 8500*g* for 10 min. The resultant supernatants (extracts) were used for immunoblot and ELISA assays. Concentration of protein in the extract was determined using the BCA assay.

ELISA Assay of End-Product Adducts in Raw and Roasted Peanuts. A direct ELISA was performed for each of the antibodies to end-product adducts. Briefly, a microtiter plate was coated with peanut proteins (presumably containing end-products) by incubating a diluted peanut extract (100 *μ*L; 20 *μ*g protein/mL of 0.1 M sodium bicarbonate, pH 9.6) in the well at 37 °C for 90 min. After coating, the plate was washed and blocked with SuperBlock as described above. A rabbit preimmune serum (control) or antiserum against AGE, CML, MDA, or HNE (1:250) (100 *μ*L) diluted in SuperBlock:PBS/Tween (1:1) was added and incubated at room temperature for 30 min. After washing, the secondary antibody conjugate and substrate solution were added as described above. Absorbance was read at 405 nm.

Immunoblot Assay of End-Product Adducts in Raw and Roasted Peanuts. SDS−polyacrylamide gel electrophoresis (PAGE) (4−20% gel in Tris-glycine) and Western blots were performed in the Xcell II Mini-Cell and Blot Module, respectively, according to the manufacturer's instruction. Briefly, 20 *μ*g of proteins from raw or roasted peanut extract was applied to each well of the gel. This was followed by transferring the proteins to an Immobilon-P membrane in the blot module. After blocking with SuperBlock, the membrane was incubated for 30 min with a diluted rabbit preimmune serum or antiserum, washed, and then incubated for another 15 min with an anti-rabbit IgG alkaline phosphatase conjugate (1:15 000). After washing and incubating the membrane in a substrate solution (a kit from BioRad Lab) containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT), purple bands developed. The appearance of band(s) on the membrane is indicative of the presence of end product adducts.

ELISA Assay of IgE Binding to Raw and Roasted Peanuts. IgE binding to peanuts was determined in a direct ELISA as described above. In this case, a pooled patient serum (IgE antibodies: 1:20) or a normal serum was used and incubated for 60 min in the plate coated with a peanut extract. A rabbit anti-human IgE peroxidase conjugate (1:1000) was used as the secondary antibody (30 min incubation). The substrate solution was *o*-phenylenediamine (0.5 mg/mL), 0.03% hydrogen peroxide in 0.1 M citrate buffer, pH 5.5. Color development was terminated after 30 min by adding 50 *μ*L of 4 N sulfuric acid. Absorbance was read at 490 nm.

Competitive ELISA Assay of IgE Binding to Adducts. In this assay, IgE binding to the adducts was indicated by an

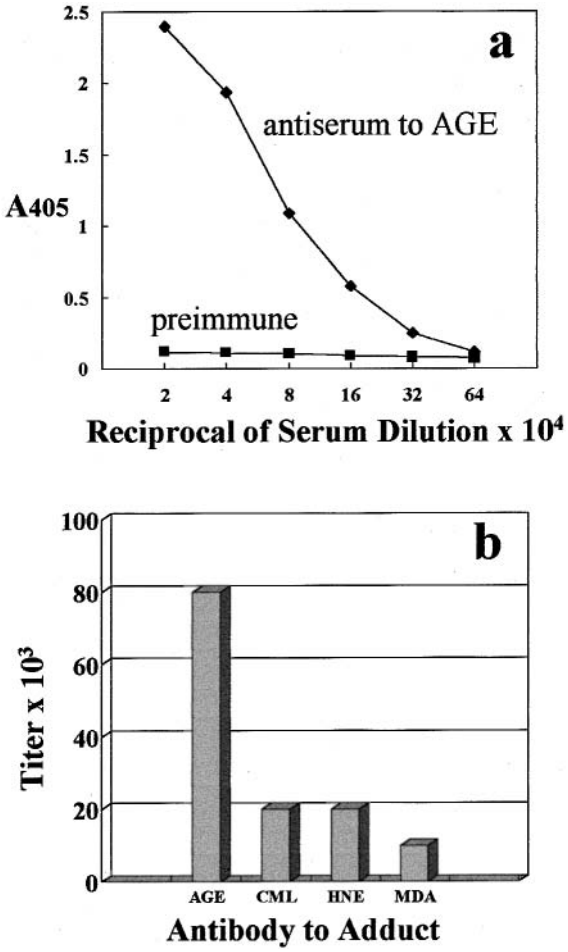

Figure 1. (a) Titration of antibody against AGE (at week 6th) in a plate coated with AGE-ovalbumin conjugate (20 μg/mL). Antibody was detected using an anti-rabbit IgG alkaline phosphatase conjugate (1:12 500) plus the substrate, ρ-nitrophenyl phosphate. (b) Maximum titers for antibodies against AGE, CML, HNE, and MDA at weeks 6, 8, 8, and 10, respectively.

inhibition of IgE by IgG antibodies. Briefly, plates were coated with raw or roasted peanut extracts and blocked as described above. A pooled patient serum (IgE) (1:50, final) was mixed in equal volume with a rabbit antiserum (IgG) or preimmune serum (control) at various serial dilutions (from 1:200 to 1:6400) The mixture (100 μL) was then added to the plate and incubated for 60 min. The plate was washed. A rabbit anti-human IgE peroxidase (1:1000), substrate and 4 N sulfuric acid were added separately as described above. Inhibition (%) of IgE by IgG was defined as $(1 - B/B_o) \times 100$, where B is the absorbance value of a sample containing IgE and antibodies to adducts, and B_o the absorbance value of a control containing IgE and preimmune serum.

RESULTS AND DISCUSSIONS

Titer and Cross-Reactivity of Polyclonal Antibodies to End-Product Adducts. Polyclonal antibodies against AGE, CML, MDA, and HNE adducts were generated in rabbits and checked for titer and cross-reactivity before use as a tool to detect adducts in peanuts. Typical titration curves for the antiserum against AGE and the preimmune serum are shown in Figure 1a. The significant difference in the titration curves of preimmune and antiserum indicates that antibodies against AGE were present in the antiserum. Also, antibodies against CML, MDA, and HNE were successfully produced (data not shown). Antibodies

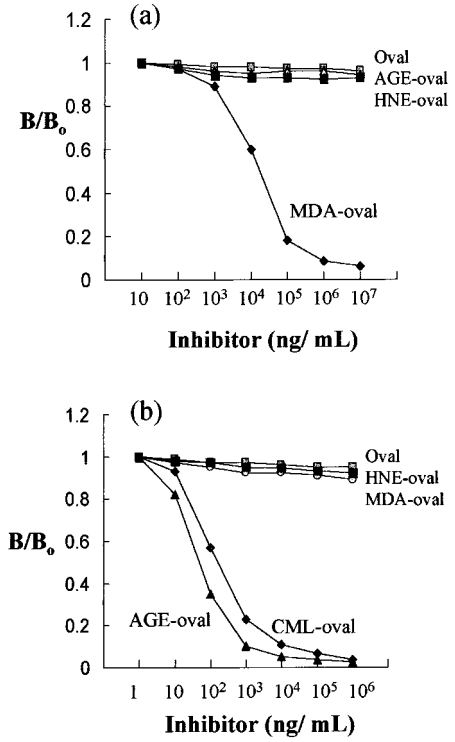

Figure 2. Cross-reactivity of antibodies to (a) MDA and (b) AGE toward ovalbumin adducts. After incubation of a mixture of antiserum and end product-ovalbumin conjugate (i.e., adduct) in a plate coated with a specific end-product conjugate, inhibition of antibodies was detected using an anti-rabbit IgG alkaline phosphatase conjugate and substrate ρ-nitrophenyl phosphate. B = absorbance of sample containing antibodies and adduct; B_o = absorbance of control containing antibodies only.

produced at weeks 6, 8, 8, and 10 against AGE, CML, HNE, and MDA, respectively, had the highest titer. These were 1:80 000, 1:20 000, 10 000, and 1:20 000 for AGE, CML, HNE, and MDA antibodies, respectively (Figure 1b).

The cross-reactivity of each antibody was then tested in a competitive ELISA. A typical cross-reactivity profile of the MDA antibody is shown in Figure 2a. The antibody was inhibited by MDA-ovalbumin, but not by HNE-, AGE-, or CML-ovalbumin. Neither was it inhibited by the native ovalbumin. This suggests that the MDA antibody recognizes MDA and not AGE, HNE, or CML. Similarly, the HNE antibody recognized HNE only (data not shown). By contrast, the antibody against AGE (or CML) was shown to be inhibited not only by AGE-ovalbumin but also by CML-ovalbumin (Figure 2b). This suggests that AGE and CML adducts have a common site that is recognized by both antibodies. Neither of these antibodies were inhibited by MDA/HNE-ovalbumin or the native ovalbumin.

Detection of End-Product Adducts in Peanuts by Antibodies. ELISA and immunoblots were respectively performed to determine if the adducts exist in raw and roasted peanuts. In ELISA, adducts of AGE, CML, MDA, and HNE were detected not only in roasted peanuts but also in raw peanuts (Figure 3a). As compared to raw peanuts, roasted peanuts gave a much higher level (~2-fold) of AGE and MDA. In contrast, there is no significant difference in CML and HNE levels between raw and roasted peanuts. The results indicate that roasting results in an increase of AGE and MDA adducts.

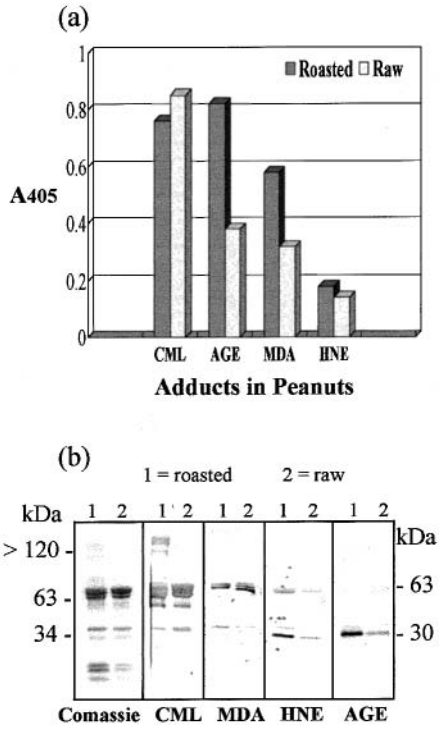

Figure 3. Detection of adducts in raw and roasted peanuts. (a) ELISA ($n = 3$). After incubation of the antiserum in a plate coated with a raw or roasted peanut extract, adducts were detected using an anti-rabbit IgG alkaline phosphatase conjugate and substrate *p*-nitrophenyl phosphate. (b) Immunoblot assay. Adducts as indicated were detected on membranes, using the same antibody reagents but substrate BCIP/NBT. Coomassie = Coomassie blue staining.

In addition, immunoblot assays were performed. Several proteins were detected using the antibodies. Proteins (major bands) that carry AGE, MDA, HNE, and CML in both raw and roasted peanuts were 30, 63, 30/63, and 34/50/63 kDa, respectively (Figure 3b). In Coomassie Blue staining, two additional high molecular-weight proteins (>120 kDa) were seen in roasted peanuts but not in the raw. These two proteins were detected strongly by the antibodies against CML and weakly by the other antibodies. These results indicate that end products tend to cross-link preferably with certain proteins. The detection of high molecular-weight proteins (>120 kDa) supports previous findings that heating or roasting causes the formation of high molecular-weight products (*31*, *32*). Overall, protein bands (detected by antibodies) from roasted peanuts were darker in density than those from raw peanuts. The data suggests that roasting or heating favors the formation of end product adducts. While differences between raw and roasted peanuts exist, it is not obvious that these small differences (i.e., 2−4-fold) will be important clinically.

IgE-Binding of Roasted and Raw Peanuts. Previously, using a competitive ELISA, this laboratory showed that proteins in roasted peanuts bound increased amounts of IgE, as compared to raw peanuts (*31*, *32*). This increase was thought to be due to the increased level of end product adducts. However, matrix effect (i.e., interference by other peanut components due to incubation of the peanut extract with the pooled patient serum or normal serum) could also have contributed to the increase of IgE binding. To prevent matrix effect, a direct ELISA was performed in the present study. In the assay, a pooled patient serum (IgE) or normal serum was added to a plate coated with proteins from a raw or roasted peanut extract rather than incubated with a peanut extract. Data (Figure 4) shows that IgE-binding was higher to proteins in roasted peanuts than to proteins in raw peanuts. This is consistent with our previous findings (*31*, *32*). The increase in IgE-binding of roasted peanuts coincided with the increased level of adducts (i.e., AGE and MDA) as detected by ELISA. It was, therefore, possible that AGE and/or MDA adducts are responsible for the increased IgE binding to proteins in roasted peanuts. If so, the adducts should have an affinity for IgE. To support this postulation, the ability of IgE to bind to the adducts was examined.

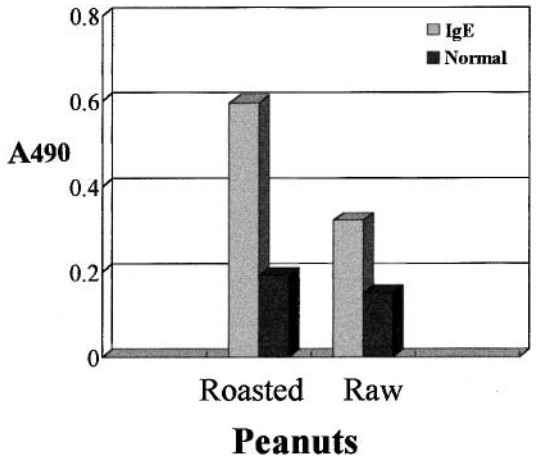

Figure 4. Direct ELISA assay of IgE binding of raw and roasted peanuts. After binding ($n = 3$) to the plate coated with raw or roasted peanut extract, IgE from a pooled patient serum (1:20) was detected using a rabbit anti-human IgE peroxidase conjugate (1:1000) and substrate *o*-phenylenediamine dihydrochloride (0.5 mg/mL).

IgE Binding to Adducts in Competitive ELISA. In this assay, IgG from an antiserum and IgE from a pooled patient serum were allowed to compete with each other for binding to the plate coated with peanut proteins (containing adducts). In the absence of IgG, IgE binds to the plate (proteins plus adducts) and an absorbance value is obtained. However, if there is an affinity between IgE and the adducts in the plate, the absorbance value would be lowered upon addition of IgG. This is because IgG binds to the adducts, thus preventing IgE from binding to the adducts in the plate and consequently lowering the absorbance value. Figure 5a shows the effect of antiserum against AGE on IgE binding to the plate coated with roasted peanuts. The absorbance (normalized) decreased from 100% to approximately 80% when the antiserum against AGE was added. This represents a 20% inhibition or reduction of IgE binding displayed by roasted peanuts (Figure 5b). By contrast, the preimmune serum (containing no IgG) showed little decrease in the absorbance value. Also, no effect on the absorbance was seen, using normal human serum. This suggests that IgE has an affinity for peanut AGE adducts. Also, this is in agreement with our previous finding, using a sugar−protein model system (*31*). In the case of CML, MDA, or HNE, little inhibition of IgE binding by the antiserum was seen (data not shown). Therefore, of the four adducts examined, only AGE was found to bind to IgE from patients allergic to peanuts. As compared to roasted peanuts, raw peanuts which have a lower level of AGE shows only a minor decrease in the absorbance or approximately 6% inhibi-

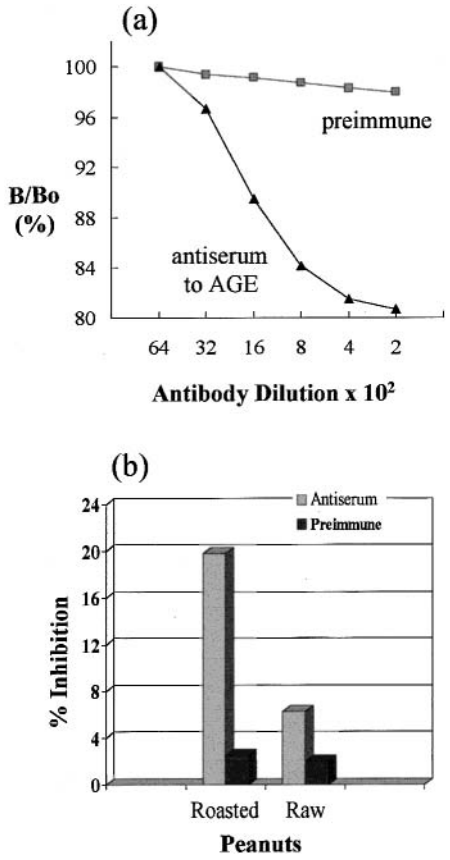

Figure 5. Inhibition of IgE binding by antibody against AGE in a competitive ELISA. (a) After incubation of a mixture of a pooled patient serum (IgE) and antiserum (IgG) against AGE at the dilution indicated in a plate coated with a roasted peanut extract, inhibition of IgE binding was detected using a rabbit anti-human IgE peroxidase conjugate (1:1000) and substrate *o*-phenyl-enediamine dihydro-chloride. (b) % inhibition of IgE binding exhibited by roasted and raw peanuts. % Inhibition = 100 − B/B_o (%), where B = absorbance value of sample containing IgE and antiserum; and B_o = absorbance value of sample containing IgE and preimmune serum.

tion by AGE antibodies (Figure 5b). This suggests that the % inhibition or the degree of IgE binding is related to the level of AGE adducts. In other words, the higher the level of adducts, the higher the IgE binding. Therefore, AGE adducts are considered a potential factor contributing to the increased IgE binding in roasted peanuts.

Discussions. Our study shows that peanuts contain end-product adducts (AGE, CML, MDA, and HNE) and that the level of AGE and MDA adducts in roasted peanuts is higher than in raw. Of the four adducts investigated, AGE was shown to bind to IgE. IgE binds to AGE probably because IgE, like the scavenger receptors, has a specificity for the anionic character and alkylated lysine provided by AGE adducts (*35*, *36*). Since AGE adducts could be correlated with IgE binding, it is possible that AGE could be in part responsible for the increased IgE binding in roasted peanuts. However, question still remains as to whether AGE adducts are indeed allergenic (i.e., causes allergic reaction). Unless a clinical trial is carried out, this study only indicates that AGE adducts possess IgE-binding property (i.e., may or may not cause allergic reaction) and not allergenic property. However, the assumption that they are potentially allergenic can be made based on several studies relating Maillard reaction products to milk allergy. For instance, Bleumink and Young (*37*) showed that coupling of lactose to the lysine residue of β-lactoglobulin increased skin reaction of patients with cow's milk allergy. Kaminoagawa et al. (*38*) showed that the sugar−protein complex from a reagent grade lactose was responsible for the high positive skin test of patients with cow's milk allergy. All these implicate Maillard reaction products in allergy. Therefore, it is possible that AGE adducts from peanuts are allergenic.

Additionally, AGE adducts have been shown to be capable of eliciting IgG immune responses (*25*, *28*, *39*). In this study, we showed that IgG (antibody to AGE adducts) inhibited IgE binding to the protein adducts. Question has been raised as to whether immunization with AGE adducts (thus producing IgG antibodies) could reduce the risk of peanut allergy. Little research has been done in this area. However, there have been studies relating such immunization to the improvement of other diseases such as atherosclerosis. For instance, injection of MDA adducts to rabbits with atherosclerosis has been shown to reduce atherosclerotic lesions (*27*, *40*). This is because injection results in the production of antibodies against MDA, which then bind to the adducts on LDL, thus preventing LDL from adhering to the arterial wall and consequently reducing atherosclerostic lesions. With peanut allergy, the same approach (i.e., injection with AGE adducts and subsequent inhibition of IgE binding to the adducts by IgG) may be applied and probably could reduce allergic responses. Before this, it is important that the present findings are linked to data, if any, from in vivo studies (e.g., using mouse models). Establishment of this linkage is the goal of our future research.

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