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High-Oleic Peanuts Are Not Different from Normal Peanuts in Allergenic Properties

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High-oleic peanuts are known for a high content of oleic fatty acid. However, it is not known whether high-oleic peanuts are different from normal chemistry peanuts in levels of allergenicity and end-product adducts (i.e., products cross-linked with proteins). For this purpose, four different peanut cultivars (Florunner, Georgia Green, NC 9, and NC 2) were evaluated and compared with high-oleic peanuts (SunOleic 97R). Adducts such as AGE/CML from Maillard reactions and MDA/HNE from lipid oxidation were determined, respectively, in ELISA, using polyclonal antibodies. Allergenicity was determined based on IgE binding and T-cell proliferation. Results showed that raw high-oleic peanuts were not different from normal peanuts in adduct levels. After roasting, CML and HNE levels remained unchanged, but an increased and similar amounts of AGE adducts were found in all peanuts. MDA also increased but not in high-oleic peanuts. This suggests that high-oleic peanuts are more stable to lipid oxidation than others during heating. Despite this, high-oleic peanuts did not differ from normal peanuts in IgE binding and T-cell proliferation. It was concluded that a high content of oleic fatty acid has no effect on peanut allergenicity and that high-oleic peanuts do not give a higher or lower risk of allergy than normal peanuts.

KEYWORDS: High-oleic peanuts; allergenicity; Maillard reaction adducts; lipid oxidation adducts; AGE; CML; MDA; HNE; IgE antibodies; T-cells; polyclonal antibodies; ELISA

INTRODUCTION

High-oleic peanuts such as SunOleic 97R have oil with 80% oleic fatty acid and 2% linoleic fatty acid as compared to 50% oleic and 25% linoleic in oil of normal peanuts (*1*). Two recessive genes are responsible for the high-oleic trait (*2*, *3*). Incorporation of these genes into Florida breeding lines has resulted in two peanut varieties, SunOleic 95R (*4*) and SunOleic 97R (*5*). This oil chemistry has been shown to lower cholesterol levels in hypercholesterolemic women (*6*), decrease the level of monounsaturated fatty acids in swine (*7*), enhance peanut shelf life, and reduce rancidity (*8*−*10*).

Because incidences of peanut allergies are being increasingly reported (*11*), questions have been raised as to whether high-oleic peanuts are different from other peanuts in their level of allergenicity. In other words, does a high ratio of oleic to linoleic acid have any influence on allergenicity? Effects of dietary fatty acids such as linolenic acid (an $n-3$ fatty acid) and linoleic acid (an $n-6$ fatty acid) on allergic responses in mice have been reported (*12*). Diets high in a ratio of linolenic to linoleic acids ($n-3/n-6$) are reported to suppress allergic responses and anaphylactic shock in mice (*13*, *14*). In one study (*15*), mast cell degranulation, which results in histamine release and allergic responses, was shown to be inhibited by a diet high in a ratio of linoleic to oleic fatty acids. All of these (in vivo studies) indicate an association between fatty acids (the ratio in particular) and allergy.

To date, several major peanut allergens (e.g., Ara h 1, 2, and 3) have been identified (*16*). Roasted peanuts are reported to have a higher level of IgE binding (i.e., allergenicity) than raw peanuts (*17*, *18*). In addition, several end-product adducts (i.e., products cross-linked with proteins) have been identified. Adducts such as advanced glycation end-products (AGE) and carboxymethyllysine (CML) from protein−sugar (*19*, *20*) or protein−lipid (*21*) reactions have been implicated in milk allergy (*22*, *23*). More recently, AGE have been shown to be potentially associated with immunoglobulin E (IgE) binding (i.e., allergenicity) of peanuts (*18*, *24*). Other adducts such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) from lipid oxidation (*25*, *26*) are also implicated in allergy. For instance, they stimulate allergy-related T-cell responses (*27*, *28*) and render proteins more resistant to digestion (*29*, *30*). Such

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proteins that are stable to digestion tend to be the major allergens of foods (*31*). Because high-oleic peanuts are unique in the high ratio of oleic to linoleic acid, it is possible that adducts such as those described above (MDA and HNE in particular) may change and thus alter the allergenicity of high-oleic peanuts.

In this study, in vitro assays relating to the levels of adducts and allergenicity of high-oleic peanuts and other peanut cultivars (e.g., Florunner, Georgia Green, NC 2, and NC 9) were performed. The objective was to determine if high-oleic peanuts are different from these cultivars in levels of adducts, IgE binding, and T-cell proliferation (i.e., a measure of allergenicity). The results will provide insights into whether a high ratio of oleic to linoleic acid has a role in allergenicity and adduct formation.

MATERIALS AND METHODS

Apparatus. A CERES 900C microtiter plate reader was purchased from Bio-Tek Instruments, Inc. (Winooski, VT).

Reagents and Materials. Anti-rabbit IgG alkaline phosphatase conjugate, *p*-nitrophenyl phosphate, rabbit anti-human IgE−peroxidase conjugate, *o*-phenylenediamine, [^{3}H]thymidine, bovine serum albumin (BSA), Tween 20, ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and phosphate-buffered saline (PBS) were purchased from Sigma Chemical Co. (St. Louis, MO). HNE was purchased from Cayman Chemical (Ann Arbor, MI). Normal human serum, serum from a pool of three patients with peanut allergy, and patient T-cells 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 (Florunner, Georgia Green, NC 2, and NC 9) were provided by Drs. Tim Sanders and Christopher L. Butts, respectively, from USDA-ARS, North Carolina State University, Raleigh, NC, and National Peanut Research Laboratory, Dawson, GA. Commercial high-oleic peanut seeds (raw and roasted) were provided by Dr. Kenneth L. Buhr, University of Florida, Gainesville, FL.

Production of Antibodies to AGE, CML, MDA, and HNE. Antibodies were produced in rabbits as previously described (*18*), using immunogens such as BSA−glucose (after incubation for 90 days at 37 °C), BSA−CML, BSA−MDA, and BSA−HNE.

Preparation of Peanut Protein Extracts. Extracts were prepared as previously described from defatted meals of raw and roasted peanuts (*18*). Briefly, meals (40 mg) were stirred in 3 mL of 0.02 M sodium phosphate, pH 7.4, and 10 mM EGTA for 30 min at 4 °C, followed by centrifugation at 8500*g* for 10 min. The resultant supernatants (extracts) were used for enzyme immunosorbent assays (ELISA). Concentration of proteins in the extract was determined using the BCA assay.

Assay of End-Product Adducts in Raw and Roasted Peanuts. A direct ELISA ($n = 3$) was performed according to the method of Chung and Champagne (*18*). Briefly, a microtiter plate was coated with a peanut extract (20 μg/mL) (100 μL per well) in 0.1 M sodium bicarbonate, pH 9.6, at 37 °C for 90 min. The plate was washed four times with PBS/Tween 20 and blocked three times (5 min each time, followed by washing) with a 200 μL SuperBlock solution. A rabbit preimmune serum (control) or antiserum (1:200) against AGE, CML (1:500), MDA, or HNE (100 μL each) [diluted in SuperBlock/PBS/Tween (1:1)] was then added to the plate and incubated for 30 min at 25 °C. The plate was washed four times with PBS/Tween. An anti-rabbit IgG alkaline phosphatase conjugate (1:12500) (100 μL) was added and incubated for 15 min at 25 °C. The plate was washed and incubated with a substrate solution (*p*-nitrophenyl phosphate in 10% diethanolamine, pH 9.8, 0.5 mM $MgCl_2$; 1 mg/mL) (100 μL) for 30 min at 37 °C. The absorbance was then read at 405 nm, using the Ceres 900C BioTek microplate reader.

Assay of IgE Binding to Raw and Roasted Peanuts. Direct and competitive inhibition ELISAs were carried out ($n = 3$). In each case, the plate was coated with a peanut extract as described above. In a direct ELISA, a normal or pooled patient serum (IgE) (1:20) (100 μL) [diluted in SuperBlock:PBS/Tween (1:1)] was added to the plate, whereas in a competitive inhibition ELISA, the plate was filled with a mixture of the pooled patient serum (IgE) (1:20) (50 μL) and a peanut extract (i.e., inhibitor) (50 μL) at various protein concentrations (0.1−1000 μg/mL). After incubation for 60 min at 25 °C, the plate was washed four times with PBS/Tween. A rabbit anti-human IgE−peroxidase conjugate (1:500) (100 μL) was added and incubated for 30 min at 25 °C. The plate was then washed. A substrate solution (100 μL) of *o*-phenylenediamine (0.5 mg/mL) and 0.03% hydrogen peroxide in 0.1 M citrate buffer, pH 5.5, was added and incubated for 60 min at 37 °C. The reaction was stopped with 50 μL of 4 N sulfuric acid. Absorbance was then read at 490 nm. In the competitive ELISA, the absorbance value of a sample containing IgE and a peanut extract (inhibitor) was represented by B, whereas B_0 represented the absorbance value of a control containing IgE only.

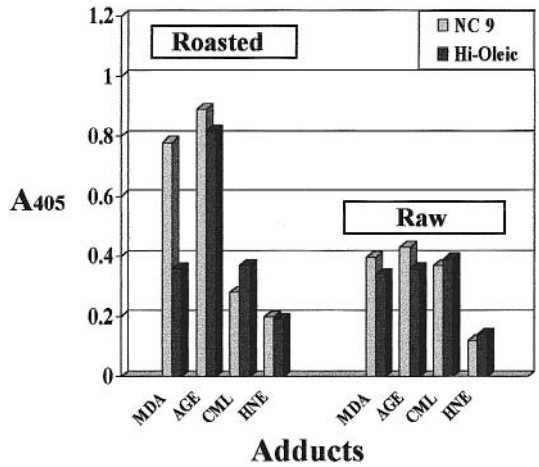

Figure 1. Adducts in NC 9 and high-oleic peanuts (raw and roasted). Adducts as indicated were detected in a direct ELISA ($n = 3$), using specific polyclonal antibodies and an anti-rabbit IgG alkaline phosphatase conjugate.

Measurement of T-Cell Proliferation. The peripheral blood lymphocytes (PBLs) of four peanut-sensitive individuals were isolated from whole blood by Ficoll−Hypaque gradient centrifugation (Pharmacia, Piscataway, NJ) (*32*). Cells were washed and suspended in media at the concentration of 4×10^6 cells/mL. Aliquots (3×1 mL) of T-cells were placed in 24 well tissue culture plates and stimulated with peanut extracts (50 μg/mL) to establish peanut-specific T-cell lines. For the proliferation assays, 9 wells of a 96 well plate at 2×10^5 PBLs/well were stimulated in triplicates with media (control), peanut extracts in both raw and roasted forms (50 μg/mL), and incubated at 37 °C. The cells in the 96 well plates were allowed to proliferate in the absence (media) and presence of the stimulant (i.e., peanut extracts in raw or roasted forms) for 6 days. On day 6, the cells were treated with [^{3}H]-thymidine (1 μCi/well) and reincubated at 37 °C for 6−8 h and harvested onto glass fiber filters (Packard, Meriden, CT). T-cell proliferation was estimated by quantitating the [^{3}H]thymidine incorporation into the DNA of proliferating cells (*32*). [^{3}H]Thymidine incorporation is reported as the stimulation index (SI), which is defined as the stimulation above media-treated cells (control).

RESULTS AND DISCUSSION

Adducts in High-Oleic versus Other Cultivars. Using polyclonal antibodies, adducts of AGE, CML, MDA, and HNE were determined, respectively, in high-oleic and four other peanut cultivars (Florunner, Georgia Green, NC 2, and NC 9). For illustration, typical adduct profiles of NC 9 and high-oleic peanuts are presented (**Figure 1**). Raw, high-oleic peanuts showed little difference from NC 9 in levels of MDA, AGE, CML, and HNE. However, when roasted, NC 9 exhibited an increased amount of MDA (~2-fold), whereas a change in MDA level was hardly seen in high-oleic peanuts. This difference in MDA between NC 9 and high-oleic peanuts suggests that high-

Table 1. Comparison of Peanuts in IgE Binding and Adduct Levels

	IgE binding		AGE		MDA		CML		HNE	
cultivar	raw	roasted	raw	roasted	raw	roasted	raw	roasted	raw	roasted
Florunner	–[a]	+[b]	–	+	–	+	–	–	–	–
Georgia Green	–	+	–	+	–	+	–	–	–	–
NC 2	–	+	–	+	–	+	–	–	–	
NC 9	–	+	–	+	–	+	–	–	–	–
SunOleic	–	+	–	+	–	–	–	–	–	–

[a] No increase, compared to roasted. Levels are similar between peanuts (raw or roasted). [b] Increase in level, compared to raw. Note that MDA did not increase in roasted SunOleic.

oleic peanuts are more stable to lipid oxidation than NC 9 during heating and that a high content of oleic acid may be responsible for the heat stability. It should be noted that oleic acid is a monounsaturated fatty acid rather than a polyunsaturated fatty acid and, therefore, is less susceptible to oxidation. Such heat stability explains why high-oleic peanuts have a lesser rancidity problem and a longer shelf life than normal peanuts (*8*−*10*).

Although a high content of oleic acid appears to help prevent an increase of MDA during roasting, data show that oleic acid does not have a preventive effect on AGE adducts. As shown in **Figure 1**, AGE increased ~2-fold in NC 9 after roasting. Unlike MDA, this increase of AGE also occurred in high-oleic peanuts. The reason AGE increased during roasting is because heat enhances sugar−protein or Maillard reactions, which in turn produce high levels of AGE. Raw or roasted, NC 9 and high-oleic peanuts showed no difference in the level of AGE. In the case of CML and HNE, roasting did not result in a significant change in the levels of these two adducts. This suggests that levels of CML and HNE, as determined in the direct ELISA, are not greatly affected by heating and that CML and HNE are not the major Maillard reaction and lipid oxidation adducts produced during roasting. Overall, the data show that AGE and MDA are the primary adducts produced by roasting and that the difference between NC 9 and high-oleic peanuts is in MDA, which increases in NC 9 and not in high-oleic peanuts during roasting.

Further investigation of other peanut cultivars (i.e., Florunner, Georgia Green, and NC2) revealed that there was no difference among these cultivars and NC 9 or high-oleic peanuts in levels of AGE, CML, HNE, and MDA adducts (**Table 1**). However, when roasted, these peanut cultivars, like NC 9, exhibited increased and similar levels of AGE and MDA adducts. In this case, the normal chemistry cultivars were different from high-oleic peanuts because the latter did not give an increase of MDA during roasting. Overall, the data (**Table 1**) indicate that high-oleic peanuts are not different from other peanut cultivars in levels of adducts except MDA, which increases during roasting but not in high-oleic peanuts.

IgE Binding of High-Oleic versus Other Cultivars. Direct and indirect competitive inhibition ELISAs were carried out. Typical profiles of IgE binding to NC 9 and high-oleic peanuts (raw and roasted) are presented in **Figure 2**. No difference was found between NC 9 and high-oleic peanuts (raw or roasted) in the level of IgE binding by direct ELISA (**Figure 2a**). Similarly, in a competitive ELISA (**Figure 2b**), no difference in IgE binding, based on the IgE inhibition curve, was found for raw or roasted peanuts. Other peanut cultivars (i.e., Florunner, Georgia Green, and NC 2) also showed similar results (**Table 1**) and were not different in IgE binding from high-oleic peanuts when roasted. It should be noted that all of these cultivars, when roasted, had a higher level of MDA than high-oleic peanuts. This finding suggests that a difference in the MDA

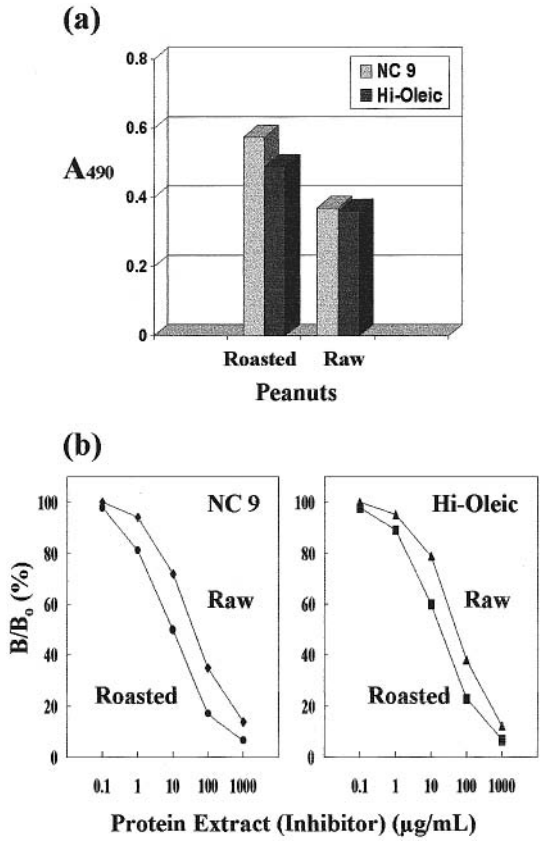

Figure 2. IgE binding of NC 9 and high-oleic peanuts (roasted and raw): (a) direct ELISA ($n = 3$) (a plate was coated with a raw or roasted peanut extract; IgE antibodies from a pooled serum of allergic patients were added to the plate and detected, using a rabbit anti-human IgE HRP conjugate); (b) competitive inhibition ELISA ($n = 3$) [IgE antibodies in the coated plate were incubated with a peanut extract (i.e., inhibitor) and inhibited at the protein concentrations indicated; detection was the same as described in (a)].

level has no effect on IgE binding. In all cases, roasted peanuts demonstrated a higher IgE binding or a steeper inhibition curve than the raw (**Figure 2**). This increase in IgE binding probably is due to an increased level of AGE adducts. Correlation such as this between the increases of AGE and IgE binding has previously been demonstrated (*18*). 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 (*33*). Overall, the data indicate that high-oleic peanuts are not different from other peanut cultivars in the level of IgE binding or allergenicity.

T-Cell Proliferation Stimulated by High-Oleic and NC 9. T-cells from four patients were each stimulated in vitro with raw/roasted NC 9 and high-oleic peanut extracts. The resultant T-cell proliferation or stimulation index (SI) is shown in **Figure**

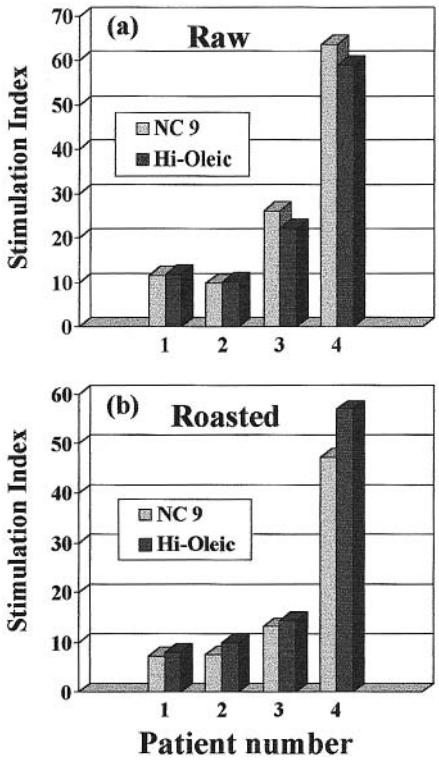

Figure 3. T-cell proliferation of NC 9 and high-oleic peanuts (roasted and raw). Proliferation of T-cells from different patients as indicated by the numerical number was achieved by stimulating T-cells in vitro with a raw or roasted peanut extract.

3. Although T-cell responses varied among patients (patients 1−4), the SI values for NC 9 and high-oleic peanuts (raw or roasted) appeared to be similar in each patient. For instance, SI values for NC 9 and high-oleic peanuts, when raw, were approximately 10 and 11, respectively, in patient 1 (**Figure 3a**); when roasted, they were 6 and 7, respectively (**Figure 3b**). Similar patterns (with different SI values) were also obtained with patients 2, 3, and 4 (**Figure 3**). This finding indicates that there was no difference in T-cell proliferation between NC 9 and high-oleic (raw or roasted) peanuts. This means that high-oleic and NC 9 peanuts are not different in allergenic properties. Although not performed, other peanut cultivars (Florunner, Georgia Green, and NC 2) were thought to give similar T-cell results. This is based on the above finding that the level of IgE binding between these cultivars and NC 9/high-oleic was similar (**Figure 2**).

Summary. The aim of this study was to determine if high-oleic peanuts are different from normal oil chemistry peanut cultivars (e.g., Florunner, Georgia Green, NC 9, and NC 2) in levels of allergenicity (based on IgE binding and T-cell proliferation) and adducts such as AGE, CML, HNE, and MDA. Adducts are important because previously it was shown that there was an association between adducts and food allergy or T-cell proliferation. Also, adducts such as MDA and HNE could vary between peanuts due to a difference in fatty acid contents (i.e., oleic and linoleic acids). Results showed that adduct levels were very similar among raw peanuts, including high-oleic. After roasting, an increased and similar amount of AGE was found in all peanuts. Also, MDA increased but not in high-oleic peanuts. This suggests that high-oleic peanuts are more stable to lipid oxidation than others during heating. Despite this, little difference was found between high-oleic and other peanuts in IgE binding and T-cell proliferation. This suggests that a high ratio of oleic to linoleic fatty acid or the MDA itself has little effect on peanut allergenicity. In other words, high-oleic peanuts have no greater additional adverse allergenic effects than normal peanuts.

It should be noted that the above was an in vitro study. Whether high-oleic peanuts would behave differently in allergenicity in in vivo studies (i.e., utilizing mice or humans) remains to be seen. One unique feature of high-oleic peanuts is, in addition to its high level of oleic acid, their low content of linoleic acid (2% compared to 25% in normal peanuts). Evidence that dietary linoleic acid is associated with allergy has been documented. For instance, a decrease in the intake of dietary linoleic acid or a diet high in the ratio of linolenic (an $n-3$ fatty acid) to linoleic acid (an $n-6$ fatty acid) has been shown to suppress allergic responses in mice (*13*, *14*). Also, differences in the consumption of linoleic acid are reported to be responsible for the 20−60-fold variation in the frequency of allergic symptoms between Asian−Eastern European countries and America−Australia−Northern Europe (*34*). The countries (e.g., Asian) with a low frequency of allergic diseases reportedly tend to have a lower intake of linoleic acid in the diet. According to the Food and Agricultural Organization (*35*), the grams of fat per capita per day obtained from vegetable oils (which are the main source of linoleic acid) in Russia, India, and China in 1995 were 15.2, 18.2, and 15, respectively. This compares with values for the United Sates, the United Kingdom, and France of 62.4, 45.8, and 46.2, respectively. All of these indicate that a low intake of linoleic acid is beneficial, especially for people who have potential allergic symptoms. The mechanism behind this probably lies in the fact that linoleic acid is a precursor of prostaglandin E_2 in humans, which in turn may promote allergic sensitization.

Although the present in vitro study showed that high-oleic peanuts were not different from other peanut cultivars in allergenicity, the above discussion indicates that high-oleic peanuts could be less allergenic, if analyzed in vivo (i.e., in mice or humans), than other peanuts. This is because intake of linoleic acid is lower with high-oleic peanuts than with other peanuts. Although this is only a hypothesis, the high-oleic peanuts warrant further investigation.

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