

Research Note

Effects of Gamma Radiation on the Allergenic and Antigenic Properties of Milk Proteins

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MS 00-235: Received 11 July 2000/Accepted 17 September 2000

ABSTRACT

This study was carried out to evaluate the application of food irradiation technology as a method for reducing milk allergies. Bovine α -casein (ACA) and β -lactoglobulin (BLG) were used as milk proteins. Using milk-hypersensitive patients' immunoglobulin E (IgE) and rabbit IgGs individually produced to ACA and BLG, the changes of allergenicity and antigenicity of irradiated proteins were observed by competitive indirect enzyme-linked immunosorbent assay. Allergenicity and antigenicity of the irradiated proteins were changed with different slopes of the inhibition curves. The disappearance of the band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and increase of the turbidity showed that solubility of the proteins decreased by radiation, and this decrease might be caused by agglomeration of the proteins. These results indicated that epitopes on milk allergens were structurally altered by gamma irradiation.

Cow's milk allergy is probably the most common food allergy during infancy and early childhood, and for milk-allergic infants, the circulating immunoglobulin E (IgE) specific for casein and whey proteins is responsible for many adverse reactions to milk (5, 7, 19, 21). The primary allergens in cow's milk are casein proteins, β -lactoglobulin (BLG), α -lactalbumin, bovine serum albumin, and bovine serum IgG (25, 28). BLG is considered the most important allergen because it is not present in human milk (1, 17, 21) and gives the highest rate (66%) of positive oral challenges in children with a milk allergy, although casein shows the highest rate for skin test reactivity (68%) (2, 9, 10). The most common means for reducing the allergenicity of cow's milk is enzymatic hydrolysis with various proteolytic enzymes (1, 22, 24). However, these hypoallergenic formulas have an unsatisfactory taste caused by the presence of bitter peptides and amino acids. The cases of an allergy to cow's milk hydrolysate were reported, and the use of the proper inhibition method was necessary (18, 26, 27). Also, with the exception of a cow's milk allergy, protein hydrolysate is not widely used in the treatment of food allergies (22).

In the previous studies, the structural modification of food allergens by gamma irradiation was observed, and several results have indicated that ionizing radiation could reduce allergenicity by the destruction of human IgE-binding epitopes in food allergens (3, 8, 12, 16). The purpose

of this study was to evaluate the usability of gamma radiation for reducing milk allergies through the observation of the changes of allergenicity and antigenicity of the major milk allergens, α -casein (ACA) and BLG.

MATERIALS AND METHODS

Milk proteins. Isolated ACA (lyophilized powder, chromatographically purified; approximately 85% by electrophoresis) and BLG (crystallized and lyophilized; approximately 95% by Lowry and biuret methods) were purchased from Sigma Chemical Co. (St. Louis, Mo.) at the highest available purification level and with the following chemical characteristics to use milk proteins. Each protein was dissolved in 0.01 M sodium phosphate-buffered saline (PBS) with NaCl of 0.15 M, pH 7.4, and adjusted to a final concentration of 2.0 mg/ml.

Human sera. Sera were obtained from 20 patients (younger than 3 years; 13 boys and 7 girls) diagnosed as having IgE-mediated milk allergy after open cow's milk challenges and/or by a convincing history of acute urticaria or diarrhea after milk ingestion. Fifteen patients experienced atopic dermatitis and/or acute urticaria, three patients experienced skin and gastrointestinal symptoms (diarrhea and vomiting), and two patients had only chronic diarrhea. All patients showed elevated cow's milk-specific IgE levels (score 2 or more than 2) measured by AlaSTAT RIA (DPC Co., Los Angeles, Calif.), and the sera were pooled for determining the IgE-binding ability to gamma-irradiated milk proteins. Ten age-matched subjects provided serum for control. None had food allergy or any other atopy-related skin, respiratory, or gastrointestinal symptoms.

Production of polyclonal antibody (Ab). Polyclonal Abs were produced from rabbits immunized to each protein to study

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the conformational changes of epitopes on the antigen (Ag). Before immunizing, 1 ml of normal blood from a noninjected rabbit was collected by the ear-bleeding method as a normal serum blank. Two rabbits per protein were individually immunized by intradermal injection (1 ml) of a concentration of 100 $\mu\text{g}/\text{ml}$ as the injection concentration emulsified with an equal volume of Freund's complete adjuvant (GIBCO Laboratories, Grand Island, N.Y.). Subsequent injections of each Ag in an incomplete Freund's adjuvant were performed three times at 2-week intervals, and the rabbits were checked for their Ab responses by indirect enzyme-linked immunosorbent assay (ELISA). The rabbits were finally boosted with Ag without adjuvant. After 1 week, blood was collected by a heart puncture, and each antiserum was separated by centrifuging at 3,000 rpm for 10 min at 4°C.

Each antiserum was affinity chromatographed on a protein A high trap column (5 ml, Sigma) equilibrated in PBS (pH 7.4) to separate IgG. The IgG fractions were eluted with 0.1 M acetic acid (pH 4.0). The fractions were pooled into dialysis tubing (pore < 12,600, Spectrum, Medical Industries, Inc., Houston, Tex.), dialyzed in 0.01 M PBS (pH 7.4), and concentrated on polyethylene glycol (20,000). The concentrations of all the IgG solutions were adjusted to 2.0 mg/ml with 0.01 M PBS. All the Ab solutions were mixed with an equal volume of glycerol and stored at -20°C.

Gamma irradiation. Each protein solution (10 ml) was put into a glass tube (ϕ 1.0 cm) with a cap and irradiated in a cobalt-60 irradiator (IR-79, Nordion International Ltd., Ontario, Canada) equipped with 100-kCi activity at $10 \pm 0.5^\circ\text{C}$ and operated at a dose rate of 10 kGy h^{-1} . The applied dose levels were 3, 5, and 10 kGy. The absorbed dose was monitored with both free-radical and ceric/cerous dosimeters. After irradiation, the tubes were stored at 4°C. Nonirradiated solution was used as a control.

Competitive indirect ELISA (Ci-ELISA) with patients' IgE. The binding abilities of the patients' IgE and rabbit IgG to the irradiated proteins was tested by Ci-ELISA (16). Briefly, polystyrene flat-bottom microtiter plates (Maxisorp, Nunc, Kamstrup, Denmark) were coated with 100 μl of each protein solution (5.0 $\mu\text{g}/\text{ml}$) in a 0.2 M bicarbonate buffer (pH 9.6) overnight at 4°C. All subsequent steps were performed at 37°C. Plates were washed three times with PBS containing 0.05% (vol/vol) Tween 20 (PBST). To reduce nonspecific binding, the plates were blocked by incubation for 1.5 h with 120 μl of PBS containing 1% (wt/vol) gelatin. After washing, 50 μl of different diluents of sample solution serially diluted from 200 to 0.2 $\mu\text{g}/\text{ml}$ in PBS were added to three coated and blocked wells, and then 50 μl of the patients' sera diluted 50 times in PBS was added. The plates were incubated for 2 h and then washed three times with PBST. After the addition of 100 μl of 1:1,000 diluted horseradish peroxidase conjugated rabbit anti-human IgE IgG solution (Sigma) to the wells, the plates were incubated for 1.5 h. The plates were then washed, and 100 μl of 0.04% α -phenylenediamine (Sigma) in 0.1 M phosphate-citrate buffer (pH 5.0) with 0.04% hydrogen peroxide (vol/vol, 35% H_2O_2) was added for color reaction for 20 min before stopping it with 2.0 M H_2SO_4 (50 $\mu\text{l}/\text{well}$). The absorbance was measured at 492 nm by an ELISA reader (CERES UV-900C, BIO-TEK Instruments Inc., New York, N.Y.). Formation of Ci-ELISA to rabbit IgG was the same as that to human IgE, except for the dilution rate of the rabbit IgG solution (1:5,000) and goat anti-rabbit IgG conjugated horseradish peroxidase (Sigma) solution (1:20,000).

To quantify irradiated milk proteins and to evaluate the changes of binding ability of Abs, irradiated ACA and BLG

solutions were individually diluted to 20 $\mu\text{g}/\text{ml}$ in PBS. The concentration was also determined by Ci-ELISA as mentioned above.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE (5 to 15% gradient gel) for irradiated milk proteins (1.0 mg/ml) was performed by Laemmli's method (15) using a Hoefer vertical electrophoresis apparatus SE-26 (Hoefer Scientific Instruments, San Francisco, Calif.), and the staining and destaining method was done as described previously (4). A molecular weight marker was purchased from Bio-Rad Laboratories (Hercules, Calif.) to determine the molecular weights. The marker contains myosin (200 kDa), β -galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa).

Turbidity and colorimetric reaction of irradiated milk protein solution. The turbidity and protein concentration of an irradiated milk protein solution were determined by an absorbance test at 280 nm and by using a bicinchoninic acid protein assay kit (Sigma) with bovine serum albumin solution as a standard at 562 nm with a spectrophotometer (UV-1600PC, Shimadzu Corp., Kyoto, Japan) by the method described previously (23).

Statistical analysis. All samples were done in triplicate, and the experiments were repeated five times. The means and standard deviations were used to evaluate the binding abilities of human IgE and rabbit IgG to milk protein. The data were analyzed by general linear procedures, least square means, and Duncan's multiple range test. SAS software (20) was used to achieve the above-mentioned studies.

RESULTS AND DISCUSSION

Binding abilities of patients' IgE and rabbit IgG to irradiated milk protein. The binding ability of patients' IgE to irradiated protein was determined by Ci-ELISA. The patients' milk-specific IgE did not recognize well the irradiated ACA and BLG, depending on the dose (Fig. 1). Using rabbit IgG, binding ability to irradiated ACA and BLG was also obtained (Fig. 2). The binding ability of anti-ACA IgG increased to 3 kGy and then decreased. The binding ability of anti-BLG IgG increased to 5 kGy and then decreased. These results showed that ACA and BLG were structurally changed with different allergenicity and antigenicity and/or that the epitopes on the proteins were masked by aggregation of the molecule (12). The slopes of the curves obtained by competition between coated standard Ag and irradiated proteins offered important clues regarding conformational changes that may occur in the epitopes following irradiation. Increase of the slope indicates the reduction of binding ability of Ab to irradiated protein. If the slopes were similar, as seen only at binding of anti-ACA IgG (Fig. 2A), then the epitopes were not likely to be altered in structure. However, the concentration of the epitopes in Fig. 2A appeared to change, suggesting alterations in solubility or agglomeration that might mask some epitopes. However, in Figures 1A, 1B, and 2B, the comparative slopes of the treatments did differ. In these cases, the evidence suggested that the epitopes were being structurally altered, although masking solubility may also contribute to the result (11). There are several factors on the

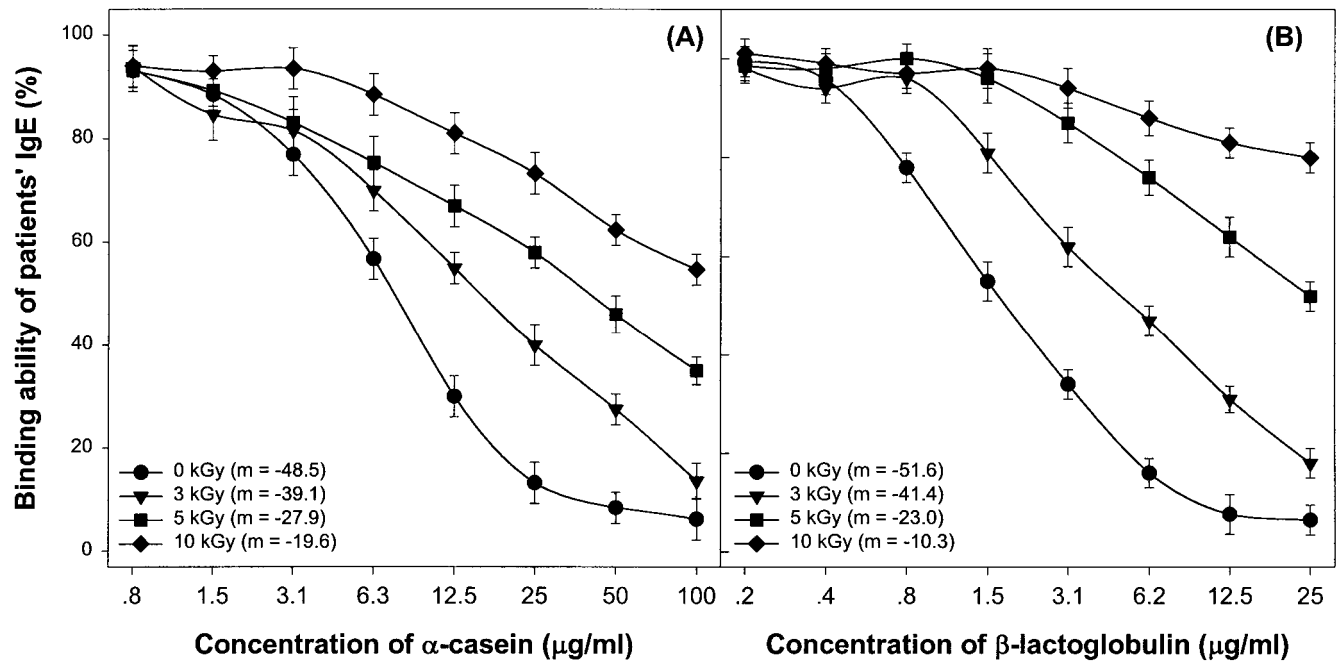


FIGURE 1. Binding ability (allergenicity) of milk-allergic patients' IgE to irradiated ACA (A) and BLG (B) by Ci-ELISA. The protein solutions (2.0 mg/ml) were irradiated, serially diluted, and then tested. m is the slope of the curve obtained from binding rate of IgE to coated standard protein.

denaturation of protein in an aqueous solution by radiation: the local conformation of an amino acid in the peptide chain, its accessibility to the water radiolysis products, and the amino acid sequence itself, where some particular amino acid residues may be more susceptible to radiolysis (6, 8, 12–14).

SDS-PAGE. From the SDS-PAGE (5 to 15% gradient gel) of irradiated BLG and ACA, it appeared that the main bands disappeared with increase of the irradiation dose and were not detected at doses of 3 kGy and above in ACA and at a dose of 10 kGy in BLG (Fig. 3). Traces spread from the starting line of separating gel at doses of 3 kGy

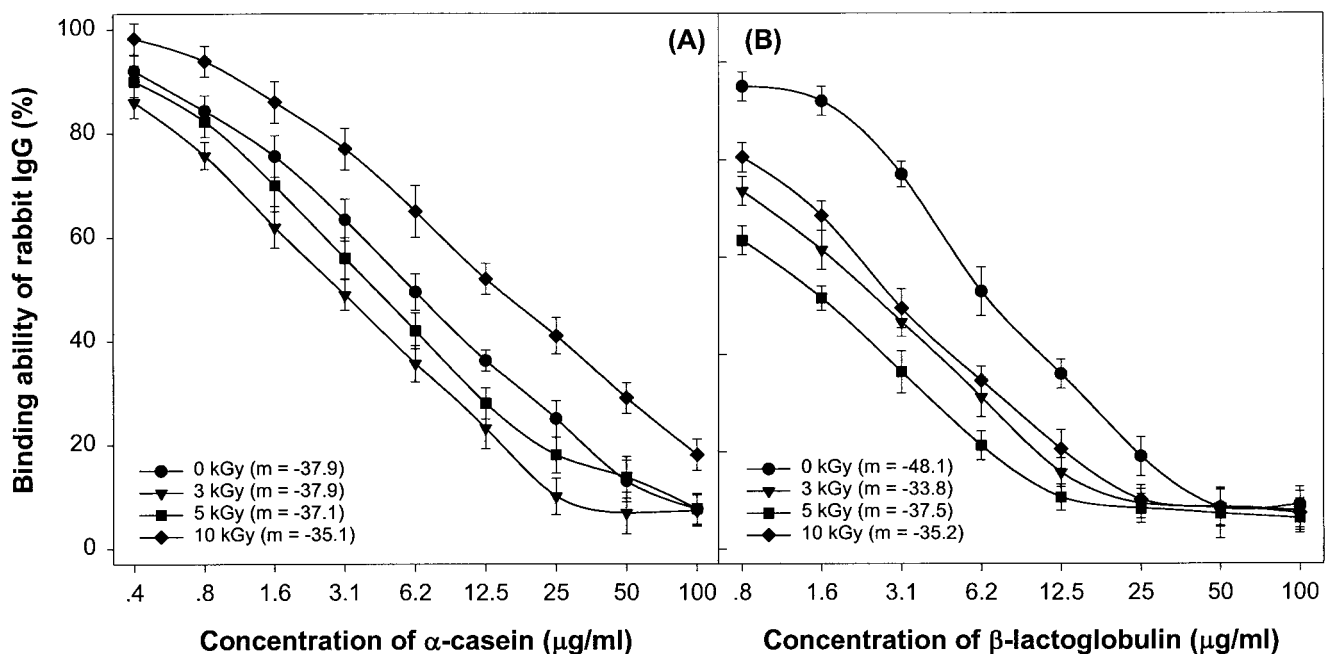


FIGURE 2. Binding ability (antigenicity) of rabbit anti-ACA IgG (A) and rabbit anti-BLG IgG (B) to irradiated ACA and BLG, respectively, by Ci-ELISA. The protein solutions (2.0 mg/ml) were irradiated, serially diluted, and then tested. m is the slope of the curve obtained from binding rate of IgG to coated standard protein.

TABLE 1. Turbidity and colorimetric reaction by bicinchoninic acid assay of irradiated milk protein solutions (2 mg/ml)^a

Protein		Irradiation dose (kGy)			
		0	3	5	10
α -Casein	Optical density at 280 nm	1.841 A ^b	2.039 B	2.094 C	2.173 D
	Optical density at 562 nm	0.319 A	0.311 A	0.318 A	0.319 A
β -Lactoglobulin	Optical density at 280 nm	1.649 A	1.922 B	2.027 C	2.232 D
	Optical density at 562 nm	0.307 A	0.305 A	0.309 A	0.308 A

^a Ten milliliters of milk protein solution was irradiated at the designated dose and individually measured at the experiment items.

^b Means in the same row with unlike letters differ significantly ($P \leq 0.05$).

and above were observed. The results depicted in Fig. 3 could be explained by a change in solubility as a result of irradiation treatment. By gamma irradiation, a protein molecule can be broken down to smaller molecules or coagulated to larger molecules by interaction among the molecules (3, 11, 16). ACA and BLG were coagulated by gamma irradiation in the presence of oxygen. This result was similar to the changes of irradiated food proteins (3, 8, 11). These changes in irradiated protein could cause the change of Ab reactivity and protein solubility.

Turbidity and colorimetric reaction of irradiated milk proteins. Table 1 indicates the results of the turbidity and colorimetric reaction of the irradiated BLG and ACA solutions. The turbidity of the irradiated BLG and ACA solutions increased, depending on the increase of the dose, when observed by a spectrophotometer (280 nm). However, there were no significant differences in the protein concentration in the irradiated BLG and ACA solutions when determined by the bicinchoninic acid method. These results showed that the structure of protein was released and coagulated by gamma irradiation (3, 13, 16). The results also supported the increase of binding ability of rabbit IgG to

3- or 5-kGy irradiated proteins compared with a nonirradiated protein (Fig. 2) and that the structural modification of milk proteins by irradiation decreased the solubility of the proteins (Figs. 2A and 3).

In conclusion, the allergenicity of milk allergens can be reduced by gamma irradiation.

ACKNOWLEDGMENT

This project has been carried out under the Nuclear Research and Development Program by the Korean Ministry of Science and Technology.

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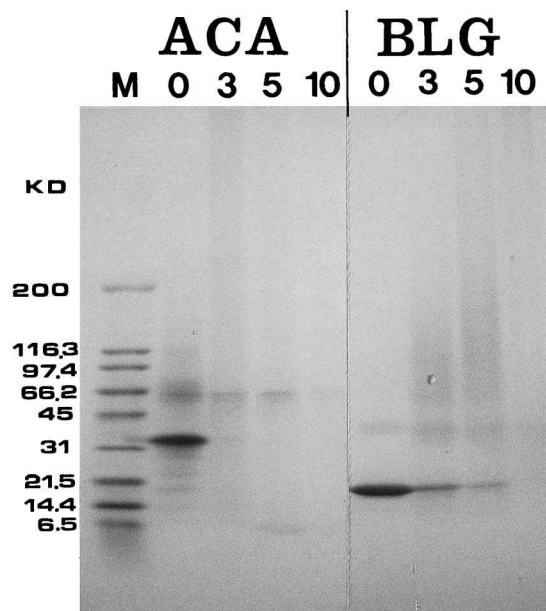


FIGURE 3. SDS-PAGE (5 to 15% gradient acrylamide gel) of the irradiated ACA and BLG (2 mg/ml). M and numerals indicate molecular weight standard and the irradiation doses (kGy), respectively.

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