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Deleterious effects of electron beam radiation on allergen extracts

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Background: The recent threat to the public posed by the dissemination of *Bacillus anthracis* through the US postal system has resulted in increased security measures, including electron beam irradiation for the sterilization of some mail. The deleterious effects of electron beam radiation on biological products are not fully understood.
Objective: The purpose of this investigation was to assess the effect of electron beam radiation, as currently used to sterilize packages and mail in the United States, on several standardized or characterized allergen extracts.
Methods: Selected irradiated extracts were analyzed for allergen content and potency by SDS-PAGE, immunoblot, and ELISA (including inhibition) and compared with untreated extracts.
Results: The compositions and immunochemical potencies of these products were altered significantly by irradiation treatment. Physical changes to native protein structures observed after electrophoretic separations coincided with near-complete loss of allergenic and antigenic epitopes present on major and minor allergens, according to ELISA and immunoblot comparisons with untreated extracts.
Conclusions: These results indicate that extracts subjected to electron beam sterilization conditions are likely to contain modified component structures and properties that might compromise the clinical effectiveness of these products. (J Allergy Clin Immunol 2002;110:215-9.)

Key words: Electron beam radiation, ionizing radiation, allergen extracts

The recent threat to the public posed by the dissemination of *Bacillus anthracis* through the US postal system has resulted in increased security measures, including the use of ionizing radiation for the sterilization of some mail. Ionizing radiation for commercial use can be produced in 3 forms: gamma, x-ray, and high-speed electron accelera-

Abbreviations used
AP-Avidin: Alkaline phosphatase–labeled avidin
BCIP/NBT: Bromochloroindoxyl phosphate/nitroblue tetrazolium
e-beam: Electron beam
pNPP: Para-nitrophenyl phosphate
USACAEL: US Army Centralized Allergen Extract Laboratory
USPS: US Postal Service

tion (electron beam [e-beam]). E-beam irradiation, previously used for sterilizing medical equipment, is now being used by the US Postal Service (USPS) for sterilizing certain mail to eradicate anthrax spores. The deleterious effects of e-beam radiation on biological products are not fully understood, particularly at doses required to sterilize microbial spores (30-50 kGy).[1]

The US Army Centralized Allergen Extract Laboratory (USACAEL) sends and receives vials of allergen extracts from facilities throughout the world. Most of these are shipped through the US postal system. USACAEL is located in the area of Washington, DC, where incoming mail to federal agencies is subjected to ionizing radiation.

The purpose of this study was to evaluate the effects of e-beam irradiation on allergen extract composition and potency.

MATERIALS AND METHODS
Extracts studied and e-beam irradiation

Two identical volumes of each of the following glycerinated extracts were placed in 10-mL vials: timothy (100,000 BAU/mL), perennial rye (100,000 BAU/mL), *Dermatophagoides pteronyssinus* (5000 AU/mL), *Alternaria alternata* (1:10 w/v), and ragweed mix (1:10 w/v; equal parts of giant and short ragweed). In addition, 1:10 v/v dilutions of each of these extracts (1 mL extract in 9 mL human serum albumin) were studied (with the exception of perennial rye). All extracts were obtained from Hollister Stier (Spokane, Wash) except the dust mite extract, which was manufactured by ALK-Abello (Wallingford, Conn). One set of extracts and dilutions was packaged in standard containers made of Styrofoam (Dow Chemical Company, Midland, Mich) and padded envelopes and sent via the USPS to the e-beam contractor, Titan Technologies (Lima, Ohio) for irradiation. The entire package was handled according to postal service protocol and returned within 2 days after undergoing e-beam sterilization. The package was subjected to 2

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passes through the radiation beam for maximal sterilization, which is standard procedure for the mail. The control set of extracts and dilutions was held at USACAEL. One milliliter of each extract pair was aliquotted and sent to Greer Laboratories (Lenoir, NC) for specific allergen measurements, ELISA inhibition (relative potency) determinations, and immunoblot analyses. All samples tested were blinded as to which had been irradiated. The control and irradiated extract pairs were also evaluated by SDS-PAGE.

Gel electrophoresis

Extract proteins were analyzed with the Novex NuPAGE SDS-PAGE system (Invitrogen Life Technologies, Carlsbad, Calif) under reducing conditions through use of NuPAGE 4-12% Bis-Tris gels with a MOPS (3-[N-morpholino] propane sulfonic acid) buffer according to the manufacturer's protocol. Gels were stained with either Coomassie blue or the Gelcode silver stain kit (Pierce, Rockford, Ill). Bradford protein assays were conducted through use of BSA standards and dye concentrates from Pierce; the manufacturer's instructions were followed.

Immunoblot

SDS-PAGE immunoblotting analyses were performed with 12% acrylamide gels through use of a Mini-Protean II assembly (Bio-Rad, Hercules, Calif) at 20°C to 25°C under nonreducing conditions and transferred to Immobilon-P^{SQ} polyvinylidene difluoride membranes (Millipore, Bedford, Mass) through use of passive migration. After blocking, membranes were incubated with allergic human sera (1:20-1:50 v/v final dilutions) in a high-salt TBS buffer, washed 3 times, and probed with goat antihuman IgE-alkaline phosphatase conjugate. IgE binding proteins on blots were visualized by addition of bromochloroindoxyl phosphate/nitroblue tetrazolium (BCIP/NBT).

Allergen content by ELISA and ELISA inhibition

A 2-site (double-bind, sandwich) ELISA procedure was used to measure the following allergens: grass group 1 antigens (perennial rye and timothy), Der f 1 (*Dermatophagoides farinae*), Der p 1 (*D pteronyssinus*), Alt a 1 (*A alternata*), and Amb a 1 (short ragweed). Either monoclonal mouse IgG antibodies (Der f 1 and Der p 1) or polyclonal rabbit IgG (Alt a 1, Amb a 1, grass group 1 antigens) was used as both capture and probe. Unmodified and biotinylated antibodies specific for Der f 1 and Der p 1 were obtained from Indoor Biotechnologies (Charlottesville, Va). Rabbit antisera directed against short ragweed Amb a 1 or group 1 grass antigens (perennial rye Lol p 1 and homologs) were produced at Greer Laboratories according to standard immunization procedures through use of Freund's complete and incomplete adjuvants. Rabbit anti–Alt a 1 antiserum was purchased from Dr Hari Vijay (Health Canada, Ottawa, Ontario, Canada). Allergenic extracts used as ELISA reference preparations were produced at Greer Laboratories and included *D farinae*, *D pteronyssinus*, *A alternata*, short ragweed, timothy, and perennial rye grass. Alkaline phosphatase–labeled avidin (AP-Avidin) was acquired from Zymed Laboratories (South San Francisco, Calif). Alkaline phosphatase substrates para-nitrophenyl phosphate (pNPP) and BCIP/NBT were purchased from Amresco (Solon, Ohio). Buffers, salts, detergent (Tween 20), and goat antihuman IgE-alkaline phosphatase conjugate were sourced from Sigma (St Louis, Mo).

ELISA microtiter plates (either Immulon 2 HB [Dynex Technologies, Chantilly, Va] or Costar [Corning Inc, Corning, NY]) were coated with sera or antibodies (1:100-1:5,000 dilutions) in carbonate (pH 9.6) or phosphate buffer for 12 to 24 hours, then washed 3 times with PBS containing 0.05% Tween 20. Coated plates were incubated with reference or test antigens at variable concentrations (starting

dilutions, 1:5-1:2,430; 2- or 3-fold serial dilutions) in a high-salt PBS buffer for 3 to 6 hours, washed, and probed with biotinylated antibodies in PBS buffer. Biotinylated antibodies bound to the plates were detected by successive incubations with AP-Avidin and pNPP. Absorbances were measured at 405 nm and 600 nm through use of a Bio-Tek Elx808 (Winooski, Vt) microplate reader.

ELISA inhibition analyses of grass pollen and dust mite extracts were performed by coating microplates (Costar) with reference extracts (Greer standardized concentrates) diluted 1:500 in carbonate buffer for 12 to 18 hours at 2°C to 8°C; this was followed by incubations with allergic human serum pools (in-house or US Food and Drug Administration reagents; 1:30-1:50 dilutions) in the presence of varying concentrations of treated or untreated extracts. Antigen-specific IgE molecules were detected through use of biotinylated goat antihuman IgE (Kirkegaard and Perry, Gaithersburg, Md), AP-avidin, and pNPP. Absorbance measurements and rates of reaction were determined as described above for double-bind ELISA. The extent of inhibition was calculated for each test or reference sample, relative potencies being determined through use of a parallel line bioassay algorithm.

Statistical methods

Pre- and post-irradiation gel banding patterns were compared through use of a McNemar test; allergen and total protein contents before and after irradiation were analyzed through use of a Wilcoxon signed-rank test.

RESULTS

The irradiated packages underwent 2 cycles of e-beam irradiation (at 46.8 kGy and 46.1 kGy). According to sources at Titan Technologies, package temperatures can reach as high as 71°C to 82°C, though the specific temperature of our package was not measured. On visual inspection, the glass vials were observed to have changed from clear to amber (Fig 1), and a few drops of extract had leaked out of several vials, suggesting a compromise of the butyl rubber stopper. On removal of the rubber stopper there was a release of pressurized air and a strong pungent odor from each of the irradiated vials; this was not observed with any of the control vials. All of the extracts were also discolored. Fig 1 shows a sample of the *D pteronyssinus* extract placed in a clear plastic vial. The ragweed extract had developed a precipitate (not shown). Minimal changes in total protein levels were observed after irradiation (Table I); these were not statistically significant ($P = .508$ by Wilcoxon signed-rank test). Gel electrophoresis patterns reveal the dramatic loss of essentially all major protein bands after irradiation (Fig 2; P values ranged from <.0005 to .004). (Not all gels are pictured, but results were similar with all extracts.) Immunoblot results on *D pteronyssinus*, timothy, and perennial rye extracts confirmed the loss of relevant IgE binding (a representative *D pteronyssinus* blot is shown in Fig 3). The major allergen contents of the extracts are listed in Table I. All extracts (full-strength and diluted) showed degradation of the major allergen to the point at which in some cases it was not detectable ($P = .004$ by Wilcoxon signed-rank test). A marked decrease in relative potency of the irradiated extract was noted on the ELISA inhibition study (Table II) for *D pteronyssinus* and timothy extracts (only these extracts were evaluated by inhibition).
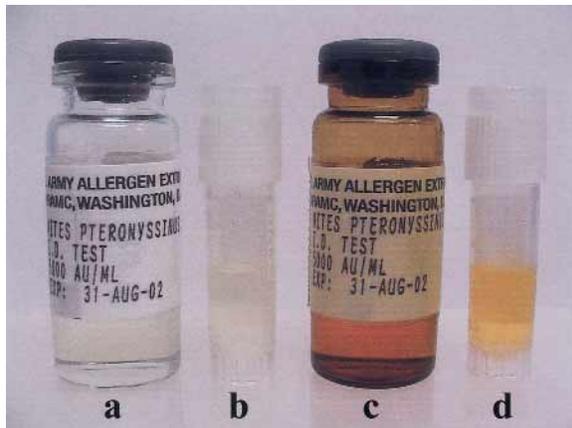
FIG 1. a, Uuntreated *D pteronyssinus* vial. **b,** Extract removed from control vial and placed in a clear plastic vial for comparison purposes. **c,** *D pteronyssinus* vials after irradiation show amber color change in the glass. **d,** Extract removed from the irradiated vial and placed in a clear plastic vial to show discoloration of the extract after irradiation.

DISCUSSION

The sterilizing quality of ionizing radiation is due to high-energy transfer that disrupts the DNA chains and breaks protein bonds.[2] The energy imparted from the radiation beam not only has sterilizing effects on organic material but also affects the physical properties of inert materials such as glass. The high energy produces ionization in the molecules of the irradiated material, and this results in a series of molecular dissociations and addition reactions that lead to a new state of stabilization, which can be more or less durable. The physical and chemical changes resulting from the stabilization process can include the production of chromophores, which are responsible for discoloration.[3] In the case of glass products, the mineral content of the glass determines the color change (amber in our borosilicate extract vials). Color changes can occur in advance of any loss in physical property of the material; thus the change to amber does not necessarily indicate a deleterious effect on the glass or the extracts.[3] However, we cannot be absolutely certain that there were no physical changes in the glass that might have affected the leachability properties. The odor generated from irradiation is the result of specific radiostabilizing chemical reactivity; again, however, this is not necessarily a sign of degradation. The seal of the butyl rubber stopper in several, but not all, vials had been compromised; this is not surprising, inasmuch as the literature on radiation effects reports butyl rubber as having poor radiation stability.[3] The compromised stoppers could pose an additional risk by allowing bacterial contamination, but this was not investigated in the present study.

E-beam radiation tends to be a low-temperature process at low doses; however, doses at the level used in this study might raise temperatures to 71°C to 82°C (oral communication from Titan Technologies, March 2002). The occurrence of deleterious thermal effects on the studied extracts could not be determined, because the actual package tem-
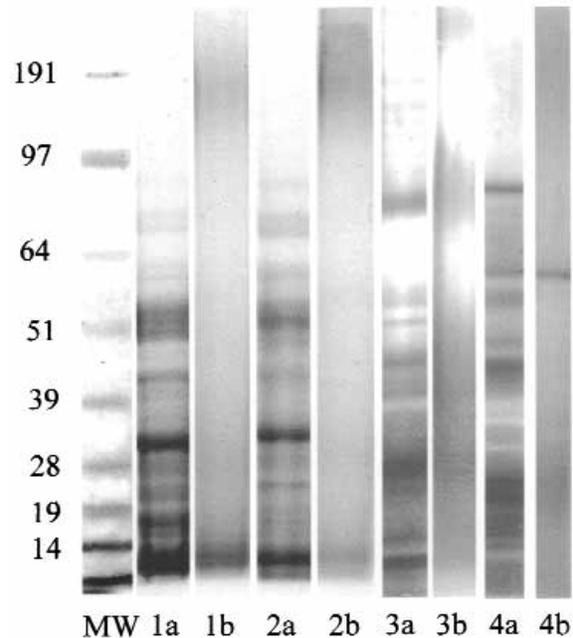
FIG 2. SDS PAGE of 5 selected gels. *1a,* Control timothy. *1b,* Irradiated timothy; loss of all bands; $P < .0005$. *2a,* Control rye. *2b,* Irradiated rye; loss of all bands; $P = .004$. *3a,* Control Der p. *3b,* Irradiated Der p; loss of all bands; $P < .0005$. *4a,* Control *Alternaria*. *4b,* Irradiated *Alternaria*; loss of all bands except one; $P < .0005$. *MW,* Molecular weight markers (kilodaltons). To confirm results, Der p control and irradiated samples were run 4 times and *Alternaria* control and irradiated samples were run 2 times, all with consistent findings.
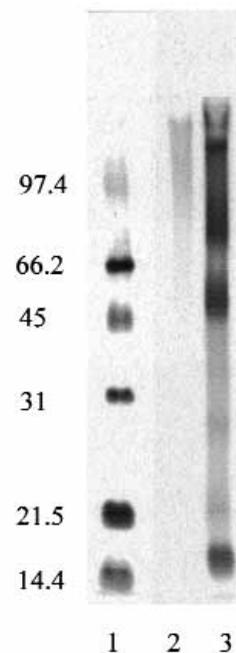
FIG 3. Representative IgE immunoblot of *D pteronyssinus*. *Lane 1,* Molecular weight markers (kilodaltons). *Lane 2,* Irradiated sample shows loss of IgE binding. *Lane 3,* Control extract. $P = .031$. (Similar results with timothy and perennial rye are not shown.)

TABLE I. Major allergen content and total protein of the studied extracts before and after e-beam irradiation

Extract (stock concentration)	Allergen measured	Amount of allergen (µg/mL)		Total protein (µg/mL)	
		Control	Irradiated (% of control)	Control	Irradiated
Alternaria (1:10 w/v)	Alt a 1	2.26	<0.08 (<3.5)	248.4	305.8
Alternaria (1:100 w/v)	Alt a 1	0.186	<0.009 (<4.8)	306.6	334.5
D pteronyssinus (5000 AU/mL)	Der p 1	34.23	0.21 (0.6)	277.1	225.8
D pteronyssinus (500 AU/mL)	Der p 1	1.88	0.029 (1.5)	299.3	216.0
Perennial rye grass (100,000 BAU/mL)	Group 1 ag (Lol p 1)	1105.77	273.03 (24.7)	2978.4	2997.4
Timothy grass (100,000 BAU/mL)	Group 1 ag (Lol p 1)	2420.11	205.91 (8.5)	2663.8	2901.0
Timothy grass (10,000 BAU/mL)	Group 1 ag (Lol p 1)	95.43	8.89 (9.3)	457.7	399.6
Ragweed (1:10 w/v)	Amb a 1	145.19	0.62 (0.4)	2800.7	2950.1
Ragweed (1:100 w/v)	Amb a 1	20.02	0.09 (0.4)	558.9	699.9

TABLE II. Relative potency of *D pteronyssinus* and timothy extracts by ELISA inhibition

Extract (stock concentration)	Relative potency*		Potency of irradiated extract as percent of control value
	Control extract	Irradiated extract	
D pteronyssinus (5000 AU/mL)	0.546	0.017	3.1
Timothy grass (100,000 BAU/mL)	0.916	0.048	5.2

*Relative potency determined by parallel line bioassay algorithm. Results are a ratio of test sample concentration divided by standard concentration.

perature was not available. However, it is unlikely that thermal factors contributed to the protein degradation, inasmuch as the maximum temperatures reported are 71°C to 82°C, which are at or below the temperatures (approximately 70°C to 100°C) used to process samples for gel electrophoresis. Thus the changes seen in Figs 2 and 3 were due to irradiation rather than temperature effects, because control extracts showed banding even after heating.

Although many factors contribute to the negative radiation effects on material, one of the primary components is radiation dose. The doses of e-beam radiation required to sterilize *B anthracis* have not been formally determined, but gamma radiation data show denaturing at a dose of 41.5 kGy.[1] The current e-beam sterilization dose for US mail (30-50 kGy) was based on the gamma radiation data. However, to ensure complete sterilization, the USPS protocol includes 2 passes through the beam. The effects of the 2 passes are additive, resulting in a total dose for our package of 92.9 kGy.[2,4] In comparison, the doses allowed by the US Food and Drug Administration for food sterilization are 1 kGy for fruits and vegetables, 3 kGy for eggs, and 3 to 7 kGy for meats.[5] Thus the dose used for anthrax is approximately 100-fold greater, perhaps explaining why the results in this study were so dramatic.

The degradation of the proteins was suggested by the color change of the extracts and precipitate in the ragweed vial and confirmed by the significant reduction in the amount of major allergen measurable after irradiation and the disappearance of most bands on gel electrophoresis and immunoblot. The concentration of the extracts did not have any effect on their stability; both full-strength and diluted extracts were degraded. Not only were the proteins degraded, allergenicity was lost. This was evident from the loss in IgE binding noted on immunoblots and

from the relative loss of potency as determined by ELISA inhibition. The effects of e-beam irradiation on allergenic extracts have not previously been studied, and very little literature exists on radiation effects on allergenic products in general. One report was found in the literature that evaluated the effects of gamma radiation on heat-stable shrimp allergen. The authors reported a dose-dependent reduction in the shrimp allergen, but they only evaluated up to 10 kGy. Even at this relatively low dose, they noted degradation of the protein.[6]

There are no data on the effects of e-beam irradiation on other biologicals (such as vaccines and monoclonal antibodies); however, given that the protein degradation seen in the allergen extracts was extensive, one can postulate that other biologicals would be similarly affected. An interesting question for future studies is how T-cell epitopes are affected by e-beam irradiation.

Although USPS irradiation is limited at this time to certain mail going to zip codes 202— through 205—,[7] future bioterrorism threats might lead to more widespread use of this sterilization measure.

In conclusion, we have demonstrated that the e-beam radiation dose used by the USPS to sterilize mail has significant degrading effects on allergenic proteins regardless of the concentration. Not only are the proteins degraded, there is also loss of allergenicity. All health care providers using allergen extracts for testing and treatment should become aware of the local mail policy regarding e-beam irradiation for microbial sterilization.

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