

Hypersensitivity to bacterial enzymes

I. Atopic hypersensitivity induced in rhesus monkeys

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The present study demonstrates that intracutaneous inoculations of bacterial enzymes (Novo alcalase and Monsanto DA-10) used in laundry detergents induce significant levels of homocytotropic antibody in rhesus monkeys. None of the animals immunized with these bacterial enzymes developed either Arthus or delayed skin reactions during the duration of this study. The ability to induce atopic reactions in rhesus monkeys with bacterial enzymes used in laundry detergents provides an animal model which permits evaluation of: (1) the relative allergenicity of bacterial enzymes and (2) the changes in bronchial or alveolar functions at various stages after sensitization.

Since the addition of bacterial enzymes to household detergents, several reports¹⁻⁵ have been made of respiratory difficulties among workers in plants manufacturing laundry detergents. The symptoms reported included nasal stuffiness, rhinorrhea, throat irritation, wheezing, breathlessness, and chest tightness. These symptoms and the results of skin and provocation tests²⁻⁴ suggest the involvement of atopic (type I) or possible type III reactions. Serum precipitins directed against bacterial enzymes have been demonstrated^{2, 4} in both exposed and unexposed factory workers. Serum precipitins are usually found in organic dust diseases (e.g., farmer's lung), and considerable attention has been drawn to their possible role in the pathogenesis of observed bronchial and alveolar inflammatory lesions.

Although the incidence of sensitization to bacterial enzymes is highest among employees manufacturing enzyme-containing laundry detergents, the danger of sensitization may not only be restricted to them but also may represent a potential hazard for the consumer.

Previous studies in our laboratory demonstrated that intracutaneous injections of either a low molecular weight fragment of timothy pollen (antigen D)⁷ or an aqueous extract of *Ascaris suum*⁸ induced atopic hypersensitivity in rhesus monkeys. The present study was undertaken to determine if intracutaneous immunization of rhesus monkeys with two partially purified bacterial enzymes (Novo alcalase and Monsanto DA-10) used in laundry detergents would induce atopic hypersensitivity in rhesus monkeys. The availability of an animal model

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exhibiting atopic hypersensitivity to these bacterial enzymes would permit studies of: (1) relative allergenicity and cross-reactivity of various enzymes, (2) incidence of serum precipitins upon repeated exposure to enzymes, and (3) the pathophysiology of bronchial and alveolar inflammatory lesions.

MATERIALS AND METHODS

Antigens

Novo alcalase (subtilopeptidase A derived from the Carlsberg strain of *B. subtilis*) and Monsanto DA-10 (subtilopeptidase B derived from BPN strain of *B. subtilis*) bacterial enzymes used in this study were provided by Dr. E. V. Buehler, Procter and Gamble Company, Cincinnati, Ohio. Both the Novo alcalase and Monsanto DA-10 represent partially purified enzyme preparations: Monsanto DA-10 contains three different classes of enzymatic activities (α -amylase, alkaline, and neutral proteases), and Novo alcalase possesses alkaline protease activity.

Animals

Macaca mulatta monkeys weighing 2 to 4 kilograms were kept in a closed colony for one year prior to the initiation of this experiment. Guinea pigs (250 to 300 grams) used in passive cutaneous anaphylaxis experiments were of the Hartley and Campbell-Trapani (C-T) strains.⁹

Antisera

Human sera obtained from patients showing direct skin reactions (wheal and flare) upon intradermal challenge with either Novo alcalase or Monsanto DA-10 bacterial enzyme preparations were provided by Dr. E. V. Buehler. These serum samples were utilized to establish the optimum antigen concentration required for the release of histamine from passively sensitized monkey lung tissue. Rabbit antisera directed against either Novo alcalase or Monsanto DA-10 bacterial enzymes were provided by Dr. E. V. Buehler. Each antiserum was used to establish the optimum antigen concentration (5 to 10 μ g protein) required to demonstrate positive passive cutaneous anaphylaxis reactions in guinea pigs. Control sites included: (1) rabbit antisera directed against the appropriate bacterial enzyme, (2) rhesus antiovine serum albumin, and (3) saline.

Sensitization of monkeys

Twelve rhesus monkeys were divided into four groups of 3 animals and were bled prior to sensitization (Day 0). Sensitization was accomplished by intradermal injections of 0.1 ml. of an appropriate dilution of the bacterial enzyme preparation. Groups I and II animals were immunized, respectively, with either 10 μ g protein or 1 μ g protein of the Monsanto DA-10 bacterial enzyme preparation at 7 or 14 day intervals. Group III and Group IV animals were immunized, respectively, with either 10 μ g protein or 1 μ g protein of the Novo alcalase bacterial enzyme preparation at 7 or 14 day intervals. Animals of Groups I and III were skin tested with 1 μ g protein of the appropriate antigen prior to receiving their immunizing injections (10 μ g protein) of antigen. All the animals of Groups I and III gave positive cutaneous reactions following the third sensitization injection (15 days after the initial sensitization).

In vitro anaphylactic histamine release

Passive sensitization. Fragments of rhesus monkey lung tissue were passively sensitized with sera obtained from bacterial enzyme-sensitized animals, and the lung fragments were then incubated with the appropriate antigen. The amount of histamine released was determined by a spectrofluorometric method previously described.^{10, 11}

Direct sensitization. Rhesus monkey No. 4305 was killed, and the lung tissue perfused with 500 ml. of fresh Tyrode's solution. Lung tissue fragments of Animal 4305 were incubated

TABLE I. Skin test reactions of rhesus monkeys immunized with bacterial enzymes

Group	Antigen	Dose (μ g)	Animal No.	Skin reactions*							
				3/26	4/2	4/9	4/23	4/30	5/7	5/14	5/21
I	Monsanto DA-10	1	4535	-	-	2+	-	+	-	-	-
		10		-	-	2+	±	+	+	+	2+
		1	3567	-	-	-	±	±	±	-	-
		10		-	-	+	±	±	+	+	+
		1	3305	-	-	2+	±	2+	2+	-	+
		10		-	+	3+	3+	3+	3+	2+	3+
II	Monsanto DA-10	1	3769	-	-	±	-	±	-	-	-
		1	3943	-	-	±	-	±	-	±	-
		1	3952	-	-	-	-	-	+	-	-
III	Novo alcalase	1	3958	-	-	-	-	±	-	±	±
		10		-	±	3+	3+	+	+	3+	3+
		1	3975	-	-	+	+	+	2+	+	+
		10		-	±	3+	3+	3+	3+	2+	3+
		1	4305	-	+	+	+	+	+	2+	2+
		10		-	2+	2+	2+	3+	3+	3+	2+
IV	Novo alcalase	1	4321	-	±	±	±	-	±	±	-
		1	4322	-	±	-	-	-	-	-	±
		1	3191	-	-	±	-	-	+	2+	±

*Reactions were read 10 to 15 minutes after intradermal injection of antigen. Reactions were based on the diameter of the wheal size and graded as follows: 4+, greater than 20 mm. diameter; 3+, 16 to 20 mm. diameter; 2+, 11 to 15 mm. diameter; +, 5 to 10 mm. diameter; ±, 0 to 5 mm. diameter; -, negative.

with optimal concentrations of either Novo alcalase or Monsanto DA-10 for 15 minutes at 37° C. The supernatant fluid was removed and analyzed for the amount of histamine released as described above.

Protein determinations were carried out using the Nessler reaction as described by Campbell and associates.¹²

RESULTS

Table I shows the sensitization schedule, the onset of skin reactivity, and the extent of skin reactions developed by the four groups of sensitized rhesus monkeys. All of the animals immunized with 10 μ g protein of either Novo alcalase or Monsanto DA-10 bacterial enzyme preparations had developed wheal and flare reactions 14 days after beginning sensitization. Wheal and flare skin reactions occurred 20 to 30 minutes after injection of antigen and were identical to skin reactions (wheal and flare) previously described in *Ascaris*-sensitized rhesus monkeys.⁹ No skin reactions were observed at 4 to 6, 24, or 48 hours. Animals (Groups II and IV) immunized with 1 μ g protein of bacterial enzymes did not develop sensitivity throughout the 2 month (8 injections) immunization schedule. However, once animals (Groups I and III) showed positive skin reactions with 10 μ g protein of either bacterial enzyme preparation, these animals also showed reactivity with the skin test dose (1 μ g protein) of the bacterial enzyme preparation. Attempts to demonstrate precipitating antibody in the sera of either the

TABLE II. Comparison of direct skin reactivity and histamine release from monkey lung tissue

Antigen	Sensitizing dose (μ g)	Animal No.	Date	Skin reaction	Histamine* release
Monsanto DA-10	10	3305	3/10†	-‡	0.14
			4/15	3+	4.5
			5/12	2+	2.3
Monsanto DA-10	1	3952	3/10†	-	-
			4/15	-	-
			5/12	-	-
Novo alcalase	10	3958	3/10†	-	2.5
			4/15	3+	1.8
			5/12	3+	3.0
Novo alcalase	10	3975	3/10†	-	1.2
			4/15	2+	1.6
			5/12	3+	2.3
Novo alcalase	1	4322	3/10†	-	-
			4/15	-	-
			5/12	-	-
Novo alcalase	10	4305	3/10†	-	-
			4/15	2+	-
			5/12	3+	-

*Micrograms histamine release per milliliter serum.

†Preimmunization bleeding.

‡Negative.

Novo alcalase- or Monsanto DA-10-sensitized animals by gel diffusion and passive cutaneous anaphylaxis in guinea pigs were unsuccessful.

In vitro histamine release studies employing monkey lung tissue passively sensitized with sera from bacterial enzyme-sensitized animals showed various degrees of histamine release. Table II compares the direct skin reactivity and the quantity of histamine released from lung tissue passively sensitized with sera from the same animals. Animals immunized with 1 μ g protein of either Novo alcalase or Monsanto DA-10 preparations showed neither direct skin reactions nor antibodies in the sera that passively sensitized monkey lung tissue to release histamine upon antigen challenge. The sera from animals immunized with 10 μ g protein of either bacterial enzyme preparation contained antibodies that passively sensitized monkey lung tissue for release of histamine upon antigenic challenge. The histamine released by sensitization with the sera from these animals loosely correlates with the direct skin reactions observed in these animals. Preimmunization sera of some monkeys showed the presence of antibody that binds to monkey lung tissue resulting in histamine release after antigenic challenge. In general, the capacity of the sera from these animals to sensitize for histamine release increased following immunization. Animal 4305 gave positive skin reactions with 10 μ g protein of Novo alcalase. However, the sera from this animal failed to passively sensitize monkey lung tissue. Animal 4305 was killed, the lung tissue perfused with 500 ml. of fresh Tyrode's solution, and tissue slices (200 mg. wet weight) incubated with Novo alcalase (5 μ g protein), Monsanto DA-10 (5 μ g protein), or 5 μ g of dialyzed extract of timothy pollen.¹³ Tissue

TABLE III. Skin test reactions of rhesus monkeys immunized with bacterial enzymes

Animal No.	Sensitizing antigen	Challenging antigen*	Skin reactions†	
			5/22	9/22
3305	Monsanto DA-10	Monsanto DA-10	3+	+
3958	Novo alcalase	Novo alcalase	3-	+
3975	Novo alcalase	Novo alcalase	3+	+
4305	Novo alcalase	Novo alcalase	2+	+

*Ten micrograms protein used as challenging antigen.

†Skin reactions based upon diameter of wheal size and were graded as follows: 4+, greater than 20 mm.; 3+, 16 to 20 mm.; 2+, 11 to 15 mm.; +, 5 to 10 mm.

challenged with either Monsanto DA-10 or the dialyzed extract of timothy pollen showed no histamine release; on the other hand, tissue challenged with Novo alcalase showed 0.51 μ g histamine released per gram of wet weight.

Table III shows that once an animal develops sensitivity to a particular bacterial enzyme, direct skin reactivity is retained without further exposure to antigen for at least four months. Animals sensitized to one bacterial enzyme (Novo alcalase or Monsanto DA-10) did not show cross-reactivity with the other bacterial enzyme either by direct skin tests or by the histamine release method.

Recent studies have been directed toward elucidating the nature of the immunoglobulin produced in rhesus monkeys following sensitization with either Novo alcalase or Monsanto DA-10 sensitization. Adsorption experiments with specific rabbit antirhesus immunoglobulins (IgG, IgA, IgM) failed to remove capacity of the sera from Animals 3305, 3958, and 3975 to sensitize for antigenic histamine release.

DISCUSSION

The results (Table I) of the present experiments show that rhesus monkeys sensitized with 10 μ g protein of either Novo alcalase or Monsanto DA-10 enzyme preparations by intradermal inoculations developed wheal and flare reactions upon intradermal challenge. Persistence of the skin reactivity in the sensitized animals varied between about 90 and 120 days (Table III). However, all animals responded to restimulation with 10 μ g protein of the sensitizing antigen and attained their previous level of sensitivity. Animals given intradermal inoculations of 1 μ g protein of either bacterial enzyme preparation (skin test dose) at weekly intervals for 8 to 12 weeks did not develop wheal and flare reactivity. None of the sensitized rhesus monkeys showed any sign of either Arthus or delayed hypersensitivity reactions following intradermal challenge during the duration (5 months) of this study. Sera obtained from the sensitized rhesus monkeys failed to show precipitins directed against either bacterial enzyme preparation by either gel diffusion analysis or passive cutaneous anaphylaxis in guinea pigs.

The histamine release from passively sensitized lung tissue demonstrates that the wheal and flare reactions observed (Table II) were associated with circulating antibody. Previous studies¹⁰ showed that neither heterologous (rabbit) nor homologous (rhesus) precipitating antibody would passively sensitize rhesus monkey lung tissue fragments for histamine release. Passive sensitization and

histamine release have been observed only with human reaginic sera and with sera from either *Ascaris*- or antigen D-sensitized rhesus monkeys. The data (Table II) suggest that the antibody mediating these reactions is homocytotropic antibody directed against the bacterial enzymes, Novo alcalase and Monsanto DA-10.

Although all animals showed negative skin tests upon initial inoculation with either enzyme preparation, preimmunization sera obtained from a number of the rhesus monkeys (Tables I and II) showed varying levels of capacity to sensitize for histamine release. In general, tissue controls were negative. Consequently the results with the preimmunization sera cannot be explained by enzymatic action of the antigens upon the tissue fragments. However, in a few cases monkey lung tissue incubated for 2 hours in Tyrode's solution (tissue control) showed some histamine release. When tissue controls were positive, tissue passively sensitized with sera from sensitized rhesus monkeys released significantly more histamine than the tissue controls upon antigenic challenge. The above observation would suggest that some of the animals in this study had developed sensitivity to these bacterial enzymes by natural exposure or to a cross-reacting material. Repeated exposure of these animals to the bacterial enzymes showed increased reactivity upon specific challenge and a correlation between direct skin reactivity and capacity of the sera to sensitize for histamine release. Animals developing skin reactivity to one of the bacterial enzymes do not show reactivity with the other bacterial enzyme. Thus, there was no antigenic cross-reactivity between Novo alcalase and Monsanto DA-10 enzymes.

In general, the histamine release from monkey lung tissue is a more sensitive index of homocytotropic antibody than are direct skin tests. However, Animal 4305 (Table II), which showed strong skin tests in three separate experiments, failed to show circulating antibody in the serum. Direct antigen challenge of perfused lung tissue fragments of Animal 4305 showed significant levels of histamine release, indicating that this monkey possessed significant levels of homocytotropic antibody bound to its own lung tissue. These observations suggest that Animal 4305 had no circulating homocytotropic antibody and resembles reported human allergic patients exhibiting direct skin reactions but little or no demonstrable reagin when assayed by Prausnitz-Küstner reactions.

The present study clearly demonstrates that both bacterial enzymes (Novo alcalase and Monsanto DA-10) induce significant levels of homocytotropic antibody in the rhesus monkey. The time of onset of the homocytotropic antibody response to these enzymes is approximately the same as that previously reported with antigen D- and *Ascaris*-sensitized animals.^{7, 8} The level of homocytotropic antibody produced in the enzyme-sensitized animals (1 to 4.5 μg histamine release [HR] per milliliter) is intermediate between the antigen D-sensitized rhesus monkeys (up to 1.5 μg HR per milliliter) and *Ascaris*-sensitized rhesus monkeys (5 to 15 μg HR per milliliter).

The ability to induce atopic hypersensitivity in the rhesus monkey directed against bacterial enzymes provides an animal model which will permit evaluation of: (1) other enzymes for their allergenic potential, (2) changes in bronchial or alveolar functions during sensitization, (3) reduction of allergenic activity resulting from chemical modification of bacterial enzymes with minimum loss of

enzymatic activity, and (4) relative allergenicity of bacterial enzymes based on capacity of the sera obtained from sensitized animals to passively sensitize monkey lung for antigenic histamine release.

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