

Induction of IgE antibodies with predefined specificity in rhesus monkeys with recombinant birch pollen allergens, Bet v 1 and Bet v 2

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Background: Recombinant birch pollen allergens Bet v 1 and Bet v 2 (birch profilin) have been characterized in vitro previously.

Objective: To establish a close-to-man model of type I allergy, recombinant birch pollen allergens were injected into rhesus monkeys.

Methods: The allergens were expressed in *Escherichia coli*, purified to homogeneity and injected into rhesus monkeys with aluminium hydroxide as adjuvans. The development of type I allergy was monitored by measurement of specific IgE, in vitro histamine release tests, cellular proliferation assays, skin testing, and bronchial provocation tests.

Results: Immunized rhesus monkeys displayed symptoms of type I allergy comparable to those of allergic patients, and cross-reactivity of IgE antibodies with Bet v 1 and Bet v 2 homologous allergens was observed. Systemic application of corticosteroids during secondary immunizations suppressed specific antibody responses.

Conclusion: Recombinant birch pollen allergens (Bet v 1 and Bet v 2) were effective to establish a close-to-man model of natural type I allergy in rhesus monkeys, allowing study of specific IgE regulation in vivo. (*J ALLERGY CLIN IMMUNOL* 1996;97:95-103.)

Key words: Type I allergy, recombinant tree pollen allergens, Bet v 1, profilin Bet v 2, IgE, rhesus monkeys

More than 15% of the population in industrialized countries has allergic symptoms (rhinitis, conjunctivitis, and bronchial asthma) caused by type I allergies.^{1,2} Considerable progress has been made in the last few years regarding the characterization of allergens by using recombinant techniques.³ Several allergens have been cloned and expressed

Abbreviations used

PBMC: Peripheral blood mononuclear cells
SDS: Sodium dodecylsulfate

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Supported by grant 3/9677 of the "Fonds zur Förderung der Gewerblichen Wirtschaft" and by grant S06703-MED of the Austrian Science Foundation.

Received for publication Nov. 15, 1994; revised April 4, 1995; accepted for publication April 4, 1995.

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0091-6749/96 \$5.00 + 0 1/1/65315

in *Escherichia coli*^{4,5,6} and were shown to possess properties similar to their natural counterparts.⁷⁻¹³ Because of extensive immunologic cross-reactivities of relevant allergens, it appears that recombinant allergens might be useful candidates to establish allergen-specific in vivo models to investigate the effects of therapeutic approaches on the regulation of specific IgE antibody responses in vivo.

Previous studies showed that pollen allergy can occur spontaneously in subhuman primates.^{14,15} It was also shown that IgE from monkeys can be detected with anti-human IgE antibodies, and allergic symptoms could be induced in monkeys with crude pollen extracts.¹⁶⁻¹⁸ This encouraged us to

attempt the induction of type I allergy in rhesus monkeys with recombinant allergens. For this purpose, two well-characterized pollen allergens were used: purified recombinant Bet v 1,^{6,7,10} the major birch pollen allergen, and purified recombinant birch profilin (Bet v 2).^{8,12,19} Bet v 1 is a target for IgE antibodies in 95% of all patients allergic to tree pollen^{7,20} and also represents an important food allergen.^{21,22} Profilins belong to a novel class of panallergens, which because of their ubiquitous distribution as actin-binding proteins¹² and similar IgE-binding capacity, are responsible for allergic symptoms in about 20% of all patients allergic to pollen and a high proportion of patients allergic to food.^{8,19,23,24} In addition to the potential of both recombinant allergens to induce type I allergy in a primate model, the effects of corticosteroids on specific IgE responses were studied in rhesus monkeys during secondary immunizations.

METHODS

Immunization of rhesus monkeys

With 100 μ l aluminium hydroxide (Serva, Heidelberg, Germany) as adjuvant, allergens or phosphate-buffered saline (negative control) were injected once a month subcutaneously at a dose of 1 or 10 μ g of allergen per animal into a genetically heterogenous group of male and female adult rhesus monkeys. Blood samples were taken from the monkeys 1 week before and 1 week after the immunizations, and sera were stored frozen at -20° C until use.

To study the effects of corticosteroids on specific IgE responses during reimmunization, a group of six rhesus monkeys was first immunized for a period of 6 months in monthly intervals with 1 μ g of recombinant Bet v 1 adsorbed to 100 μ l of aluminium hydroxide. The immunization was then stopped for 4 months. Two monkeys were then reimmunized with 1 μ g recombinant Bet v 1 adsorbed to Al (OH)₃ without corticosteroids, two monkeys received a parallel intramuscular injection of corticosteroids (Volon A; 40 mg) on the same day, and two monkeys received corticosteroids 4 days after each immunization. The immunization protocol was repeated 4 times in 14-day intervals. Blood samples were obtained before each immunization.

Antibody detection immunoblotting

Natural birch pollen allergens were separated by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis²⁵ and were blotted on to nitrocellulose.²⁶ The monkeys' sera were diluted 1:10 for IgE, 1:100 for IgM and IgA, and 1:1000 for IgG binding. Bound monkey immunoglobulins were detected with 1:1000 diluted rabbit anti-human IgE, anti-IgM, anti-IgG, and anti-IgA antibodies (Dako, Glostrup, Denmark) and with a 1:1000 diluted ¹²⁵I-labeled donkey anti-rabbit

antibody (Amersham, Buckinghamshire, U.K.). When Bet v 1-specific IgE binding in serum samples from animals that were reimmunized with Bet v 1 with and without corticosteroids were determined, care was taken to dilute serum samples in the same way (1:10), and incubations were done with nitrocellulose strips containing equal amounts of Bet v 1 (antigen was present in excess to IgE in all these experiments) to allow a comparison of Bet v 1-specific IgE reactivity among the different serum samples.

Basophil histamine release assays

Blood samples were obtained and heparinized 1 week after the immunizations, and granulocytes were prepared by dextran sedimentation as described.²⁷ Granulocytes were then incubated with various doses of the recombinant allergens (recombinant Bet v 1 or recombinant birch profilin Bet v 2), and the percentage of histamine release compared with the total histamine release obtained by cell lysis was measured in the cell-free supernatants by radioimmunoassay (Immuno-tech, Marseille, France). The spontaneous release was subtracted from the values. All data reflect means of triplicate determinations, and the standard deviations were always less than 15%.

Proliferation assays

Monkeys' peripheral blood mononuclear cells (PBMC) (10^5 /well) were prepared by Ficoll (Pharmacia, Uppsala, Sweden) density gradient and incubated in serum-free medium AIM-V (Gibco, Grand Island, N.Y.) supplemented with 2 mmol/L glutamine and 2×10^{-5} mol/L β -mercaptoethanol with the respective allergen, a control allergen (negative control), or interleukin-2 as a positive control for 6 days followed by 12 hours of incubation with tritiated thymidine (Amersham, Buckinghamshire, U.K.).²⁸ Results, in counts per minute, reflect means of duplicate determinations. The standard deviation in all experiments was <20%.

Skin tests

Skin testing was done as described.²⁹ Monkeys were anesthetized intramuscularly with ketamine hydrochloride (10 mg/kg) and xylazine (0.5 mg/kg). Fifteen minutes after an intravenous injection of Evans blue dye (0.1% to 1 ml/kg), rhesus monkeys were injected intradermally in the abdomen with the allergens. Recombinant Bet v 1 and recombinant birch profilin (100 ng/100 μ l) and commercially available skin prick test solutions (ALK, Copenhagen, Denmark) were used. 100 μ g of histamine dihydrochloride (Sigma Chemical Co., St. Louis, Mo.) served as a positive control. The skin reactions were read 15 minutes after histamine or antigen injections by measuring two diameters of the blue spot, calculating the mean in millimeters, and assessing the color intensity by means of a scoring system (0 to 4). The score established for the color intensity was multiplied by the mean diameter.

Bronchial provocation tests

A representative monkey (D18) sensitized with recombinant Bet v 1 was tested for airway reactivity to birch pollen protein extract 1 week after the seventh immunization. The monkey was anesthetized intramuscularly with ketamine hydrochloride (10 mg/kg) and xylazine (0.5 mg/kg). Birch pollen extract containing Bet v 1 (10 μ g/ml) was nebulized (Bird micronebulizer; Bird Products, Palm Springs, Calif.) with compressed air and administered by intermittent positive pressure breathing (Bird Mark 7A respirator) consisting of 15 inhalations in approximately 1.5 minutes. Specific lung resistance and specific lung conductance were measured before and after challenge with the birch pollen extract by an endotracheal tube connected to an airway mechanics analyzer (Buxco Electronics Inc., New York, N.Y.). All computations were done on a breath-by-breath basis and expressed as changes from prechallenge values. The following parameters were measured: F_1 = frequency, PEF_1 = peak expiratory flow, TV = tidal volume, CDYN = compliance, and RL_1 = resistance.

RESULTS

Induction of specific IgE antibodies in rhesus monkeys with purified recombinant Bet v 1 and Bet v 2: Cross-reactivity of monkeys' IgE antibodies with Bet v 1 and Bet v 2 homologous proteins

Immunization of rhesus monkeys with recombinant Bet v 1 and Bet v 2 led to the induction of IgE, IgG, IgA, and IgM antibodies reacting with nitrocellulose-blotted natural Bet v 1 and Bet v 2, respectively. In the case of recombinant Bet v 1, doses of 1 to 10 μ g per animal per injection were sufficient to induce specific IgE antibodies, whereas 10 to 20 μ g per animal per injection Bet v 2 were needed to induce IgE reactivity to Bet v 2. The IgM and IgA reactivity faded in the course of the immunization, whereas specific IgE and IgG antibodies could be detected even after 1 year of continuous immunization.

As in allergic patients, Bet v 1- and Bet v 2-allergic rhesus monkeys displayed IgE cross-reactivity with homologous allergens. Fig. 1, A shows the IgE reactivity of two representative Bet v 1-allergic monkeys (A, B) with the homologous allergens in hazel pollen and apple. In Fig. 1, B, the IgE reactivity of two Bet v 2-allergic monkeys (A, B) with timothy grass profilin³⁰ is displayed.

Specific histamine release from monkeys' blood basophils on incubation with recombinant Bet v 1 and Bet v 2

In previous studies, it was demonstrated that recombinant Bet v 1 and Bet v 2 were able to

induce dose-dependent and specific histamine release from basophils of sensitized patients. Likewise, basophils from Bet v 1- and Bet v 2-allergic monkeys released histamine in a dose-dependent manner on stimulation with the recombinant allergens. Fig. 2 shows the dose responses done with basophils from three representative Bet v 1- and three representative Bet v 2-allergic rhesus monkeys. No histamine release was observed with basophils from control monkeys that were immunized with phosphate-buffered saline and aluminum hydroxide (data not shown). Recombinant Bet v 1 did not release histamine from basophils of Bet v 2-allergic monkeys, and vice versa (data not shown).

Skin reactivity of Bet v 1- and Bet v 2-allergic monkeys

To determine whether rhesus monkeys with IgE antibodies specific for Bet v 1 and Bet v 2 display positive skin test results on intradermal application of allergens, skin tests with commercial skin test solutions containing natural allergens with recombinant Bet v 1, recombinant Bet v 2, and controls (histamine-positive control, 0.9% sodium chloride) were performed. Table I summarizes the results of the skin testing. Monkeys that had been immunized with recombinant Bet v 1 showed skin reactivity with recombinant Bet v 1, birch pollen extract, and hazel pollen extracts, which contain Bet v 1 or Bet v 1 homologous allergens (Cor a 1). The Bet v 2-allergic monkey (888) displayed skin reactivity with recombinant Bet v 2, natural birch extract, and natural allergen extracts containing Bet v 2 homologous allergens (hazel pollen, timothy grass pollen, celery). Both control monkeys that had been immunized with PBS without allergen and aluminium hydroxide showed no significant skin reactivity with any allergen preparation, although histamine led to a positive reaction.

Recombinant Bet v 1 and Bet v 2 induce specific proliferation of monkeys' PBMC

To demonstrate T-cell reactivity of monkeys' PBMC with recombinant Bet v 1 and Bet v 2, PBMC were incubated with the recombinant allergens, the medium (negative control), and interleukin-2 (positive control), respectively. The proliferation of monkeys' PBMC were measured by tritiated thymidine incorporation in two representative animals (Fig. 3). PBMC from a Bet v 1-allergic monkey (D9) proliferated upon stimulation with Bet v 1 but not when Bet v 2 was used, and PBMC from a Bet v 2-allergic monkey

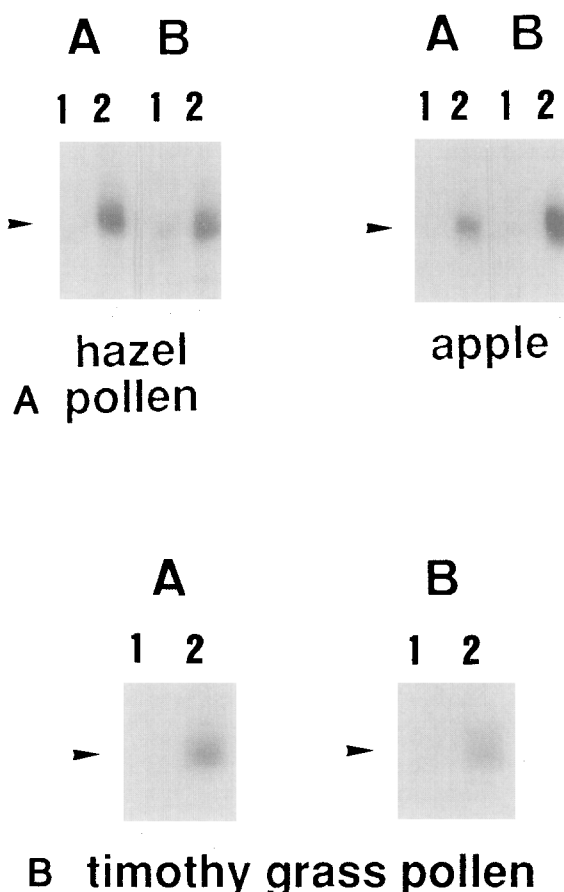


FIG. 1. Allergic monkeys display IgE cross-reactivity with Bet v 1- and Bet v 2-profilin homologous proteins. Serum IgE from two Bet v 1-allergic monkeys (A and B) was used to detect the major hazel pollen allergen, Cor a 1 and the major apple allergen (A). In lane 1 nitrocelluloses were tested with the preimmune sera, whereas in lane 2 immune sera were used. Two of the profilin-allergic monkeys (A and B) were tested for IgE reactivity with timothy grass (*Phleum pratense*) profilin (B). In lane 1 the preimmune serum was used, and in lane 2 the immune serum was used. Arrows indicate IgE binding to natural allergens.

(Vi56) showed specific tritium uptake on stimulation with recombinant Bet v 2. Human interleukin-2 was capable of inducing proliferation of monkeys' PBMC (positive control), whereas low spontaneous proliferation was obtained when PBMC were cultivated in the medium alone (negative control).

Airway responses to aerosolized birch pollen extract in a Bet v 1-allergic monkey

For measurement of respiratory responses, a representative Bet v 1-allergic monkey with positive skin prick test reactivity was selected and tested for airway responsiveness to nebulized birch pollen extract 1 week after the seventh immunization. After inhalation of birch pollen extract, the monkey's breathing frequency increased up to 90%, and bronchial resistance increased to 40%—

whereas compliance decreased up to 50% compared with values before the challenge (data not shown).

Systemic application of corticosteroids suppresses Bet v 1-specific IgE responses during secondary immunization

Corticosteroids are known to effectively reduce allergic symptoms in patients. Having established a close-to-man model of specific type I allergy in primates, we were interested in investigating the in vivo effects of systemic corticosteroid application on specific IgE responses in allergic rhesus monkeys during reimmunization. An experiment which was thought to mimic the situation in patients allergic to Bet v 1 who have contact with Bet v 1 during the birch pollen season, was performed. New contact with Bet v 1 in the next pollen season

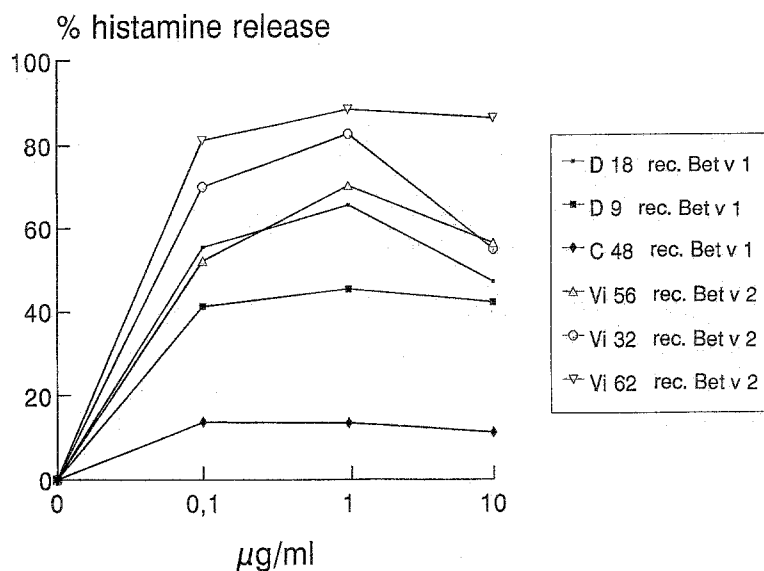


FIG. 2. Blood basophils of Bet v 1 and monkeys allergic to profilin (Bet v 2) release histamine on allergen stimulation. The representative histamine releases demonstrated in three different Bet v 1-allergic monkeys (*D18*, *D9*, and *C48*) and three profilin (Bet v 2)-allergic monkeys (*Vi56*, *Vi32*, and *Vi62*) were dose-dependent. The horizontal axis indicates the concentration of recombinant allergens in micrograms per milliliter, and the vertical axis shows the percentage of histamine release.

TABLE I. Cutaneous reactions in monkeys immunized with recombinant Bet v 1 or recombinant birch profilin (Bet v 2)

Immunogen	Dose (µg/animal)	Monkey No.	Test substance						
			rBet v 1	rBet v 2	Birch pollen	Hazel pollen	Timothy grass pollen	Celery	Histamine
rBet v 1	1	D18	1.8	0.1	1.8	1.5	0	0	2.1
	1	A61	1.4	0.7	2.0	1.8	0	0	2.4
	10	D9	1.4	0	1.4	1.0	0	0	2.4
	10	C48	1.3	0.2	1.3	1.2	0	0.3	1.9
rBet v 2	10	888	0	0.9	1.3	1.5	1.4	1.4	2.4
Control		1	0	0	0	0	0	0	1.8
		645	0	0	0	0	0	0.4	1.9

Rhesus monkeys were immunized once a month with doses of antigens (recombinant Bet v 1 or recombinant birch profilin, Bet v 2) as indicated. Skin tests were performed 10 days after the seventh immunization.

usually increases Bet v 1-specific IgE. A group of six rhesus monkeys was immunized for 6 months with recombinant Bet v 1 to induce specific IgE antibodies. The immunization was then stopped for 4 months so that Bet v 1-specific IgE reactivity was barely detectable in the blood samples obtained at that time (Fig. 4, lane 1 in animals A through F). Two monkeys were then reimmunized with 1 µg of recombinant Bet v 1 (animals A and B; Fig. 4), two animals were reimmunized with 1 µg of recombinant Bet v 1 and received a parallel injection of corticosteroids on the same day (ani-

mals C and D; Fig. 4), and two animals received 1 µg of Bet v 1 and corticosteroids 4 days after the immunization (animals E and F; Fig. 4). The animals were immunized according to this protocol in 14-day intervals four times. Although little or no Bet v 1-specific IgE could be detected before the immunizations in all animals, Bet v 1-specific IgE reactivity increased in the two monkeys that were immunized without corticosteroids (animals A and B; Fig. 4) and in those animals that received corticosteroids 4 days after immunization (animals E and F; Fig. 4). In monkey F Bet v 1-specific IgE

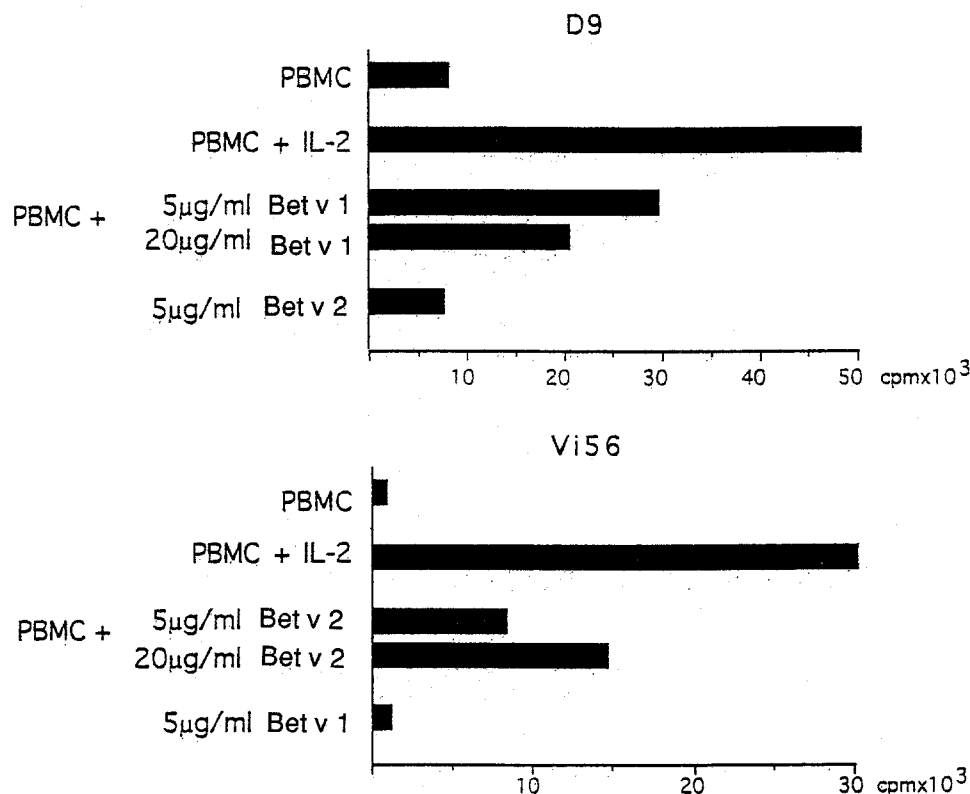


FIG. 3. Cellular proliferation assay with PBMC from a Bet v 1-allergic monkey (*D9*) and a birch profilin (Bet v 2)-allergic monkey (*Vi56*). PBMC were incubated without agonist (PBMC), with interleukin-2 (positive control), and the recombinant allergens (recombinant Bet v 1 or recombinant birch profilin-Bet v 2), respectively. Thymidine incorporation (cpm) after 6 days of culture is displayed on the horizontal axis.

reactivity seemed to be at least partially reduced by the corticosteroid treatment. However, when corticosteroids were applied on the day of the immunization with Bet v 1, specific IgE reactivity was significantly suppressed. In the two animals (C and D; Fig. 4) that were treated according to this protocol, little or no Bet v 1-specific IgE reactivity could be detected, despite continuous reimmunization.

DISCUSSION

The regulation of IgE synthesis, as well as the events during the degranulation of mast cells and basophils leading to the release of biologic mediators, have been studied in great detail,³¹ whereas the characterization of allergens has been initially hampered by the fact that protein and immunochemical techniques used for this purpose were rather time-consuming and laborious. Because of the introduction of complementary DNA cloning techniques, a number of recombinant allergens has become available.³² When tested in vitro, many of

the recombinant allergens behaved similarly to their natural counterparts. Because of extensive structural and immunologic similarities, it appeared that the number of different IgE-binding structures against which patients are sensitized is less complex.³³

To provide experimental evidence for the capacity of recombinant allergens to induce IgE responses in vivo, the major birch pollen allergen, Bet v 1,^{6,7,10} and birch profilin, an actin-binding protein (Bet v 2) representing a cross-sensitizing allergenic component,^{7,8,12,19} were injected into a group of genetically heterogeneous adult rhesus monkeys with aluminium hydroxide as adjuvants.

As previously demonstrated for allergic patients,³⁸ dose-dependent and specific histamine release could be induced in basophils isolated from Bet v 1- and profilin-immunized monkeys but not from basophils of the control animals. PBMC and, in particular, T cells derived from the peripheral blood of allergic patients proliferated on stimulation with natural, as well as recombinant, allergens.^{10,28}

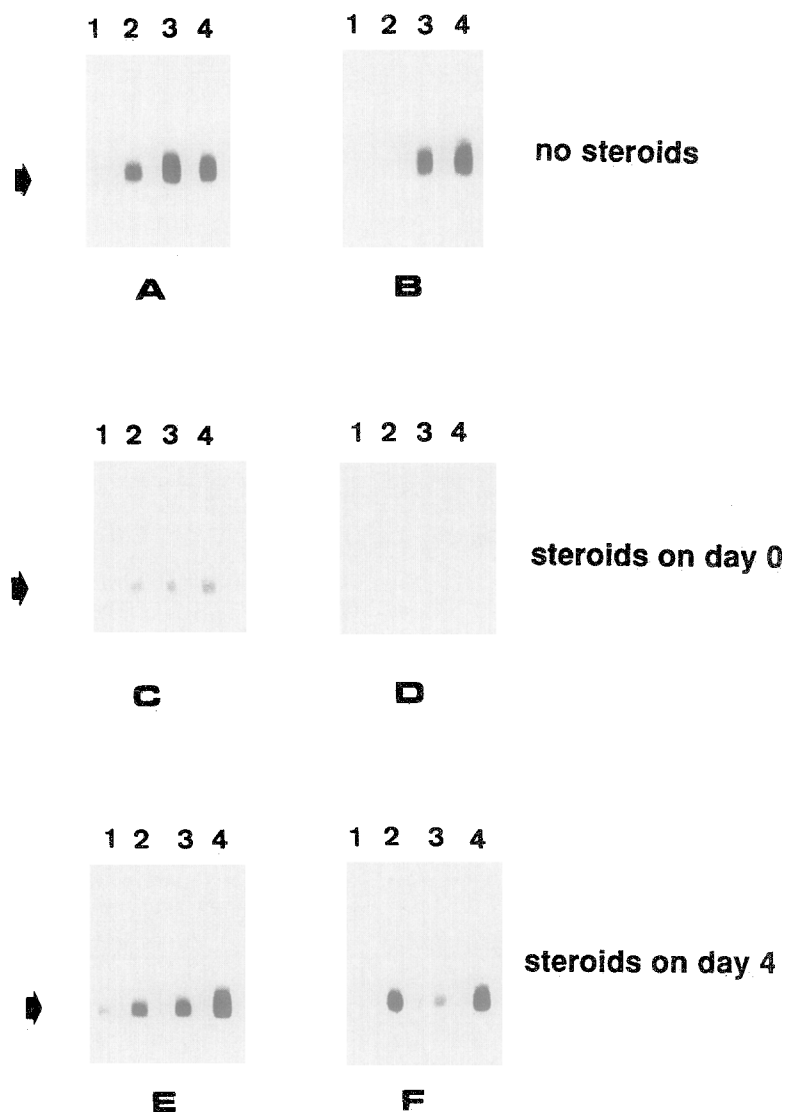


FIG. 4. Influence of systemic corticosteroid application on the IgE reactivity to pollen-derived Bet v 1 in rhesus monkeys reimmunized with recombinant Bet v 1 + Al(OH)₃. A group of six rhesus monkeys (A through F) was immunized for 6 months in monthly intervals with 1 µg of recombinant Bet v 1 adsorbed to Al(OH)₃ to induce Bet v 1-specific IgE antibodies. The immunization was stopped for approximately 4 months until the Bet v 1-specific IgE antibodies dropped almost beyond the detection limit (*lane 1*). Two monkeys were then reimmunized with 1 µg of recombinant Bet v 1 adsorbed to Al(OH)₃ without (A, B) and with a parallel intramuscular injection of corticosteroids (Volon A; 40 mg per animal) on the same day (C, D) or 4 days after each immunization (E, F). Lanes 2 through 4 represent blood samples obtained at the second, third, and fourth immunization, respectively. IgE binding to nitrocellulose-blotted natural Bet v 1 is indicated by arrows.

As described for patients allergic to pollen, IgE cross-reactivity was found in the Bet v 1-allergic monkeys with other homologous tree pollen allergens³⁴⁻³⁷ and food allergens.^{21, 22} Similarly, birch profilin-allergic monkeys showed IgE cross-reactivity with tree, grass, and weed pollen profilins,⁸ as well as with food-derived profilins.^{23, 24} All monkeys immunized with re-

combinant Bet v 1 showed positive skin reactions to recombinant Bet v 1 and commercially available standardized allergen extracts from birch, alder, and hazel pollen, which contain Bet v 1 homologous proteins. In a profilin-immunized monkey, positive skin reactions with recombinant birch profilin and protein extracts from birch, alder, hazel, timothy grass pollen, and celery

were observed. All extracts contained profilin as allergenic component.

In a second step, the usefulness of the specific primate model for in vivo studies of specific IgE regulation could be demonstrated by testing the effects of systemic corticosteroid application on Bet v 1-specific IgE responses during reimmunization. The application of corticosteroids during reimmunization significantly suppressed the induction of a Bet v 1-specific IgE-antibody production, suggesting that in addition to their antiinflammatory potential, corticosteroids may be effective in reducing allergen-specific IgE levels in vivo.

The immunization experiments suggest that because of immunologic similarities, sensitization against only a few allergens is sufficient to initiate type I allergy against a number of different allergen sources. The fact that in genetically different male and female rhesus monkeys type I allergy could be induced indicates that the onset of specific IgE responses against a given antigen is unlikely to be controlled by the genetic background of the individual.

We gratefully acknowledge the skillful assistance of Barbara Stumper in the animal experiments. All animal experiments were in accordance with the local guidelines for animal care.

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