

Induction of both reaginic and blocking antibodies with a low molecular weight fraction of timothy pollen extract

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In preliminary studies to evaluate the potential of the nonprecipitating antigen D for treatment of timothy-sensitive patients, a group of 8 nonallergic individuals was given a series of intracutaneous injections of antigen D at weekly intervals for 4 weeks. Each of the persons had no clinical history of allergy and gave negative skin reactions to antigen D, crude pollen extract (WST), and allergens A and B of timothy pollen. Four weeks after the last injection of antigen D, 6 of the 8 volunteers gave positive skin reactions when skin tested with various timothy allergens. Passive transfer studies with sera obtained from these volunteers not only showed significant blocking antibody titers but also varying amounts of reaginic antibody titers up to 1/25. The successful induction of homocytotropic antibodies in rhesus monkeys by repeated intracutaneous injections with antigen D was demonstrated by both direct skin reactions and histamine release from passively sensitized monkey lung fragments. The homocytotropic antibodies produced in both man and rhesus monkeys are heat labile, persist at sensitized sites for at least 48 hours, and can be passively transferred by sera.

Previous studies^{1, 2} with a low molecular weight fraction of timothy pollen extracts (antigen D) demonstrated a number of biological and antigenic properties suggesting that antigen D may have therapeutic value for the treatment of timothy-sensitive patients. Prior to the use of antigen D in treating such allergic patients, an effort was made to determine the immunogenic properties of antigen D in both man and rhesus monkeys.

Earlier studies indicated that repeated or even single injections of certain materials (Ascaris extracts, diphtheria toxoid, insulin, and animal serum) may

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induce immediate reactions, but repeated injections of aqueous pollen extracts did not produce immediate reactions in either atopic or nonatopic individuals.³⁻⁵ However, the incorporation of pollen extracts in an emulsion results in the induction of immediate reactions in both atopic and nonatopic volunteers.^{4, 6} Cooke, Loveless, and Stull⁵ demonstrated that repeated subcutaneous injection of ragweed pollen extracts resulted in the production of blocking antibody but no reaginic antibody. Consequently, we decided to ascertain whether intracutaneous immunization of nonallergic human volunteers with antigen D would lead to the production of blocking antibody.

The results of these studies indicate that intracutaneous immunization of nonallergic human volunteers resulted not only in significant levels of blocking antibody but also in the production of varying amounts of reaginic antibody in 6 of 8 volunteers. A similar immunization schedule in rhesus monkeys resulted in the production of low levels of homocytotropic antibody but no precipitating antibody. The degree of sensitization of both man and rhesus monkey resulting from antigen D immunization reflects the degree of antigenicity observed in earlier studies and indicates that the antigenic determinant expressed on antigen D is capable of inducing both reaginic and blocking antibody.

MATERIALS AND METHODS

Antigens

The immunochemical and biological properties of a low molecular weight fraction of timothy pollen extract (antigen D) and a detailed description of the methods of isolation of antigen D, crude pollen extract (WST), and purified allergenic fractions (A and B) of WST have been described elsewhere.^{1, 2}

Selection of volunteers

A group of 8 nonallergic volunteers from the Oregon State Penitentiary was selected on the basis of no clinical history of allergy and negative skin tests (10 μ g protein) with allergen A and B, WST, and antigen D.

Immunization schedule

Each volunteer was immunized by a series of intracutaneous injections of 1.5 mg. protein of antigen D at weekly intervals for 4 weeks. Volunteers were skin tested with 5 μ g protein of antigen D prior to each immunization injection, and reactions were noted 15 minutes later. Blood samples were taken from each volunteer prior to the start of immunization and at 2 week intervals thereafter. Sera obtained from these volunteers were used to determine both reagin and blocking antibody titers.

A group of 4 rhesus monkeys (4 to 6 kilograms) was immunized by a series of intracutaneous injections of 1 mg. protein of antigen D at weekly intervals for 6 weeks. Sera obtained from these animals were tested for antibody content by passive sensitization of normal monkey lung-tissue fragments.⁸

Antibody determinations

The presence of reaginic antibody in the sera of the antigen D-treated human volunteers was determined by passive transfer into (1) additional non-

allergic human volunteers at the Oregon State Penitentiary, and (2) rhesus monkeys.⁹ Blocking antibody determinations were performed by a modification of the method of Loveless¹⁰ described elsewhere.¹¹

The presence of a reagin-like antibody in the sera of rhesus monkeys immunized with antigen D was determined by specific histamine release from passively sensitized normal rhesus monkey lung tissue.⁸

Skin reactions

Wheal and flare reactions were recorded 15 minutes after intracutaneous challenge with antigen in the human volunteers and 30 minutes after challenge in the rhesus monkey. Reactions (wheal size) were graded as follows: 4 +, 20 mm. diameter; 3 +, 15 mm. diameter; 2 +, 10 mm. diameter; +, 5 mm. diameter; -, negative.

Protein determinations were made by the Nessler method as described by Campbell and associates.¹²

RESULTS

In preliminary studies to evaluate the potential of antigen D for treatment of timothy-sensitive patients, a group of 8 nonallergic persons was given a series of intracutaneous injections of antigen D at weekly intervals for 4 weeks. Each of the subjects had no clinical history of allergy and gave negative skin reactions to antigen D, allergen A and B, and WST. By the second week of injections 3 of the 8 volunteers developed indurated injection sites which appeared about 24 hours after injection and persisted for 7 to 10 days. One of the 8 volunteers showed a positive wheal and flare reaction prior to his fourth injection. Four weeks after the last injection of antigen D, 6 of the 8 volunteers gave positive skin reactions when skin tested with various timothy allergens, and blood samples were taken from all 8 volunteers. Table I shows the extent of their direct skin reactions with various allergens of timothy pollen and the induced reagin and blocking antibody titers. Preinjection serum samples of the 8 volunteers did not contain any demonstrable reagin or blocking antibody.

Blocking antibody titrations conducted with the sera obtained 4 weeks after the last injection of antigen D indicate that significant levels of blocking antibody were produced in all 8 volunteers. Passive transfer of the sera of these subjects into nonallergic human volunteers and rhesus monkeys indicated that their sera contained reaginic antibody titers up to $\frac{1}{25}$. None of those persons giving positive direct skin reactions with the various allergens of timothy pollen and possessing circulating reaginic antibody experienced clinical symptoms during the pollen season.

The successful induction of reagin by repeated intracutaneous injections with antigen D in man prompted a similar experiment in rhesus monkeys. Four rhesus monkeys (4 to 6 kilograms) were injected with 1 mg. protein of antigen D at weekly intervals for 6 weeks. Prior to each injection animals were skin tested with 35 μ g protein antigen D, and reaction sites were observed for 30 minutes. Two to 3 weeks after the initiation of the immunization schedule, these animals gave positive wheal and flare reactions. The sera obtained from the animals showed no precipitating antibody (ring test and PCA in guinea

Table I. *Reagin and blocking antibody titers in nonallergic human volunteers*

Subject	Direct skin reactions*			P-K titer†	PCA titer‡	Blocking antibody titer§
	WST (0.5 µg)	D (5 µg)	B (0.5 µg)			
C. B.	+	±	+	10	Undiluted	> 100
L. U.	+	-	+	5	Undiluted	> 100
L. S.	3+	+	3+	10	Undiluted	> 100
D. R.	-	-	-	-	-	> 100
L. D.	2+	±	2+	25	10	> 100
J. H.	+	-	+	10	5	> 100
L. S.	3+	+	3+	25	10	> 100
L. E.	-	-	-	-	-	> 100

*Wheal and flare reactions recorded 15 minutes after challenge. Reactions (wheal size) were graded as follows: 4+, 20 mm. diameter; 3+, 15 mm. diameter; 2+, 10 mm. diameter; +, 5 mm. diameter; ±, less than 5 mm. diameter; -, negative.

†Titers represent reciprocal of the last dilution giving a positive reaction. Challenging antigen was WST (0.5 µg protein).

‡Passive transfer titers in normal rhesus monkeys represent reciprocal of the last dilution giving a positive reaction. Challenging antigen was WST (1 mg. protein) administered intravenously 10 minutes after an intravenous injection of 5 ml. of 1 per cent Evans blue dye.

§Blocking antibody titer represents average of titrations carried out on 3 separate subjects.

Table II. *Comparison of skin reactions and histamine release of antigen D-sensitized rhesus monkeys*

Animal No.	Days after stimulation	Direct skin reactions*	Histamine release†
1162	0	-	< 0.01
	14	2+	0.4
	45	4+	1.0
1159	0	-	< 0.01
	21	+	0.17
	45	4+	1.2
1326	0	-	< 0.01
	21	+	0.17
	45	2+	0.36
1357	0	-	< 0.01
	14	+	0.12
	45	3+	0.79
Normal rhesus monkey		-	< 0.01

*Wheal and flare reactions 15 minutes after intradermal challenge, 35 µg protein of antigen D. Reactions (wheal size) were graded as follows: 4+, 20 mm. diameter; 3+, 15 mm. diameter; 2+, 10 mm. diameter; +, 5 mm. diameter; -, negative.

†µg Histamine release per milliliter per gram of wet tissue. Passively sensitized tissue challenged with 20 µg protein of WST.

pigs). An attempt to demonstrate homocytotropic antibody in their sera by passive transfer to skin of normal rhesus monkeys was unsuccessful. However, the presence of homocytotropic antibody was demonstrated by specific histamine release from normal monkey lung tissue passively sensitized with the sera of antigen D-treated animals (Table II). Previous studies⁸ indicated that neither human blocking antibody nor precipitating antibody (rabbit anti-WST and

rhesus anti-BSA) could passively sensitize monkey lung tissue and release histamine. Only human reagin and rhesus homocytotropic antibody (anti-*Ascaris*) could specifically release histamine from passively sensitized lung tissue. These studies indicate that all of the antigen D-immunized rhesus monkeys produced small but significant levels of homocytotropic antibodies. The homocytotropic antibodies produced in both man and rhesus monkeys are heat labile (inactivated by heat at 56° C. for 1 hour), persist at sensitized sites for at least 48 hours, and can be passively transferred by sera.

DISCUSSION

The induction of both reagin and blocking antibody in man, by intracutaneous injections with a soluble preparation of the low molecular weight fraction of timothy pollen, further demonstrates the antigenic properties of antigen D. The relationship of antigen D to allergen B of timothy pollen was shown by the positive direct skin reactions of 6 of the 8 volunteers (Table I). Only after prolonged, repeated injections did these volunteers show direct skin reactions with allergen A. Three of the 8 volunteers showed delayed reactions following their second injection. These reactions appeared after 24 to 36 hours, and these indurated injection sites persisted for 7 to 10 days. With the appearance of wheal and flare reactions in these volunteers, the delayed reaction no longer developed.

The successful induction of wheal and flare reactivity in rhesus monkeys with *Ascaris* extracts has been reported elsewhere.¹³ These studies and those in man led us to attempt the induction of wheal and flare reactions in the rhesus monkey with antigen D. The data presented in Table II indicates that the extent of sensitization of rhesus monkeys with antigen D is low. However, the extent of sensitization observed in the 4 rhesus monkeys is comparable to that observed in both rabbits and man and reflects the weak antigenicity of antigen D. In contrast, rhesus monkeys immunized intracutaneously with *Ascaris* extracts give significantly higher yields of homocytotropic antibodies (3 to 17 μ g of histamine released per milliliter of serum per gram of wet tissue). The homocytotropic antibodies produced in both man and rhesus monkey resulting from immunization with antigen D possess properties similar to reaginic antibody.

The induction of reaginic antibody in nonallergic volunteers may be due in part to the high concentration of antigen D administered. However, the same immunization schedule in 4 rhesus monkeys with the use of WST failed to induce either positive wheal and flare reactions or homocytotropic antibody (as revealed by histamine release). Connell¹⁴ recently reported that 3 of 22 passive transfer recipients, challenged with aqueous ragweed extract on more than two occasions, developed positive ragweed skin tests. The extent of sensitization of these patients was not determined, and the one patient followed did not develop clinical symptoms during the ragweed season. Over the last 4 years, passive transfer experiments have been conducted at the Oregon State Penitentiary with 43 volunteers having neither a clinical history nor a positive intradermal skin test when challenged with either WST (10^{-5} Gm. protein per milliliter) or

antigen D (1 mg. protein per milliliter). Twenty-eight of these volunteers acted as recipients for passive transfer of human allergic sera (30 to 40 sites per recipient) and were challenged with multiple injections of WST on 3 or more occasions. None of the 43 volunteers showed positive skin reactions at control sites when challenged with either antigen D or WST. These results would indicate that sensitization to WST did not occur either during passive transfer experiments or in rhesus monkeys extensively immunized with WST. Non-allergic human volunteers were not immunized with the same injection schedule and dosage of WST as those volunteers treated with antigen D.

The ability to induce homocytotropic antibodies in an experimental animal provides an opportunity to explore many fundamental questions in allergy. Previous studies^{1, 2} demonstrating the hapten-like properties, the induction of significant levels of blocking antibody, and the absence of toxicity or side effects resulting from immunization provide additional basis for the potential therapeutic uses of the nonprecipitating antigen D in the treatment of timothy-sensitive patients. With this in mind, a pilot study with the use of antigen D in the treatment of patients has been in progress and is soon to be reported.

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