

Biological and Immunological Properties of Sugi Basic Protein-Pullulan Conjugate

I. Suppressive Effect on IgE Antibody Production and on IgE-Mediated Reactions

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Abstract. Sugi basic protein (SBP), a major allergen of Japanese cedar pollen, conjugated to pullulan acquires suppressive activities for IgE, but not for IgG antibody production, and for IgE-mediated PK reactions in a SBP-specific manner. When either normal or IgE anti-SBP antibody-producing mice were treated with the conjugate, IgE anti-SBP antibody levels scarcely increased even after immunization with SBP + alum. This suppression was shown to be SBP specific and IgE selective. The ability of SBP-pullulan conjugate to elicit PK reactions on passively sensitized rat skin was markedly reduced. Furthermore, the conjugate inhibited the reactions mediated by the IgE antibodies and native SBP when it was injected into the IgE antibody-sensitized sites 2 h prior to SBP challenge. The reduced ability of the conjugate to elicit PK reactions was not due to loss of antigenic determinants during the conjugation procedures because binding activity of the conjugate to anti-SBP antibodies was as strong as that of native SBP. These results indicate that SBP-pullulan conjugate may be useful for 'desensitization therapy' against the pollinosis.

Introduction

Patients suffering from seasonal allergic rhinitis and/or conjunctivitis caused by pollen from Japanese cedar (*Cryptomeria japonica*) have recently been increasing in numbers. For example, in Japan several million cases have been reported [1]. Desensitization immunotherapy by multiple injections of allergen over extended time periods has been effective in alleviating allergic symptoms in some but not all patients. However, there are a number of recognized problems with this type of immunotherapy: for instance, there is a risk in inducing systemic or local anaphylactic reactions, specific IgE antibody levels might be elevated, and the time course required to achieve favorable results is over many months or years [2-4].

We have previously reported that IgE class-selective and antigen-specific suppression as well as enhanced IgG antibody production could be achieved in mice by injection of an ovalbumin-pullulan conjugate [5].

In the present study, we found that the Sugi basic protein (SBP)-pullulan conjugate was also capable of inducing suppression of IgE anti-SBP antibody pro-

duction and of enhancing IgG anti-SBP antibody production; SBP being the major allergen in Japanese cedar pollinosis. Moreover, the dose of conjugate required for eliciting Prausnitz-Küstner (PK) reactions on passively sensitized rat skin is approximately 100-fold greater than that of native SBP. In addition, the conjugate was found to be capable of inhibiting the PK reaction. Thus, treatment of the patients with the conjugate rather than conventional SBP solutions may be more advantageous in the improvement of allergic symptoms.

Materials and Methods

Animals

BALB/c female mice, 8-12 weeks of age, and Sprague-Dawley (SD) rats, 9-12 weeks old, were purchased from Charles River Japan, Inc., Kanagawa. New Zealand white rabbits, 3 months old, were obtained from Yanahara Rabbit Center, Okayama.

Antigens and Reagents

SBP was prepared according to the procedure of Yasueda et al. [6] with slight modifications. In this procedure, Cetavlon (hexadecyltrimethylammonium bromide, Sigma) was used to remove polysaccharides by precipitation. Purified SBP had characteristics simi-

Table 1. Induction of IgE-selective and SBP-specific unresponsiveness in mice by treatment with SBP-pullulan conjugate

Pretreatment	Immunization	Anti-SBP		Anti-TT	
		IgE	IgG	IgE	IgG
<i>Experiment 1</i>					
SBP in saline	SBP + alum	320 (13.0)	120	ND	ND
SBP-pullulan conjugate	SBP + alum	50 (6.0)	584	ND	ND
Mixture of SBP and pullulan	SBP + alum	320 (13.0)	171	ND	ND
<i>Experiment 2</i>					
SBP-pullulan conjugate	SBP + TT + alum	40 (6.0)	6,410	640 (15.0)	13,800
Saline	SBP + TT + alum	160 (11.0)	3,050	320 (14.0)	16,200

Normal mice were treated 3 times with SBP, SBP-pullulan conjugate or a mixture of SBP and pullulan at 1-week intervals, and 7 days later, immunized with SBP or SBP + TT adsorbed onto alum.

Numbers in parentheses represent a sample score.

ND = Not done.

Immunization with SBP + TT + alum was done twice.

lar to those described by Yasueda et al. [6]. It showed a M_r 37- to 50-kdalton component by SDS-PAGE analysis, and strong antigenicities in ELISA using sera of the cedar pollen allergic patients and β -D-galactosidase-labeled antihuman IgE antibodies (Pharmacia Fine Chemicals). The amino acid composition was found to be similar to that reported by Yasueda et al. [6]. Eighty milligrams of SBP was obtained from 500 g of dried pollen. SBP protein concentration was determined by measurement of ultraviolet absorption using absorption coefficient of $E_{1\%}^{1\text{cm}}$ at 280 nm 12.6 [6]. Tetanus toxoid (TT) was kindly donated by Dr. A. Yamamoto of the National Institute of Health, Japan.

Preparation of SBP-Pullulan Conjugate

SBP-pullulan conjugate was prepared according to the method described previously [5]. Briefly, 8 mg of cyanuric chloride (Wako Junyaku, Tokyo; used after recrystallization) dissolved in 1 ml chilled acetone were mixed with 25 ml of 2% pullulan solution (M_w 390 kdaltons, Hayashibara Biochemical Laboratories, Inc., Okayama), and the mixture was stirred in an ice bath for 2 h. The pH of the reactants was maintained at 6.8–7.0 by adding 5% NaHCO_3 dropwise. Activated pullulan was mixed with 50 mg of SBP, and the mixture was incubated at 37°C for 5 h with continuous stirring. The resulting preparation was dialyzed against 0.01 M acetate buffer, pH 5.0 and applied to CM-Sephadex C-25 (Pharmacia) column equilibrated with the same buffer to remove unconjugated SBP from the preparation. Molecular weight of the SBP-pullulan conjugate was estimated to be 400–1,600 kdaltons by HPLC analyses using a GPC column. This suggests that pullulan-pullulan and SBP-pullulan-pullulan conjugates might be formed in addition to SBP-pullulan conjugate. An immunoelectrophoretic analysis using rabbit or mouse anti-SBP antiserum revealed that the electrophoretic mobility of the conjugate was altered to the anode side from that of native SBP, and that the precipitin line formed with the anti-SBP antiserum was observed as a 'clear line' indicating hydrophilicity of the precipitin line [5]. These results indicate that the conjugate of SBP with pullulan had been formed and was separated

from unconjugated SBP by the procedures described above. In this study, the dose of conjugate was expressed as concentration of SBP in the conjugate by optical absorption at 280 nm.

Determination of Antibody Titers

Mice were bled from the tail vein on the day indicated. IgE anti-SBP and anti-TT antibodies were titrated by passive cutaneous anaphylaxis (PCA) reactions in SD rats according to Mota and Wong [8], using 200 μg SBP per rat or 100 Lf TT per rat as an eliciting antigen. IgE antibody titers are expressed as reciprocal of the highest dilutions giving a 5 $\times$ 5 mm blue spot. A sample score was calculated as the sum of the strength of blue spots at each dilution of the sample, the strength of blue spots are expressed as follows: (1) + = 5 mm in diameter; (2) ++ = 6–10 mm; and (3) +++ = more than 10 mm.

IgG anti-SBP or anti-TT antibodies were measured by ELISA using horseradish peroxidase (HRPO)-labeled rabbit antimouse IgG (DAKO) and SBP- (5 $\mu\text{g}/\text{ml}$, 100 μl) or TT- (4 Lf/ml, 100 μl) coated 96-well microplates. IgG antibody titers are expressed as reciprocals of dilutions giving a half of the maximum optical density.

Preparation of Rabbit Anti-SBP Antibodies

Specific antiserum to SBP was prepared by immunizing rabbits twice at 1-week intervals with 1 mg of SBP emulsified in Freund's complete adjuvant (Difco). The rabbits were bled 7 days after the last injection, and IgG fraction of the serum was obtained by precipitation with ammonium sulfate (50% saturation) and protein A-Sepharose (Pharmacia) chromatography. A portion of the IgG fraction was labeled with HRPO (Sigma) according to Nakane and Kawaoi [9].

PK Reaction and Its Inhibition Test

0.1 ml of 1:50 diluted mouse or rat serum containing IgE anti-SBP antibodies was injected intradermally to the back of shaved normal SD rat. The antibody doses had been previously ascertained to be sufficient for sensitizing rat skin for PCA reaction eli-

Table 2. Suppressive effect of SBP-pullulan conjugate on secondary anti-SBP IgE responses

Primary immunization	Treatment	Secondary immunization	Before treatment		After treatment		After 2nd immunization	
			IgE	IgG	IgE	IgG	IgE	IgG
<i>Anti-SBP</i>								
SBP + alum	saline	SBP + alum	160 (11.7)	1,570	80 (9.0)	2,470	640 (15.0)	2,900
SBP + alum	SBP-pullulan conjugate	SBP + alum	160 (11.0)	1,010	80 (9.0)	1,820	160 (10.0)	5,840
SBP + alum	mixture of SBP and pullulan	SBP + alum	160 (9.0)	1,180	80 (9.0)	3,120	320 (14.0)	4,900
<i>Anti-TT</i>								
TT + alum	saline	TT + alum	80 (7.0)	22,000	80 (7.0)	22,600	320 (12.0)	33,400
TT + alum	SBP-pullulan conjugate	TT + alum	80 (7.0)	11,670	80 (8.0)	14,400	320 (12.0)	23,200

SBP- or TT-primed mice were treated 2–3 times/week for 3 weeks with SBP-pullulan conjugate, a mixture of SBP and pullulan, or saline. Seven days later, mice were challenged twice with SBP or TT adsorbed onto alum. Numbers in parentheses represent a sample score.

cited by 200 µg of i.v.-administered SBP solution. Twenty-four to seventy-two hours later, 0.1 ml of serial 10-fold dilution (10^2 – 10^{-2} µg/ml) of SBP or SBP-pullulan conjugate was injected intradermally to the sensitized sites. Immediately after the injections, the rat was given 1 ml of 0.5% Evans blue solution.

To study the ability of SBP-pullulan to inhibit PK reactions, shaved SD rat skin was sensitized with 0.1 ml of 1:50 diluted mouse serum containing IgE anti-SBP or anti-tetanus toxin (TT). Twenty-four hours later, SBP-pullulan conjugate, 5.0–0.1 µg/0.1 ml/site, or saline was injected intradermally to the sensitized sites. After an additional 2 h, the specific PK reactions were elicited by intradermal injections of corresponding antigen, 500 ng SBP or 0.6 Lf TT/site, to the sensitized sites. Immediately after the injections, 1 ml of 0.5% Evans blue was given to the rat.

Enzyme-Linked Immunosorbent Assay (ELISA) for SBP and SBP-Pullulan Conjugate

Various doses of SBP or SBP-pullulan conjugate were reacted onto 96-well microplates coated with 100 µl of 20 µg/ml rabbit anti-SBP IgG fraction. Two hours later, the amounts of SBP or SBP-pullulan bound onto the solid-phase anti-SBP antibodies were visualized with HRPO-labeled anti-SBP antibodies.

To study of neutralizing ability of SBP or SBP-pullulan conjugate, various doses of SBP or SBP-pullulan conjugate were reacted with a constant dose of free anti-SBP antibodies. Two hours later, the residual anti-SBP antibodies were measured using SBP-coated 96-well microplates and HRPO-labeled antirabbit IgG (DAKO).

Results

SBP-Pullulan Conjugate Induces IgE-Selective and SBP-Specific Unresponsiveness in Normal Mice

Groups of 4 mice each were treated 3 times with 1 µg of SBP in saline solution, conjugate with pullu-

lan or in mixture with pullulan, respectively, at 1-week intervals. One week after the last treatment, the mice were immunized with 1 µg SBP adsorbed onto 4 mg alum precipitates. One week later, serum antibody titers were determined by rat PCA reactions and ELISA using HRPO-labeled antimouse IgG. The experiments were performed several times using 4 different lots of SBP-pullulan conjugate. Results of a representative experiment are shown in table 1, experiment 1. In mice receiving pretreatment with SBP-pullulan conjugate, IgE, but not IgG, anti-SBP response was markedly suppressed after the immunization. By contrast, mice receiving SBP in saline or a mixture of SBP and pullulan showed good IgE and IgG anti-SBP responses. To investigate the specificity of the IgE-suppressive effect of the conjugate, SBP-pullulan-treated mice were immunized twice with 4 Lf of TT in addition to SBP. As shown in table 1, experiment 2, the conjugate treatment suppressed selectively the IgE anti-SBP response and did not affect TT responses. These results indicate that the SBP-pullulan conjugate is capable of inducing IgE-selective and SBP-specific unresponsiveness in normal mice.

SBP-Pullulan Suppresses the Secondary Anti-SBP IgE Response in Mice Producing IgE Anti-SBP Antibodies

Mice were immunized i.p. with 1 µg SBP or 4 Lf TT adsorbed onto alum 3 times at 1-week intervals. Two weeks after the last injection, 3 groups of SBP-immu-

nized mice and 2 groups of TT-immunized mice were treated 2–3 times/week for a period of 3 weeks with 200 ng of the SBP-pullulan conjugate, or a mixture of SBP and pullulan, and, as a control, saline alone, respectively. One week after the last treatment, mice were challenged with the same doses of respective antigens as the primary immunization, and, 7 days later, the challenge was repeated again. After an additional 7 days, serum antibody titers were determined. As shown in table 2, SBP-immunized mice treated with the SBP-pullulan conjugate showed little increase in IgE anti-SBP titers following allergenic SBP injections. IgG anti-SBP titers, however, increased markedly. On the other hand, in TT-immunized mice, treatment with SBP-pullulan conjugate exerted little effect on anti-TT IgE and IgG responses, suggesting that suppressive effect of the conjugate on secondary IgE responses is also SBP-specific.

Capability of SBP-Pullulan Conjugate for Eliciting PK Reactions in Rats Is Lower than That of Native SBP

The following experiments were performed to determine whether SBP-pullulan conjugate is capable of eliciting PK reaction in rat skin passively sensitized with rat or murine IgE anti-SBP antibodies. As seen in table 3, 100 ng of SBP could elicit a positive PK reaction when the murine anti-SBP serum was used for sensitization and 10 ng of SBP elicited a positive reaction when the rat antiserum was used for sensitization. It should be noted that at even 10 µg of SBP-pullulan only a faint reaction was elicited, suggesting that concentrations higher than 10 µg of the conjugate would be required for eliciting positive PK reactions.

SBP-Pullulan Conjugate Blocks the PK Reactions Mediated by SBP and IgE Anti-SBP Antibodies

To investigate whether SBP-pullulan conjugate is capable of inhibiting PK reactions mediated by SBP and IgE anti-SBP antibodies, the following experiments were performed. As shown in table 4, the anti-SBP-sensitized sites receiving no SBP-pullulan prior to SBP challenge showed strong positive reactions (+++), whereas the reactions at the anti-SBP-sensitized sites receiving SBP-pullulan conjugate were markedly reduced. It was noted that more than 0.2 µg SBP-pullulan conjugate could completely block the reactions mediated by IgE anti-SBP antibodies and 500 ng SBP. Furthermore, it was demonstrated that SBP-pullulan conjugate exerted little effect on the TT-

Table 3. Comparison of antigenicities for eliciting PK reactions between SBP and SBP-pullulan conjugate

Sensitizing antibodies	Eliciting antigens	Dose of antigens, µg/site				
		10	1	0.1	0.01	0.001
Murine IgE anti-SBP	native SBP	+++	+++	+	–	–
	SBP-pullulan conjugate	±	–	–	–	–
Rat IgE anti-SBP	native SBP	+++	+++	++	+	–
	SBP-pullulan conjugate	±	–	–	–	–

10–10^{–3} µg/0.1 ml/site of SBP or SBP-pullulan conjugate was injected intradermally at the previously sensitized rat skin site. Immediately after the injection, the rat was given 1 ml i.v. of 0.5% Evans blue solution.

Table 4. Inhibitory activity of SBP-pullulan conjugate on PK reactions mediated by SBP and IgE anti-SBP antibodies

Intradermal injections				PK reaction
1st	2nd	3rd		
Anti-SBP	saline	SBP 500 ng		+++
Anti-SBP	SBP-pullulan	0.1 µg	SBP 500 ng	±
		0.2 µg	SBP 500 ng	–
		0.5 µg	SBP 500 ng	–
		1.0 µg	SBP 500 ng	–
		2.0 µg	SBP 500 ng	–
		5.0 µg	SBP 500 ng	–
Anti-SBP	SBP-pullulan	5.0 µg	saline	–
Anti-TT	saline	TT 0.6 Lf		+++
Anti-TT	SBP-pullulan	0.2 µg	TT 0.6 Lf	+++
		1.0 µg	TT 0.6 Lf	+++
		5.0 µg	TT 0.6 Lf	+++

5–0.1 µg/0.1 ml of SBP-pullulan conjugate or saline was injected intradermally to the rat skin site previously sensitized with IgE anti-SBP or anti-TT. Two days later, SBP- or TT-specific PK reactions were elicited with SBP or TT, respectively.

specific PK reactions, indicating that the inhibitory effect of the conjugate is SBP specific. These results suggest that SBP-pullulan actually binds to IgE anti-SBP on mast cells or basophils, thus these cells might be protected from binding of allergenic native SBP, and subsequent triggering events mediated by allergenic SBP.

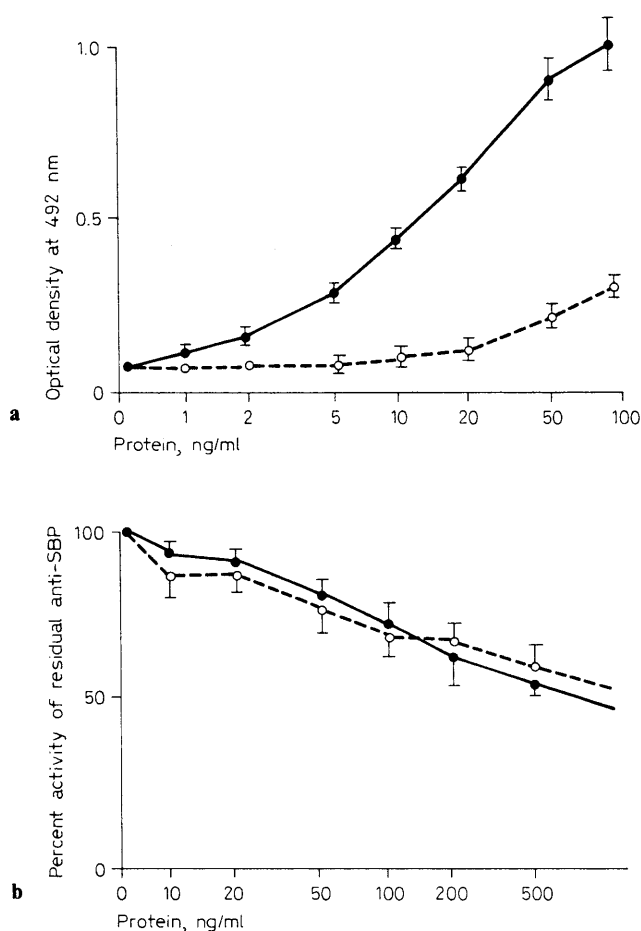


Fig. 1. Comparison of binding amounts of SBP and SBP-pullulan conjugate to immobilized or free anti-SBP antibodies. **a** The binding amounts of SBP or SBP-pullulan to anti-SBP antibodies immobilized on a 96-well microplate were determined using HRPO-labeled rabbit anti-SBP antibodies. **b** The binding amounts of SBP and SBP-pullulan to free anti-SBP antibodies were estimated by neutralizing activities to the antibodies. ● = SBP; ○ = SBP-pullulan conjugate.

Comparison of Binding Amounts of SBP and SBP-Pullulan Conjugate to Immobilized or Free Anti-SBP Antibodies

One possible reason for the reduced capability of the conjugate to elicit a PK reaction is that the SBP-pullulan conjugate might be unable to bind sufficiently to promote IgE antibody cross-linking on mast cells or other cell surfaces. Alternatively, SBP-pullulan might lose its antigenic determinants during conjugation procedures. Therefore, the amounts of

SBP and SBP-pullulan bound to immobilized or free anti-SBP antibodies were compared. As a model for cell-bound antibodies, anti-SBP antibodies coated onto 96-well microplates were used. As shown in figure 1a, the amounts of SBP-pullulan binding to solid-phase antibodies were about 100-fold less than that of native SBP solution. However, if the antibodies were not immobilized, the conjugate could bind to the antibodies as well as native SBP because the neutralization curve of SBP-pullulan against unfixed antibodies fell on that of native SBP solution (fig. 1b). These results suggest that the reduced binding of the conjugate to immobilized anti-SBP is not due to loss of its antigenic determinants, but that the binding space of SBP-pullulan conjugate to the immobilized antibodies on a solid surface is about 100-fold broader than that of native SBP.

Discussion

As mentioned, our previous studies [5] have demonstrated that administration of ovalbumin (OA)-pullulan conjugate induces in mice OA-specific and IgE-selective suppression of primary antibody responses to the antigen. In the present study, we prepared a similar pullulan conjugate with SBP, a major allergen for Japanese cedar pollen allergy and succeeded in the induction of SBP-specific and IgE-selective suppression in mice. We also found that the conjugate bears new immunobiological properties that are lacking in the native SBP: the SBP-pullulan treatment causes no increase in IgE antibody levels of normal or even IgE anti-SBP antibody-producing mice. The ability of the conjugate to elicit PK reactions on presensitized rat skin is markedly reduced, i.e. 100 times less than that of the native SBP and, moreover, such a pretreatment of the sensitized skin with the conjugate blocks subsequent PK reactions upon challenge with the allergenic SBP.

Regulation of IgE antibody responses has been extensively studied in humans and animals [10, 11]. Cells and factors involving in regulation of IgE antibody responses have been claimed [12–20] and, more recently, IL-4 and IFN- γ were also shown to be involved [21–25]. Our previous studies on the antigen-pullulan conjugates have demonstrated that the OA-specific and IgE-selective suppression could be transferred by spleen cells from the conjugate-treated mice to normals [5] and that intravenous administration of

NP-pullulan conjugate induced at least two distinct suppressor T cell populations, Ts1 (inducer) and Ts2 (transducer) [26]. In our preliminary experiments, the antigen-specific and IgE-selective suppression was also observed in mice receiving spleen cells from SBP-pullulan-treated donors (data not shown), indicating the involvement of suppressor cells in SBP-pullulan-induced suppression. Studies are now in progress in our laboratory to delineate cellular mechanisms responsible for the suppression.

Immunochemical analyses on the SBP-pullulan conjugate revealed that the antigenic determinants of SBP are presumably fully available, since the conjugate was able to neutralize free anti-SBP antibodies as effectively as the native SBP. On the other hand, the amounts of the conjugate bound to immobilized antibodies were shown to be 50–100 times less than that of the SBP, indicating that each conjugate molecule occupies a much greater space of the antibody-coated surface than the SBP molecule does. This drastically reduced binding of the conjugate to the antibody-coated surface might have been simply due to steric hindrance, because the molecular weight of the conjugate was estimated as 400–1,600 kdaltons and the conjugation ratio was calculated to be approximately 1:1. Therefore, it is reasonable to assume that such a very limited binding of the conjugate, if it took place in vivo on IgE-sensitized mast cells, might be unable to cross-link two IgE molecules resulting in its reduced ability to elicit PK reactions and also blocking of PK reactions with allergenic SBP challenge.

The above-described properties of the SBP-pullulan conjugate seem to warrant further investigations toward the development of a new immunotherapy against Japanese cedar pollen allergy, since such an improved therapy should be able to suppress specifically IgE antibody production and simultaneously reduce symptoms mediated by IgE antibodies and corresponding allergens. The data presented in this study clearly indicate that the SBP-pullulan conjugate would be a potential candidate to fulfill both requirements.

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