

Biological and Immunological Properties of Sugi Basic Protein-Pullulan Conjugate

II. Is the Reduced Ability to Elicit the Arthus Reaction Based on the Poor Activation of Complement by Immune Complex Consisting of Anti-Sugi Basic Protein and Sugi Basic Protein-Pullulan?

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Abstract. Ability of Sugi basic protein (SBP)-pullulan conjugate to elicit the Arthus reaction was found to be markedly reduced, about 100 times lower than that of native SBP. To analyze this reduced ability, activation of complement by immune complex consisting of SBP-pullulan and anti-SBP antibodies was studied. Tests for complement consumption, C3 conversion and cleavage of factor B revealed that immune complex formed with SBP-pullulan is incapable of supporting efficient activation of the complement system. Previously, we have shown data suggesting that SBP-pullulan conjugate would be a good candidate for desensitization therapy against cedar pollinosis. The results presented in this paper provide additional support for the suggestion.

Introduction

Due to the increase in Japanese cedar pollinosis, more attention has been paid to these patients in the last decade in Japan [1]. Some reasons for the increase are: the expansion of Japanese cedar-planting fields; changes in the life-style of Japanese; environmental pollution from automobile exhaust, and so on [2-4]. Effective prevention and/or therapy for these patients is urgently needed to solve this problem in Japan. In a previous paper [5], we reported that Sugi basic protein (SBP)-pullulan conjugate shows the following important properties which are different from those of native SBP: (1) when mice, either unprimed or primed with SBP, were treated with the conjugate, the anti-SBP IgE antibody response but not IgG, is scarcely enhanced by the subsequent sensitizations with SBP plus alum; (2) the ability of the conjugate to elicit Prausnitz-Küstner (PK) reactions on IgE anti-SBP-sensitized rat skin was markedly reduced, about 100 times less than that of native SBP, and (3) an adequate dose of the conjugate inhibited the PK reactions mediated by IgE anti-SBP and native SBP when it was injected into the IgE-sensitized sites 2 h prior to SBP challenge. However, SBP-pullulan was shown to bind to free anti-SBP antibodies as effectively as native SBP, indicating little reduction in the antigenicity. These properties suggest that the conjugate is a

harmless immunogen even in the sensitized animal. We suspected that the conjugate could be a potential candidate for effective immunotherapy against Japanese cedar pollinosis. Therefore, further studies on the properties of the conjugate were performed. In this paper, we present data showing that the ability of the conjugate to elicit Arthus reactions in SBP-immunized rabbits was also reduced, and that the reduction of this ability might in part be due to diminished activation of classical and alternative pathways of complement systems by immune complexes (ICs) consisting of anti-SBP and SBP-pullulan conjugate.

Materials and Methods

Animals

New Zealand white female rabbits, 2.5-3 kg body weight, were purchased from Yanahara Rabbit Center, Okayama, Japan. Female guinea pigs, 8-10 weeks of age, were purchased from Nippon Bio-Supplies Center, Tokyo, Japan.

Antigens and Antibodies

SBP and SBP-pullulan conjugate were prepared as described in the previous paper [5]. The dose of SBP-pullulan conjugate is expressed as the concentration of SBP in the conjugate calculated from optical absorption at 280 nm, $E_{1\%}^{1\text{cm}} = 12.6$. Rabbit anti-SBP antiserums were obtained from rabbits immunized twice with 1 mg SBP in Freund's complete adjuvant. The IgG fraction of the antiserum was isolated using protein A-Sepharose (Pharmacia). Rabbit

Table 1. Minimum doses of SBP-pullulan and native SBP required for eliciting the Arthus reaction

Antigens	Edema and/or erythema				
	10 µg/site	1 µg/site	0.1 µg/site	0.01 µg/site	0.001 µg/site
SBP-pullulan	+++	+	—	—	—
Native SBP	+++	+++	+++	+	—

10¹ to 10⁻³ µg/0.1 ml/site of SBP-pullulan or native SBP were injected intradermally into rabbits which had been immunized twice with 1 mg SBP emulsified in FCA. Six to 24 h later, edema and/or erythema were observed.

anti-human C3 and anti-human factor B were purchased from MBL, Japan. Sheep red blood cells (SRBC) were obtained from Japan Immunoresearch Laboratories Co., Ltd. and rabbit anti-SRBC (hemolysin) was purchased from Cappell (USA).

Buffers

GVB²⁺, isotonic veronal-buffered saline (pH 7.4) containing 0.1% (w/v) gelatin, 0.15 mM CaCl₂, or Mg²⁺ · EGTA · GVB²⁺, GVB²⁺ with 5 mM MgCl₂ and 10 mM EGTA (Sigma), was used as diluent in complement titration and hemolytic assays. For immunoelectrophoretic (IEP) analyses, 0.1 M veronal-HCl buffer (pH 8.6) was used.

Evaluation of Ability of SBP-Pullulan and Native SBP to Elicit the Arthus Reactions in SBP-Immunized Rabbits

Rabbits were hyperimmunized with SBP, and were confirmed to have produced antibodies sufficiently high to support the Arthus reaction. The rabbit abdominal skin was carefully shaved, and 0.1 ml of 10² to 10⁻² µg/ml of SBP-pullulan or SBP were intradermally injected. Six and 24 h later, edema and/or erythema were measured.

Analyses of Complement Activation by IC Consisting of anti-SBP and SBP-Pullulan Conjugate or Native SBP

Guinea pig fresh serum and human fresh serum were stored at -80°C until used as complement.

Complement Consumption Test. Titration of whole complement activity of guinea pig fresh serum was performed according to Kabat and Mayer [6] using SRBC optimally sensitized with rabbit anti-SRBC (EA) and 5 U CH50/ml complement was prepared using GVB²⁺. One volume of a constant concentration of anti-SBP IgG (100 µg/ml, previously determined in a preliminary experiment), 1 vol of 5 U CH50/ml guinea pig complement and 1 vol of various concentrations of SBP or SBP-pullulan conjugate (0.2–100 µg/ml) were mixed together in an ice bath. The mixtures were incubated at 37°C for 60 min with shaking. After incubation, residual complement activity of each mixture was estimated by hemolysis of EA. Results were expressed as the percent hemolysis of EA.

Conversion of Human C3 Component. As a dilution buffer, GVB²⁺ was used in the experiment. 40 µl of fresh human serum, 20 µl of 100 µg/ml rabbit anti-SBP IgG and 20 µl of 100, 30 or 5 µg/ml SBP or SBP-pullulan conjugate were mixed in an ice bath. The mixtures were incubated at 37°C for 30 min, followed by incubation in an ice bath for 5 min and centrifugation at 5°C. The supernatants of

the mixtures were subjected to IEP analyses using anti-human C3. Crossed immunoelectrodifusion (CIED) was performed according to Laurell [7].

Cleavage of Factor B and Conversion of C3 in Alternative Pathway (AP). Human serum was incubated with anti-SBP and SBP or SBP-pullulan under the same conditions mentioned above except for using Mg²⁺ · EGTA · GVB²⁺ as a dilution buffer instead of GVB²⁺. After the reaction, supernatants of the mixtures were analyzed by IEP using anti-human factor B and anti-human C3 antisera.

Results

Reduced Ability of SBP-Pullulan Conjugate to Elicit the Arthus Reaction

Rabbits hyperimmunized with SBP were used to estimate the ability of SBP-pullulan conjugate and native SBP to elicit the Arthus reaction. As shown in table 1, as little as 0.1 µg of native SBP could elicit a strong positive reaction, whereas 10 µg of SBP-pullulan conjugate was required to elicit a positive reaction to the same degree. Thus, the minimum dose of the conjugate required to elicit the Arthus reaction was found to be 100 times higher than that of native SBP.

No Complement-Consuming Activities of ICs Consisting of Anti-SBP and SBP-Pullulan

Since SBP-pullulan conjugate could react with anti-SBP antibodies as effectively as native SBP [5], the reduced ability of the conjugate to elicit the Arthus reaction is presumed to be due in part to the reduction in the ability to activate complement systems. To examine whether the complement activation is reduced in the case of IC formed by SBP-pullulan, a complement consumption test was performed using guinea pig complement. Throughout the experiment, GVB²⁺ was used as reaction buffer. After treatment of 5 U CH50/ml guinea pig complement with these ICs,

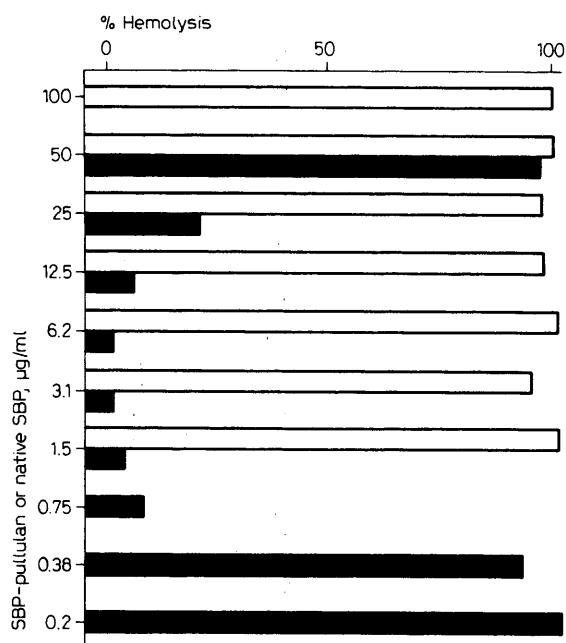


Fig. 1. Complement consumption test for immune complex composed of SBP-pullulan or native SBP. One volume of 100 µg/ml rabbit anti-SBP IgG, 1 vol of 5 U CH50/ml guinea pig complement and 1 vol of various concentrations of SBP-pullulan (□) or native SBP (■) were mixed together in an ice bath. The mixtures were incubated at 37°C for 60 min with shaking. After the incubation, residual complement activity of each mixture was determined by hemolysis of EA.

the remaining complement activity in the supernatant of each sample was evaluated by hemolysis of EA. Figure 1 shows the results. The optimal dose of native SBP to make maximum complement consumption with 100 µg/ml anti-SBP antibodies was determined to be 0.75–12.5 µg/ml. Whereas, in the case of SBP-pullulan, no positive reactions were observed in the wide range of SBP-pullulan concentrations tested (1.5–100 µg/ml). The results suggest that the IC formed by anti-SBP and SBP-pullulan had a poor ability to activate guinea pig complement via the classical pathway (CP).

Decreased Conversion of Human Complement Component C3 by Treatment with IC Composed of SBP-Pullulan

The next experiments were performed to discover whether human complement component C3 could be activated with IC consisting of SBP-pullulan and anti-SBP. 100 µg/ml of anti-SBP antibodies and 5 µg/ml of native SBP were used in the following experiments, SBP-pullulan was used at 5, 30 or

100 µg/ml. All reactants were prepared using GVB²⁺. Human fresh serum treated with IC consisting of anti-SBP and SBP-pullulan or native SBP were analyzed by IEP and CIED using rabbit anti-human C3 antibodies. As shown in figure 2a, conversion of the β1c to the β1A line was not clearly observed in the IEP pattern of serum treated with a complex of anti-SBP and SBP-pullulan as in the control serum. In contrast, fresh serum treated with complex formed with anti-SBP and native SBP showed a typical conversion pattern of the C3(β1c/A) precipitin line. Since the appearance of the β1A line indicates the formation of substantial amounts of C3c, products from C3 in the process of sequential activation of the component [10, 18], the results suggest that ICs consisting of SBP-pullulan and anti-SBP could not support activation of the human C3 component in the presence of Mg²⁺ and Ca²⁺ ions. Semiquantitative analyses by CIED showed similar results more precisely (fig. 2b).

Poor Activation of AP of Human Complement by the IC Formed with SBP-Pullulan and Anti-SBP

To discover whether the AP of human complement is activated by SBP-pullulan conjugate alone or the IC formed by the conjugate, cleavage of factor B was examined. Anti-SBP, native SBP and SBP-pullulan were diluted from a stock solution with Mg²⁺ · EGTA · GVB²⁺ in order to assay the activation of AP. The experiments were performed under the same conditions mentioned above, except when using Mg²⁺ · EGTA · GVB²⁺ instead of GVB²⁺. Results are shown in figure 3. IEP analyses of the supernatants using anti-human factor B and anti-human C3 antibodies revealed that sera treated with SBP-pullulan conjugate alone or IC consisting of the conjugate did not show the conversion of factor B and C3 lines, and that these conversions occurred only in serum treated with IC consisting of native SBP. The results indicate that the SBP-pullulan conjugate alone and the IC formed by SBP-pullulan do not support activation of factor B, but the IC formed by native SBP does support formation of a stable AP C3 convertase, C3b,Bb,P.

Discussion

The Arthus reaction is an inflammatory one induced by an antigen-antibody complex accompanying complement activation. Some complement frag-

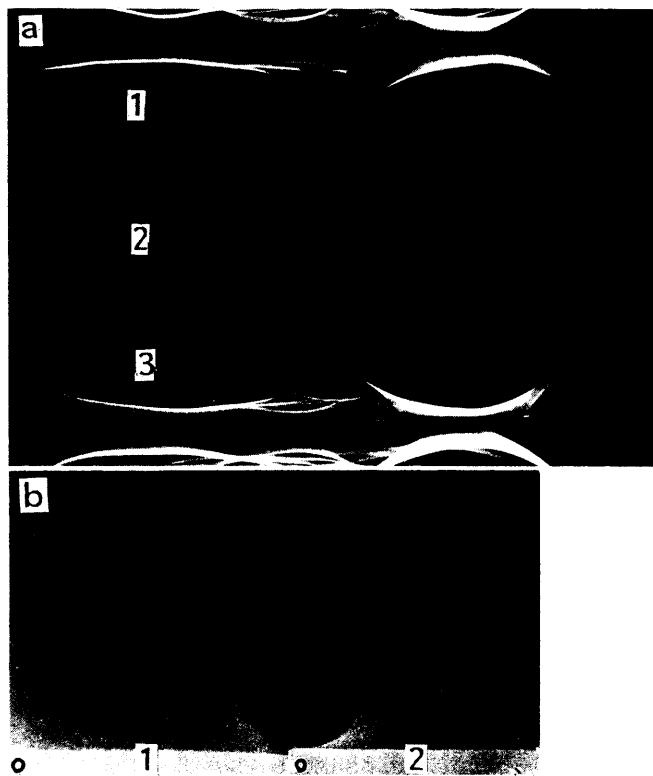


Fig. 2. Immunelectrophoretic analyses of human C3 conversion. **a** Immunelectrophoretic analyses of human fresh serum treated with: IC consisting of native SBP (1); GVB²⁺ alone (2), or IC consisting of SBP-pullulan conjugate (3). Anti-human C3 and anti-human serum were used. **b** Profiles of crossed immunoelectrodifussion. Primary electrophoresis, human fresh serum treated with IC consisting of SBP-pullulan (1) or native SBP (2). Secondary electrophoresis was performed using agarose gel containing 2% anti-human C3 antiserum.

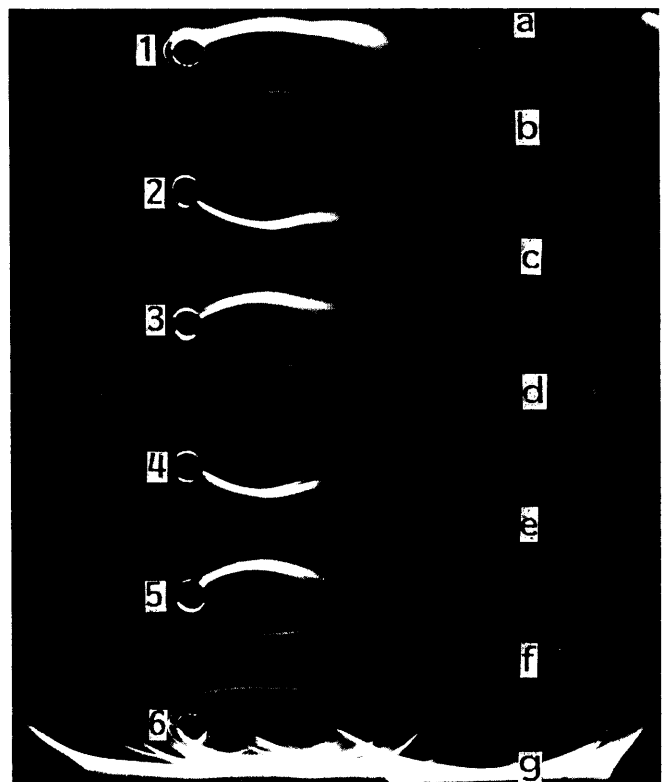


Fig. 3. Immunelectrophoretic analysis of human fresh serum treated with immune complex formed with SBP-pullulan conjugate or native SBP in the presence of Mg²⁺ · EGTA · GVB²⁺. Samples: human fresh serum treated with IC consisting of native SBP (1); IC composed of SBP-pullulan (2); SBP-pullulan alone (3); rabbit anti-SBP IgG alone (4); buffer alone (5), or none (6). Antisera: rabbit anti-human C3 (a, c, e); anti-human factor B (b, d, f), and anti-human serum (g).

ments, such as C3a or C5a known as anaphylatoxins [9, 10], are involved in the reaction. The present study shows that the ability of SBP-pullulan conjugate to elicit the Arthus reaction is markedly reduced. We could exclude the possibility that the conjugate fails to react with anti-SBP antibodies in the injection site, because the conjugate was able to neutralize free anti-SBP antibodies as effectively as native SBP, as shown in the preceding paper [5]. So, the antigen-antibody reactions could occur at the injection site of the conjugate. Therefore, it is suggested that the IC formed by SBP-pullulan conjugate might be different in physicochemical characteristics from the IC formed by native SBP. In fact, we observed that SBP-pullulan conjugate could form a precipitin line with anti-SBP antibodies in agarose gel immunodiffusion, but the line was a so-called 'clear line' (fig. 4), indicating the



Fig. 4. Immunelectrophoretic patterns of SBP-pullulan conjugate and native SBP. Upper well: native SBP. Lower well: SBP-pullulan conjugate. Antiserum: mouse anti-SBP. Arrow indicates the 'clear line' formed by the SBP-pullulan conjugate.

strong hydrophilicity of the precipitin line [11, 12]. Since the environment in which the antigen-antibody reaction occurs, or the surface nature of the particles, is known to influence the initiation of complement activation [13–16], the ability to initiate complement

activation of the IC formed with SBP-pullulan differs from that of the IC formed with native SBP. Complement activations via CP and AP are detected by several methods, for example, the complement consumption test, detection of cleavage fragments or conversion of some complement components [14, 17, 18]. In the present study, we showed that IC consisting of SBP-pullulan and anti-SBP IgG was incapable of activating the complement system effectively via both CP and AP since: (1) in the presence of Mg^{2+} and Ca^{2+} ions, the IC formed with SBP-pullulan and rabbit anti-SBP did not consume guinea pig complement at all, whereas the IC with native SBP completely consumed 5 U CH50/ml complement, and (2) in serum treated with IC consisting of SBP-pullulan, conversion of the human C3 component and cleavage of human factor B were observed as little in control serums treated with anti-SBP IgG alone or SBP-pullulan conjugate alone.

Activation of CP of complement is initiated by the binding of C1q to the Fc portions of at least two molecules of IgG antibodies bound closely to each other on antigen [18]. In the case of SBP-pullulan, two possibilities are considered: (1) the pullulan moiety of SBP-pullulan conjugate might interfere with the close approach of Fc portions of two antibody molecules on the conjugate and not allow the binding of C1q molecules, and (2) even if C1q could bind to the IC of SBP-pullulan, the surface nature of the IC could not support the formation of CP C2 convertase. For instance, Brown et al. [13] reported that erythrocyte surface molecules have an important role in modulation of CP activation, and the surface-associated CP regulation occurs at the level of cell-bound C4b. They showed that, with equal numbers of cell-bound C1 and C4, there are marked differences in C2 convertase activity on sheep, guinea pig and human erythrocytes. These two possibilities remain to be studied.

The IC consisting of anti-SBP and SBP-pullulan was found to be incapable of supporting efficient activation of AP. Cleavage of factor B was not observed in serum treated with the IC of SBP-pullulan, indicating that the formation of sufficient amounts of stable AP C3 convertase, C3b,Bb,P, could not be supported by the IC. At the present time, we do not fully understand why the IC composed of SBP-pullulan conjugate could not efficiently support the formation of C3 convertase. Hydrophilic physicochemical natures of the IC might relate to the phenomenon. It is possible that the surface of the IC could not bind AP-initiating

factors, water-hydrolyzed C3 or fluid-phase-generated C3b in normal serum, resulting in failure to support formation of AP C3 convertase, C3b,Bb,P. Another possibility is that even if the IC surface could bind these factor(s), the nature of the surface interferes with the binding of factor B to the surface-bound C3b. Studies based on biochemical analyses are required to clarify the difference in C3 fragmentation on the surfaces of ICs consisting of SBP-pullulan and of native SBP.

As far as we know, there have been no other reports indicating that allergens used for immunotherapy should have a reduced ability to activate complement systems [19]. We, however, believe that such allergens should have poor abilities to induce all types of hypersensitivities in order to minimize adverse reactions. The results reported in the previous paper [5], summarized in the Introduction, suggest that SBP-pullulan conjugate would be a good candidate for immunotherapy against cedar pollinosis. The results presented in this paper, i.e., the IC consisting of SBP-pullulan and IgG anti-SBP showed poor ability to activate complement systems, provide additional support for this suggestion because most patients with pollinosis have specific IgG antibodies in addition to IgE class. Further, we have previously reported that the cell-mediated hypersensitivity reaction to an antigen was also suppressed in mice pretreated with antigen-conjugated pullulan [20, 21]. Experiments to determine whether SBP-pullulan conjugate has such an ability are currently underway. However, all experiments mentioned in this and the preceding papers were performed using rabbit and murine anti-SBP antibodies. Experiments using human anti-SBP antibodies, IgE or IgG and both, are intended as our next studies.

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