

Regulation of Cytokine Production by Sugi Allergen–Pullulan Conjugate

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Sugi basic protein (SBP), a major allergen of Japanese cedar (*Cryptomeria japonica*) pollen, conjugated to pullulan (α -1,4'-, α -1,6'-glucan) reportedly suppresses IgE anti-SBP antibody production and enhances IgG anti-SBP antibody production in mice. We analyzed cytokine production by SBP-specific T cells after stimulation with an SBP–pullulan conjugate (SBP-P), native SBP, or a mixture of SBP and pullulan. When SBP-specific T cell lines were stimulated with the SBP-P conjugate in the presence of antigen-presenting cells (APC), the production of IFN- γ , IL-4, IL-5, and IL-10 decreased compared with the cytokine levels produced by SBP-stimulated T cells. However, when these T cells were repeatedly stimulated with the SBP-P conjugate, the production of IFN- γ increased progressively, while that of IL-4, IL-5, and IL-10 remained decreased compared with the T cells that were repeatedly stimulated with native SBP. Stimulation of the T cells with the mixture of SBP and pullulan showed little difference in the cytokine production profile from that observed after stimulation with native SBP alone. Interestingly, when the T cell lines stimulated repeatedly with SBP-P were subsequently stimulated with native SBP, a further increase in IFN- γ production was observed, while IL-10 production decreased. Inhibition of IL-4 production was also observed when SBP-specific Th2 clones were stimulated with SBP-P. These results indicate that stimulation of T cells with SBP-P up-regulates Th1 cytokine production, while down-regulating that of Th2. It is, therefore, conceivable that immunotherapeutic treatment with the SBP-P conjugate rather than with conventional SBP solutions is preferable for improving Japanese cedar pollen allergy. © 1996 Academic Press, Inc.

INTRODUCTION

CD4⁺ helper T cells regulate immune responses and, depending on the cytokines they produce, fall into two

major subsets, Th1 and Th2, both of which may differentiate from a common precursor, Th0 (1). Th1 cells selectively produce IL-2, IFN- γ , and lymphotoxin and regulate cell-mediated immune responses such as the delayed-type hypersensitivity reaction, whereas Th2 cells produce IL-4, IL-5, and IL-10 and regulate humoral immune responses such as antibody production (1, 2). Cross-regulation exists between the Th1 and Th2 subsets in which the cytokines produced by each subset reciprocally regulate the cytokine production and functions of the other (1, 2).

Protective responses to a particular type of antigen or pathogen are dependent on the development of an appropriate T helper subset. However, although the reasons are not completely clear, there have been several cases in which an inappropriate T helper subset was activated and some cytokines were overproduced by the activated T cells leading to severe consequences. For example, it is suggested that in allergic diseases and parasite infections, especially helminth infections, Th2 cells are preferentially activated (3–6) and that the IL-4 and IL-5 produced by the Th2 cells cause increased IgE production and eosinophilia, respectively. It is reasonable to speculate, therefore, that developing a new immunotherapy which inhibits the production of Th2 cytokines and preferentially activates the Th1 subset would be a useful therapeutic approach for allergic diseases.

We have previously shown that Sugi basic protein (SBP),² a major allergen of Japanese cedar (*Cryptomeria japonica*) pollen, conjugated to pullulan (α -1,4'-, α -1,6'-glucan) can induce suppression of IgE anti-SBP antibody production and augmentation of IgG anti-SBP antibody production in mice (7), although the mechanism remains to be fully elucidated. The present study was performed to determine the mechanism of IgE suppression by analyzing the cytokine production of SBP-specific murine T cells after stimulation with the SBP–

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² Abbreviations used: SBP, Sugi basic protein; SBP-P, SBP–pullulan conjugate; APC, antigen-presenting cells.

pullulan conjugate (SBP-P), native SBP, or a mixture of SBP and pullulan.

The data presented here show that repeated stimulation of SBP-specific T cells with SBP-P more effectively induced augmentation of Th1 cytokine production and suppression of Th2 cytokine production than did repeated stimulation with native SBP.

MATERIALS AND METHODS

Mice

BALB/c mice were obtained from Charles River Japan, Inc. (Kanagawa, Japan). Females aged 2 to 3 months were used throughout the experiments, either for immunization or as a source of APC.

Antigens and Reagents

SBP was prepared from Japanese cedar pollen according to Taniguchi *et al.* (7). SBP-P was prepared as described previously (7). Briefly, 8 mg of cyanuric chloride (Wako Junyaku, Tokyo, Japan; used after recrystallization) dissolved in 1 ml chilled acetone was mixed with 25 ml of 2% pullulan solution (MW 200 kDa, Hayashibara Biochemical Laboratories, Inc., Okayama, Japan) and the mixture was stirred in an ice bath for 2 hr. The pH of the reactants was maintained at 6.8–7.0 by adding 5% NaHCO₃ in drops. Activated pullulan was mixed with 50 mg of SBP, and the mixture was incubated at 37°C for 5 hr with continuous stirring. The resulting preparation was dialyzed against 0.01 M acetate buffer, pH 5.0, and loaded onto a CM-Sephadex C-25 column (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer to remove unconjugated SBP. SBP-P was recovered as an unabsorbed fraction with a single, sharp, symmetric peak. The average relative molecular weight was determined to be 4.0×10^5 by gel filtration using a G5000 PW_{XL} HPLC column (Tosoh, Tokyo, Japan). The dose of the SBP-P conjugate was expressed as the concentration of SBP in the conjugate determined by a sandwich ELISA using rabbit anti-SBP antibody and biotinylated rabbit anti-SBP antibody, both prepared in our laboratory (7). The ratio of SBP to pullulan (w/w) in the conjugate was 1:15. The endotoxin contents of both the SBP and SBP-P preparations were determined to be <0.2 ng/ml (Limulus amoebocyte lysate assay; Seikagaku Kogyo, Tokyo, Japan) at the concentrations used in this study. All cultures were maintained in complete medium composed of RPMI 1640 supplemented with 10% FCS (GIBCO BRL, Grand Island, NY), 50 μ M 2-ME, 60 μ g/ml penicillin, and 50 μ g/ml streptomycin.

Lymphokines and Abs

Human rIL-2, human rIL-1 β , and murine rIL-10 were purchased from Genzyme (Boston, MA). Murine

rIL-4 was purchased from InterGen (Purchase, NY). Murine rIL-5 was purchased from PharMingen (San Diego, CA). Murine rIFN- γ was prepared and purified in our laboratory. The activity of the rIFN- γ was calibrated against the World Health Organization–NIAID international reference reagent by bioassay using L929 cells and vesicular stomatitis virus. For determination of cytokines by sandwich ELISA, the Abs for coating the plates and the biotinylated second mAbs were as follows: for IL-4, rat mAb anti-mouse IL-4 (BVD4-1D11, PharMingen) and biotinylated mAb anti-mouse IL-4 (BVD6-24G2, PharMingen); for IFN- γ , polyclonal rabbit anti-mouse IFN- γ antibody (prepared in our laboratory) and biotinylated rat mAb anti-mouse IFN- γ (XMG1.2, PharMingen); for IL-5, rat mAb anti-mouse IL-5 (TRFK5, PharMingen) and biotinylated mAb anti-mouse IL-5 (TRFK4, PharMingen); for IL-10, rat mAb anti-mouse IL-10 (JES5-2A5, PharMingen) and biotinylated mAb anti-mouse IL-10 (SXC-1, PharMingen).

Establishment of SBP-Specific T Cell Lines and Clones

BALB/c mice were immunized four to five times by sc and ip injection with 10 μ g SBP adsorbed onto 2 mg Al(OH)₃ adjuvant (alum) every 2 weeks. Cervical, submandibular, inguinal, and mesenteric lymph nodes, used as the source of T cells, were removed 7 days after the last immunization. In the initial stimulation, lymph node cells (2.4×10^6 /well) were exposed to 10 μ g/ml SBP in 24-well plates in the presence of 6×10^6 /well syngeneic spleen cells pretreated with mitomycin C (50 μ g/ml) for 30 min at 37°C as antigen-presenting cells (APC). After 7 days, viable cells were harvested, washed twice with complete medium, and stimulated at 5×10^5 /well with fresh APC and 10 μ g/ml SBP. After 7 days, viable cells were washed and stimulated at 2.5×10^5 /well with fresh APC and 10 μ g/ml SBP. After 4–5 days, the cells were expanded threefold into medium containing 10 U/ml of rIL-2 to allow further growth. The SBP-specific T cell lines thus established were found to be composed of only CD4⁺ cells as determined by flow cytometric analysis.

SBP-specific T cell clones were obtained as follows. The lymph node cells recovered 7 days after the initial antigenic stimulation *in vitro* were cloned by limiting dilution (3, 1, and 0.3 cells/well) into 96-well flat-bottomed microtiter plates containing 5×10^5 /well APC, 10 μ g/ml SBP, 10 U/ml of rIL-2, and 10 IU/ml of rIFN- γ in 0.2 ml culture medium. After 1 and 2 weeks, half of the culture supernatants were removed and 100 μ l of fresh medium containing APC, SBP, and rIL-2 plus rIFN- γ was added to all wells. During the third week, the wells containing proliferating cells were identified visually and the cell cultures were expanded. Several of the clones obtained were immediately recloned at 0.3 cells/well. To isolate Th2 clones, 5 ng/ml of rIL-4

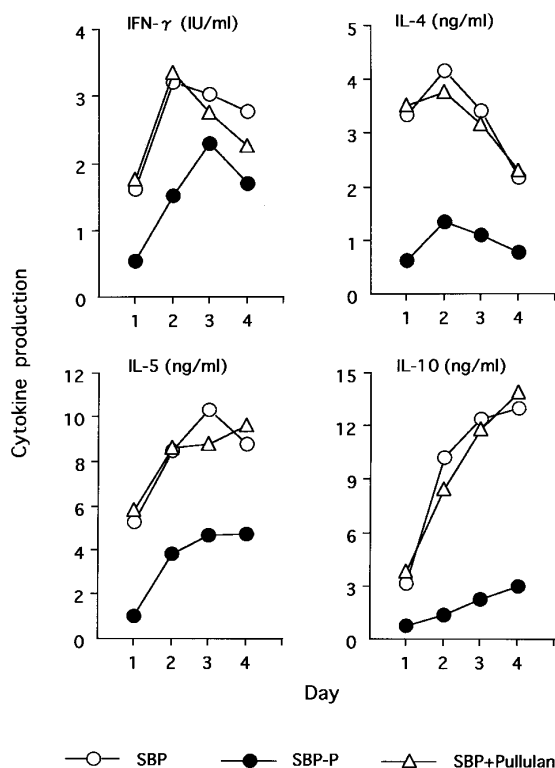


FIG. 1. Kinetics of cytokine production by SBP-specific T cell lines after stimulation with SBP, SBP-P, or SBP + pullulan. SBP-specific T cell lines (2.5×10^5 /well) were stimulated with $10 \mu\text{g/ml}$ of SBP, SBP-P, or SBP + pullulan in a total volume of 2 ml in 24-well plates in the presence of 6×10^6 /well APC. Culture supernatants were collected every day and assayed for cytokine production as described under Materials and Methods. The data shown are representative of three similar experiments.

and 10 U/ml of rIL- 1β were added. The T cell lines and clones thus obtained were maintained in culture and restimulated every 10–14 days.

Stimulation of SBP-Specific T Cell Lines and Clones for Cytokine Production

Primary stimulation of SBP-specific T cell lines (2.5×10^5 /well) in a resting state was carried out using $10 \mu\text{g/ml}$ SBP, SBP-P, or a mixture of SBP and pullulan (SBP + pullulan) and 6×10^6 /well APC in a total volume of 2 ml in 24-well plates at 37°C . These antigen doses were previously determined to be effective for cytokine production. On Day 7, the cells were expanded threefold in fresh medium and then rested for 3–5 days. Ten to 12 days after primary stimulation, the cells were harvested, washed twice, counted, and restimulated (secondary) at 2.5×10^5 /well with fresh APC and $10 \mu\text{g/ml}$ SBP, SBP-P, or SBP + pullulan. The third and fourth cultures were carried out in a manner similar to the secondary culture. In some experiments, T cells which had been stimulated with SBP-P were subsequently stimulated with native SBP.

Stimulation of SBP-specific T cell clones (1×10^4 /well) was carried out using $10 \mu\text{g/ml}$ SBP or SBP-P in the presence of 10 U/ml of rIL-2 and 5×10^5 /well APC in a total volume of 0.2 ml in 96-well plates.

To study the effects of rIL- 1β on IL-4 production by SBP-P-stimulated T cell lines or the Th2 clone, the T cells were stimulated with $10 \mu\text{g/ml}$ of SBP or SBP-P in the presence of fresh APC with or without varying concentrations of rIL- 1β .

Cytokine Assays

Culture supernatants were collected from the cultures of the T cell lines or clones at various time points after antigenic stimulation as indicated in the text and were stored at -80°C until the assays. The presence of cytokines in the culture supernatants (IL-4, IL-5, IL-10, and IFN- γ) was determined by a two-site sandwich ELISA using the mAbs described above. Standard curves were generated using recombinant cytokines.

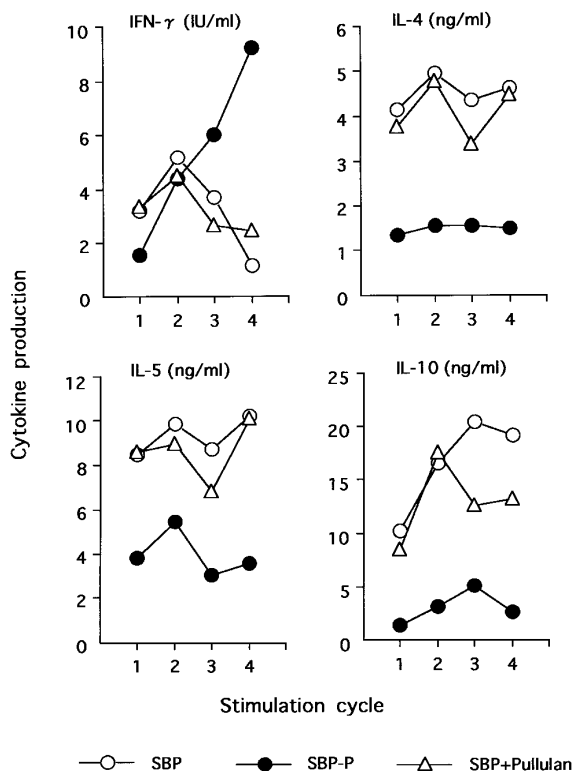


FIG. 2. Effects of repetitive stimulation with SBP, SBP-P, or SBP + pullulan on cytokine production by SBP-specific T cell lines. SBP-specific T cell lines (2.5×10^5 /well) were stimulated with $10 \mu\text{g/ml}$ of SBP, SBP-P, or SBP + pullulan in a total volume of 2 ml in 24-well plates in the presence of APC (6×10^6 /well). On Day 7, the cells were expanded threefold in fresh medium and rested for 3–5 days. Ten to 12 days after stimulation, the cells were harvested, washed twice, and restimulated with SBP, SBP-P, or SBP + pullulan. This procedure was repeated four times. Cytokine production was measured in culture supernatants collected 48 hr after each stimulation. The data shown are representative of three similar experiments.

TABLE 1
Cytokine Production by SBP-Specific T Cell Lines Subjected to Repeated Stimulations
with SBP Alone or with SBP-P Followed by SBP^a

Expt	Stimulation cycle				Cytokine production			
	1	2	3	4	IFN- γ IU/ml	IL-4 ng/ml	IL-5 ng/ml	IL-10 ng/ml
1	SBP	—	—	—	3.2	4.2	8.5	10.2
	SBP-P	—	—	—	1.5	1.4	3.8	1.4
2	SBP	→ SBP	—	—	5.0	5.2	9.9	16.5
	SBP-P	→ SBP-P	—	—	4.4	1.5	5.4	3.1
		\ SBP	—	—	13.0	4.9	11.2	10.5
3	SBP	→ SBP	→ SBP	—	3.7	4.3	8.7	20.4
	SBP-P	→ SBP-P	→ SBP-P	—	6.0	1.6	3.0	5.1
			\ SBP	—	13.6	4.4	9.4	12.7
4	SBP	→ SBP	→ SBP	→ SBP	1.2	4.6	10.2	19.1
	SBP-P	→ SBP-P	→ SBP-P	→ SBP-P	9.3	1.5	3.6	2.6
			\ SBP	—	11.6	3.9	11.1	5.1

^a Cells were cultured under the identical conditions described in the legend to Fig 2. The data shown are representative of three similar experiments.

The lower limit of detection is 25 pg/ml for IL-4, 30 pg/ml for IL-5, 200 pg/ml for IL-10, and 0.188 IU/ml (1 IU = 7 ng) for IFN- γ .

T Cell Proliferation Assays

SBP-specific T cell lines (2.5×10^4 /well) or T cell clones (1×10^4 /well) were cultured with 5×10^5 /well APC in 96-well microtiter plates, with 10 μ g/ml SBP, SBP-P, or SBP + pullulan in a total volume of 0.2 ml. Since the T cell clones obtained were low producers of autocrine growth factors, IL-2 or IL-4 (data not shown), 10 U/ml of rIL-2 was added to those cultures. Proliferation was measured by incorporation of 0.5 μ Ci [^{3} H]TdR (Dupont/NEN Research, Boston, MA) for the last 18 hr of the 72-hr culture. The amount of radioactivity was measured using a Packard Direct Beta Counter, Matrix 96 TM (Packard Instrument Co., Meriden, CT).

RESULTS

Kinetics of Cytokine Production by SBP-Specific T Cell Lines after Primary Stimulation with SBP, SBP-P, or SBP + Pullulan

The SBP-specific T cell lines established as described under Materials and Methods were stimulated with SBP, SBP-P, or SBP + pullulan, and the cytokines produced (IFN- γ , IL-4, IL-5, and IL-10) were determined by sandwich ELISA. As shown in Fig. 1, IFN- γ production by the SBP-stimulated T cell lines reached a maximum on Day 2 and then declined, whereas IFN- γ production induced by SBP-P stimulation reached a maximum on Day 3. The levels of IFN- γ production were, however, consistently slightly lower than those produced by the SBP-stimulated T cells. In contrast, IL-

4, IL-5, and IL-10 production by T cell lines stimulated with SBP-P was markedly inhibited from Day 1 through Day 4 compared with the cytokines produced by SBP-stimulated T cells (Fig. 1). There was no apparent difference in cytokine production profiles after stimulation with SBP and with SBP + pullulan. Since IFN- γ and IL-4 production after SBP stimulation reached a maximum or plateau level on Day 2, the cytokines in the 48 hr-culture supernatants were quantified in the following experiments.

Cytokine Production by SBP-Specific T Cell Lines after Repeated Stimulation with SBP, SBP-P, or SBP + Pullulan

SBP-specific T cell lines were repeatedly stimulated with antigens every 10–12 days. Forty-eight hours after each stimulation, the cytokines secreted in the culture supernatants were measured by ELISA. As shown in Fig. 2, IFN- γ production steadily increased after repeated SBP-P stimulation. There was an 8-fold increase in IFN- γ production after stimulation of the T cells four times with SBP-P compared to stimulation with SBP. In contrast, the amounts of IL-4 produced after repeated stimulation with SBP-P remained inhibited by 64–69% of that produced after SBP stimulation throughout the stimulation cycles (Fig. 2). In a manner similar to IL-4, both IL-5 and IL-10 after SBP-P stimulation decreased to 35–55% and 13–25% of the amounts produced after SBP stimulation, respectively. Collectively, in the fourth culture the ratios of IFN- γ /IL-4, IFN- γ /IL-5, and IFN- γ /IL-10 synthesis in cultures repeatedly stimulated with SBP-P resulted in 25-, 23-, and 59-fold increases, respectively, compared with those seen in the cultures after repeated SBP

stimulation. No discernible difference in cytokine production profiles was observed after stimulation with SBP and with SBP + pullulan. These results suggest that stimulation of the T cells with SBP-P up-regulates Th1 cytokine production, while down-regulating Th2 cytokine production.

Interestingly, when the T cells which had been stimulated with SBP-P one, two, or three times were subsequently stimulated with native SBP, the amounts of IFN- γ produced increased 3- to 10-fold compared to the amounts produced by T cells consecutively stimulated with SBP alone (Table 1), while IL-10 production remained more inhibited than that of cells stimulated with SBP alone.

Cytokine Production of Lymph Node Cells from SBP-Immunized Mice after Repeated Stimulation with SBP-P

To analyze cytokine production after SBP-P stimulation under conditions in which a number of cell types are included as responder cells, whole lymph node cells from SBP-immunized mice were repeatedly stimulated with antigens every week. The amounts of cytokines in culture supernatants were determined by ELISA 48 hr after each stimulation. As shown in Fig. 3, repeated stimulation with SBP-P progressively increased the amount of IFN- γ produced concomitant with a decrease in Th2 cytokines, IL-4 and IL-10, as in the case of the T cell lines. In the fourth culture, IFN- γ production by cells repeatedly stimulated with SBP-P was augmented 3.8-fold compared with that of cells repeatedly stimulated with SBP. In contrast, the IL-4 and IL-10 production levels of cells repeatedly stimulated with SBP-P were reduced to 26 and 22%, respectively, of the levels produced by cells repeatedly stimulated with SBP. Collectively, the ratios of IFN- γ /IL-4 and IFN- γ /IL-10 synthesis in cultures repeatedly stimulated with SBP-P resulted in 15- and 17-fold increases relative to those seen in cultures repeatedly stimulated with SBP. However, the levels of IL-5 produced after repeated stimulation with SBP-P or SBP were comparable.

When lymph node cells which had been repeatedly stimulated with SBP-P were subsequently stimulated with native SBP, an augmentation of IFN- γ production and a decrease in IL-10 production were observed compared with the amounts produced by cells stimulated consecutively with SBP (Table 2). These results provide evidence that the Th1-dominated cytokine response was preferentially induced after repeated SBP-P stimulation in the whole population.

Profiles of IFN- γ and IL-4 Production by SBP-Specific T Cell Clones in Response to SBP-P

To further analyze cytokine production at the clonal level after stimulation with SBP-P, we prepared SBP-specific T cell clones as described under Materials and

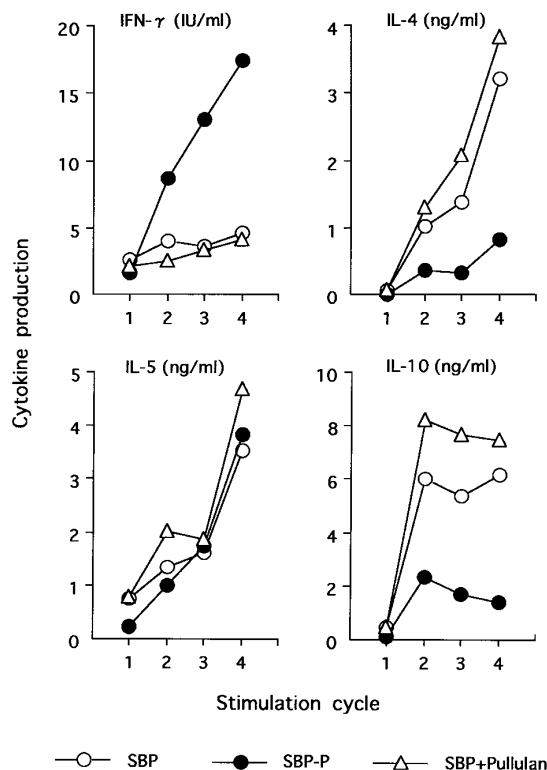


FIG. 3. Effects of repetitive stimulation with SBP, SBP-P, or SBP + pullulan on cytokine production by lymph node cells from SBP-immunized mice. Lymph node cells (2.4×10^6 /well) from SBP-immunized mice were stimulated with $10 \mu\text{g/ml}$ of SBP, SBP-P, or SBP + pullulan for 7 days in a total volume of 2 ml in 24-well plates in the presence of APC (6×10^6 /well) (first stimulation). Subsequently, viable cells were washed twice and stimulated at 5×10^5 cells/well with $10 \mu\text{g/ml}$ of SBP, SBP-P, or SBP + pullulan in the presence of fresh APC (6×10^6 /well) (second stimulation). After 7 days, viable cells were washed twice and stimulated at 2.5×10^5 cells/well with $10 \mu\text{g/ml}$ of SBP, SBP-P, or SBP + pullulan in the presence of fresh APC (6×10^6 /well) (third stimulation). The fourth stimulation was carried out under the same conditions as the third. Cytokine production was measured in culture supernatants collected 48 hr after each stimulation. The data shown are representative of two similar experiments.

Methods. Depending on the production of IFN- γ , IL-4, or both in response to SBP, the T cell clones were classified as Th1, Th2, or Th0. After stimulation with SBP-P, cytokine production was compared using these clones with that observed after SBP stimulation. Representative results are shown in Fig. 4. Among the Th1 clones, two types with different cytokine production patterns were observed. One was clones which produced higher levels of IFN- γ in response to SBP-P than the levels produced in response to SBP, and the other was clones which produced comparable levels of IFN- γ in response to either SBP or SBP-P. IL-4 production by all Th2 clones obtained in response to SBP-P was markedly reduced compared to the level observed in response to SBP (Fig. 4). No IFN- γ production by the Th2 clones was observed in response to SBP-P, even

TABLE 2
Cytokine Production by Lymph Node Cells from SBP-Immunized Mice Subjected to Repeated Stimulations with SBP Alone or with SBP-P Followed by SBP^a

Expt	Stimulation cycle				Cytokine production			
	1	2	3	4	IFN- γ IU/ml	IL-4 ng/ml	IL-5 ng/ml	IL-10 ng/ml
1	SBP	—	—	—	2.5	0.05	0.7	0.4
	SBP-P	—	—	—	1.6	<0.025	0.2	<0.2
2	SBP	→ SBP	—	—	4.0	1.0	1.4	6.0
	SBP-P	→ SBP-P	—	—	8.7	0.4	1.0	2.3
		\ SBP	—	—	6.4	0.9	1.7	5.0
3	SBP	→ SBP	→ SBP	—	3.6	1.4	1.6	5.4
	SBP-P	→ SBP-P	→ SBP-P	—	13.0	0.3	1.8	1.7
		\ SBP	—	—	10.1	0.9	2.8	3.2
4	SBP	→ SBP	→ SBP	→ SBP	4.6	3.2	3.5	6.2
	SBP-P	→ SBP-P	→ SBP-P	→ SBP-P	17.4	0.8	3.8	1.4
		\ SBP	—	—	16.6	1.6	4.8	2.3

^a Cells were cultured under the identical conditions described in the legend to Fig 3. The data shown are representative of two similar experiments.

when the clones were stimulated twice with SBP-P (data not shown). Several patterns of IFN- γ production were observed in the Th0 clones in response to SBP-P stimulation (Fig. 4) after which IL-4 production by

three of the four Th0 clones decreased to 7–47% of the levels observed after SBP stimulation. However, the remaining Th0 clone, #22, produced comparable levels of IL-4 after SBP stimulation and after SBP-P stimulation. In the experiments using T cell lines, IL-4 production in response to SBP-P stimulation never decreased to undetectable levels, even though the T cell lines had been repeatedly stimulated with SBP-P (Fig. 2). This may be accounted for by the presence of the Th0 clone.

To exclude the possibility that T cell clones stimulated with SBP-P became anergic to SBP stimulation, they were examined for their proliferative response to SBP or SBP-P. As shown in Table 3, when the clones were stimulated with SBP-P, the proliferation of most of them decreased to 16–69% of the level observed after SBP stimulation, but was never abrogated. Proliferation of Th2 clone #13 was markedly reduced. However, when these SBP-P-stimulated clones, including clone #13, were stimulated with the native SBP again after a 14-day resting culture in medium containing 10 U/ml of IL-2 for Th1 and Th0 clones and 10 U/ml of IL-2 and 5 ng/ml of IL-4 for Th2 clones, the proliferative response recovered to the level observed in response to SBP. Since it was reported that the addition of exogenous IL-2 or IL-4 did not prevent Th2 clonal anergy (8), exogenous IL-2 and IL-4 added during the resting culture would not restore Th2 clonal anergy. The results obtained with the proliferation assay therefore suggest that the T cell clones were never anergized by treatment with SBP-P.

Addition of rIL-1 β Does Not Restore Decreased IL-4 Production by SBP-P-Stimulated T Cell Lines and Clones

It is possible that the reduced IL-4 production of SBP-P-stimulated T cells might be due to insufficient

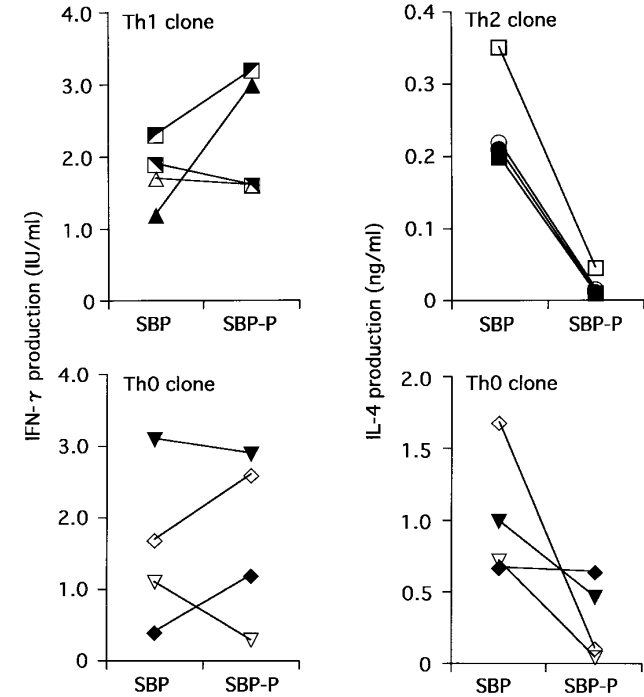


FIG. 4. Cytokine production by T cell clones after stimulation with SBP or SBP-P. SBP-specific T cell clones (1×10^4 /well) were cultured with 10 μ g/ml of SBP or SBP-P in the presence of fresh APC (5×10^5 /well) and 10 U/ml of rIL-2 in a total volume of 0.2 ml in 96-well plates. Cytokine production was measured in culture supernatants collected 48 hr after stimulation. The data shown are representative of two similar experiments.

TABLE 3

Proliferation of T Cell Clones in Response to SBP or SBP-P^a

Clone	Proliferation (cpm)			
	Primary culture		Secondary culture ^b	
	SBP	SBP-P	SBP → SBP	SBP-P → SBP
Th1				
#1	5194	2133	5579	4723
#9	2873	1974	3603	3841
#33	2144	820	ND ^c	ND
#49	5195	2688	ND	ND
Th2				
#13	799	61	1732	1596
#14	3670	1707	3840	3177
#21	3579	2722	ND	ND
#36	1553	254	ND	ND
Th0				
#20	3274	788	2803	2663
#22	1314	619	ND	ND
#25	779	194	ND	ND
#31	1326	645		

^a SBP-specific T cell clones (1×10^4 /well) were cultured with 10 μ g/ml of SBP or SBP-P in the presence of fresh APC (5×10^5 /well) and 10 U/ml of IL-2 in a total volume of 0.2 ml in 96-well plates.

^b In the secondary culture, T cell clones which had been stimulated with SBP or SBP-P and rested for 14 days in medium containing 10 U/ml IL-2 for the Th1 and Th0 clones or in medium containing 10 U/ml IL-2 and 5 ng/ml IL-4 for the Th2 clones were stimulated with 10 μ g/ml of SBP. Data are expressed as the means of duplicate samples.

^c Not done.

delivery of costimulatory signals. It is well known that IL-1 has costimulatory activity on Th2 cells (9). Therefore, we investigated the possibility that IL-1 is involved in the decreased IL-4 production. SBP-specific T cell lines or the Th2 clone were stimulated with SBP or SBP-P in the presence of APC, with or without varying concentrations of rIL-1 β . As shown in Table 4, the addition of rIL-1 β had little effect on IL-4 production by T cell lines and clone, regardless of the stimulating antigen, and could not restore decreased IL-4 production by SBP-P-stimulated T cells. These results suggest that IL-1 production does not account for the inhibition of IL-4 production by SBP-P-stimulated T cells.

DISCUSSION

In this study, we showed that repeated cycles of stimulation of SBP-specific T cell lines with the SBP-P conjugate induced a polarization of the T cell response toward a Th1-like pattern of cytokine production, essentially augmentation of IFN- γ production and inhibition of IL-4, IL-5, and IL-10 production. Furthermore, a variety of reaction patterns was observed in SBP-specific T cell clones in response to SBP-P, reflecting the results obtained with T cell lines. In particular, the

IL-4 production by all Th2 clones was almost completely abrogated in response to SBP-P. It seems unlikely that the abrogation of IL-4 production was due to anergy of the cells, since significant proliferation was observed in these T cell lines and clones in response to SBP-P. After the primary stimulation of SBP-specific T cell lines with SBP-P, IFN- γ production levels were also lower than those observed after SBP stimulation in addition to reduced IL-4, IL-5, and IL-10 production. In addition, the proliferative responses of T cell lines and clones against SBP-P were lower than those against SBP stimulation (Table 3 and data not shown). Therefore, the activation signals delivered by SBP-P stimulation are considered to be weaker than those delivered by SBP stimulation. However, the findings that IFN- γ production progressively increased after repeated SBP-P stimulation and that some Th1 and Th0 clones produced higher levels of IFN- γ in response to SBP-P than the levels produced in response to SBP invalidate the above consideration.

It has been shown that modification in the TCR contact residue of an antigen can cause qualitative differences in the effector phase of the immune response (10). For example, Evavold and Allen showed that an analog peptide of murine hemoglobin (64–76) in which Glu73 was substituted for a conservative amino acid, Asp, activated the hemoglobin (64–76)-specific Th2 clone to induce IL-4 production but that it also abrogated the proliferative response (11). Soloway *et al.* reported that substitution of a single amino acid residue in the TCR contact region of the bacteriophage λ cI repressor protein abrogated immediate-type hypersensitivity *in vivo* (12). In view of these findings, it is

TABLE 4

Effect of rIL-1 β on Cytokine Production by SBP-Specific Th2 Clone or T Cell Lines in Stimulation with SBP or SBP-P^a

Antigen	Addition	Th2 clone	T cell line	
		IL-4 pg/ml	IFN- γ IU/ml	IL-4 pg/ml
SBP	None	165	17.9	5520
	IL-1 β (2 U/ml)	158	ND ^b	ND
	IL-1 β (20 U/ml)	175	16.1	5780
	IL-1 β (200 U/ml)	172	ND	ND
SBP-P	None	<25	4.9	1400
	IL-1 β (2 U/ml)	<25	ND	ND
	IL-1 β (20 U/ml)	<25	6.0	1360
	IL-1 β (200 U/ml)	<25	ND	ND

^a Th2 clone (2.5×10^5 /well) or T cell lines (2.5×10^5 /well) were stimulated with 10 μ g/ml of SBP or SBP-P in the presence of fresh APC (6×10^6 cells/ml) with or without varying concentrations of rIL-1 β in 24-well plates. Cytokine production was measured in culture supernatants collected 48 hr after stimulation. The data shown are representative of three similar experiments.

^b Not done.

tempting to speculate, as one of the possible explanations of our results, that when pullulan binds to an amino acid residue of SBP within or near the TCR contact region, the conjugate may transmit qualitatively different signals leading to quantitative and qualitative variations in cytokine production. In one Th0 clone, #22, IL-4 production was comparable between SBP- and SBP-P-stimulated cultures, while in the other three Th0 clones IL-4 production decreased considerably after SBP-P stimulation. This suggests that clone #22 might recognize an epitope receiving no influence from the binding of pullulan to SBP. Epitope mapping of all the SBP-specific T cell clones and identification of the binding sites of pullulan to SBP are in progress to evaluate this speculation.

Since IL-1 is well known as a costimulatory signal in Th2 cells (9), we investigated the suppressive effects of SBP-P stimulation on IL-4 production to determine whether they could be accounted for by the defects in IL-1 production. However, the addition of rIL-1 β to the culture did not restore the decreased IL-4 production by T cell lines or the Th2 clone in response to SBP-P. In this regard, Lichtman *et al.* reported that IL-1 may have no effect on IL-4 production by T cells, although it acts as a costimulator for the proliferation of Th2 clones (13).

It has been shown that IL-12 enhances IFN- γ production by T cells or NK cells (14, 15), induces Th1 development *in vitro* and *in vivo* (16–20), and under some experimental conditions decreases IL-4 production (21). Therefore, the possibility that IL-12 participates in the up-regulation of IFN- γ by SBP-P-stimulated T cells should be considered.

Recently, it was shown that cAMP and cAMP-increasing reagents such as cholera toxin and forskolin up-regulate the IL-4 and IL-5 production of activated CD4 $^{+}$ T cells while decreasing IL-2 production (22). These results are reciprocal phenomena which we have observed in the present study. Therefore, it will be interesting to investigate whether cAMP levels in T cells stimulated with SBP-P decrease.

When lymph node cells from SBP-immunized mice were repeatedly stimulated with SBP-P, IL-5 production levels were comparable with those observed after SBP stimulation, while both IL-4 and IL-10 production decreased. By FACS analysis, the lymph node cells stimulated three times with SBP-P *in vitro* were found to contain 10% CD3 $^{+}$ CD8 $^{+}$ T cells, while the lymph node cells stimulated three times with SBP were shown to be composed of 100% CD3 $^{+}$ CD4 $^{+}$ T cells (data not shown). Croft *et al.* reported that CD8 $^{+}$ T cells primed with alloantigen in the presence of IL-4 produced only small amounts of IL-4, whereas substantial levels of IL-5 were produced in the secondary stimulation with the alloantigen (23). Furthermore, it has been shown that intraepithelial CD8 $^{+}$ T cells, isolated from the small intestine of mice, produce IL-5 in response to Con

A (24). Although the reason CD8 $^{+}$ T cells remained in the SBP-P-stimulated T cell population is unclear, the presence of such cells may account for our finding that the levels of IL-5 production of the lymph node cells stimulated with SBP-P were comparable to the IL-5 production of lymph node cells stimulated with SBP.

It was reported that administration to mice of OVA conjugated with pullulan resulted in the induction of a long-lasting antigen-specific IgE-selective unresponsiveness to subsequent immunization with native OVA together with alum (25). A similar phenomenon was found *in vivo* with the SBP-P conjugate (7). Since IFN- γ is known to inhibit IL-4-mediated IgE production (26, 27), the antigen-specific suppression of IgE production observed in mice treated with the antigen–pullulan conjugate may be accounted for by the speculation that the increased IFN- γ production and the decreased IL-4 and IL-10 production were induced *in vivo* after stimulation with the conjugate as observed *in vitro* in this study. However, a detailed *in vivo* study would be necessary to confirm this possibility. In this regard, Hayglass *et al.* reported that treatment of mice with glutaraldehyde-polymerized OVA inhibited the induction of IgE responses and abrogated ongoing IgE synthesis, which is as we observed (28, 29). Furthermore, they showed that administration of polymerized OVA to mice elicited preferential induction of Th1-like responses (30).

Recent investigations into the immunological basis of allergic diseases implicate Th2 cells in particular (3–6). In this study we have shown that the conjugate of SBP and pullulan down-regulates the Th2 cytokine production of SBP-specific T cells while up-regulating Th1 cytokine production. These results together with the above finding of specific IgE suppression *in vivo* may have implications for therapeutic approaches in allergic disorders. Furthermore, when the T cell lines or lymph node cells which had been stimulated once or repeatedly with SBP-P were stimulated with native SBP, an increased IFN- γ production and a decreased IL-10 production were observed compared with cytokine production by T cells stimulated with SBP. These results further suggest that the conjugate may be useful as an immunotherapy.

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