

PS80 interferes with the antiallergic effect of Cry-consensus peptide, a novel recombinant peptide for immunotherapy of Japanese cedar pollinosis, at very low concentration through modulation of Th1/Th2 balance

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Summary

Polysorbate 80 (PS80 or Tween-80) is often used as an additive to promote the rapid solubilization of pharmaceuticals in aqueous solutions. We investigated whether coinjection of a minimal amount of PS80 had a modulatory effect on the immunotherapeutic effects of Cry (Cryptomeria)-consensus peptide, a novel peptide developed for the therapeutic management of Japanese cedar pollinosis, using a Cry j 1-sensitized mouse model with experimental allergic rhinitis. Subcutaneous challenge with Cry-consensus peptide plus 50 µg/ml of PS80 did not affect the antigen-specific proliferation of splenocytes, but decreased the potency of Cry-consensus peptide to inhibit antigen-specific interleukin (IL)-5 production by the cells significantly in comparison with challenge with Cry-consensus peptide alone. However, there was no significant difference between the effect of Cry-consensus peptide administration on interferon (IFN)-γ production in the presence and absence of PS80, indicating that PS80 interfered with the T helper 1 (Th1)-dominant T helper balance induced by Cry-consensus peptide challenge. Moreover, the increase in the level of antigen-specific immunoglobulin G2a (IgG2a) induced by Cry-consensus peptide challenge was inhibited slightly but unambiguously by PS80 coinjection. These *in vitro* experiments indicated that PS80 induces Th2-type differentiation of T helper cells through preferential inhibition of IFN-γ expression relative to IL-5 expression in splenocytes in a concentration-dependent manner. In naïve mice, sensitization by Cry-consensus peptide with PS80 induced antigen-specific IL-5 production more potently than sensitization by Cry-consensus peptide alone, and when PS80 was added to bone marrow-derived dendritic cells, the endocytosis of fluorescence-labelled Cry-consensus peptide was dramatically inhibited in a concentration-dependent manner. Therefore, we conclude that PS80 has an immunomodulatory effect on the antigen-specific response resulting in a shift towards Th2 predominance with respect to the antigen recognition stage. Taken together, our findings suggest that PS80 might decrease the efficacy of Cry-consensus peptide through modulation of the efficiency of antigen endocytosis and/or of the direction of successive T helper cell differentiation.

Keywords: allergy; cytokine; immunotherapy; pollinosis; polysorbate 80

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Abbreviations: BMDC, bone marrow-derived dendritic cell; BSA, bovine serum albumin; CD, cluster of differentiation; Cry, Cryptomeria; DC, dendritic cell; EDRF, endothelium-derived relaxing factor; ELISA, enzyme-linked immunosorbent assay; EPO, eosinophil peroxidase; FCS, fetal calf serum; GM-CSF, granulocyte-macrophage colony-stimulating factor; Ig, immunoglobulin; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PS80, polysorbate 80; PWM, poke weed mitogen; SIT, specific allergen immunotherapy; Th, T helper.

Introduction

During the past few decades, the prevalence of allergic diseases such as rhinitis and asthma has dramatically increased.^{1–3} Japanese cedar pollinosis represents a major clinical problem from late February to April of every year in Japan, affecting as many as 10% of the Japanese population.⁴ Recently, it has been demonstrated that the subcutaneous or oral administration of a peptide containing at least one immunodominant T-cell epitope from a particular allergic protein can induce T-cell non-responsiveness to a subsequent allergenic protein or allergen challenge in experimental murine models *in vivo* and *in vitro*.^{5–10} The use of peptide sequences corresponding to T-cell determinants of the allergen has been postulated as an alternative to specific allergen immunotherapy (SIT) in which high molar doses of T-cell determinants can be delivered over a shorter time period and with improved safety compared with SIT. In efforts to address the problem of Japanese cedar pollinosis, clinical studies by our company and others, employing subcutaneous or sublingual administration of peptides containing major T-cell epitopes, are now underway.

Allergic diseases have been reported to be linked to an excess of the T helper 2 cytokines interleukin (IL)-4 and IL-5 relative to the T helper 1 cytokine interferon (IFN)- γ .^{11,12} Previously, we reported the concept of peptide-based immunotherapy for Japanese cedar pollinosis employing Cry (Cryptomeria)-consensus peptide (Sone *et al.*^{13,14}), which consists of six major T-cell determinants from Cry j 1 and Cry j 2, major allergens of Japanese cedar (*Cryptomeria japonica*) pollen (Fig. 1). The effect of Cry-consensus peptide challenge on allergic parameters indicated an improved T helper

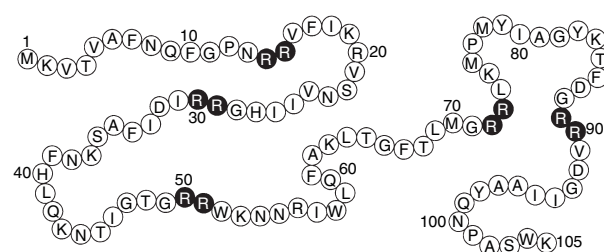


Figure 1. Amino acid sequence of the Cry (Cryptomeria)-consensus peptide. Six T-cell epitopes (1–13, 16–28 and 74–88 from Cry j 1 and 31–49, 52–71 and 91–105 from Cry j 2) are linearly linked with arginine dimers. The concept of the Cry-consensus peptide was first suggested by Sone *et al.*^{13,14} Major histocompatibility complex (MHC) class II restriction molecules for each epitope are as follows: peptides 1–13 and 52–71, DPB1*0501; peptide 16–28, DRB5*0101; peptide 31–49, DPB1*0201/DRB4*0101; and peptide 91–105, DRB5*0101 (unknown for peptide 74–88). B10.S mice sharing the N-terminal epitope peptide (1–13) with humans were used in all experiments.

pattern; for example, splenic cytokine production and the immunoglobulin E (IgE)/IgG2a antibody ratio in serum were decreased (our unpublished data). In clinical trials, Cry-consensus peptide challenge was also effective in improving the total symptom and medication scores of Japanese cedar pollinosis patients in the last pollen season (our unpublished data). However, when Cry-consensus peptide challenge was performed with 25 μ g/ml of PS80, which enabled the drug to be immediately solubilized in the vehicle, its efficacy was diminished. The mechanism by which PS80 interfered with the efficacy of Cry-consensus peptide was not known.

There have been many reports on the *in vivo* and *in vitro* effects of surfactants such as PS80, which are commonly added to pharmaceutical formulations.^{15–19} For example, PS80 inhibits the peptide transporter, as indicated in assays of glycyl sarcosine permeability in Caco-2 cell monolayer cultures, and inhibits the increases of digoxin absorption after oral administration through inhibition of the efflux mediated by P-glycoprotein in the rat intestine. However, studies of the immunomodulatory effect of PS80 have not been reported, to the best of our knowledge.

In the present study, we investigated the mechanism by which PS80 reduces the antiallergic effect of Cry-consensus peptide challenge in B10.S mice. Firstly, the effect of PS80 on the antigen recognition stage was examined by Cry-consensus peptide immunization. In experimental allergic rhinitis model mice, antigen-specific IgE and IgG2a levels, and eosinophil peroxidase (EPO) and cytokine secretion from splenocytes were compared between Cry-consensus peptide alone and Cry-consensus peptide plus PS80 challenges. Moreover, the *in vitro* effect of PS80 on the shift of the T helper balance in splenocytes from Cry j 1-sensitized mice was also investigated. Finally, the inhibitory effect of PS80 on Cry-consensus peptide endocytosis into bone marrow-derived dendritic cells was studied by confocal microscopy. In the light of these findings, possible mechanisms by which PS80 could have decreased the immunotherapeutic effect in the clinical study are discussed.

Materials and methods

Culture media and reagents

The cell culture medium used throughout was RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Filtron, Brooklyn, Australia), 1% (100 $\times$) penicillin-streptomycin-glutamine, 1% minimal essential medium (MEM) (100 $\times$) non-essential amino acid solution, 100 mM 1% MEM (100 $\times$) sodium pyruvate solution (these three reagents were obtained from Invitrogen, Grand Island, NY), and 50 μ M 2-mercaptoethanol (Sigma,

St Louis, MO). Cry j 1 was purified from Japanese cedar pollen by our group. *Escherichia coli*-expressed Cry-consensus peptide was purified by the bioengineering division of our company and provided to us as a gift. Fluorescence-labelled Cry-consensus peptide (Cry-consensus F) was synthesized using ATTO 550 (ATTO-TEC GmbH, Seigen, Germany) and purified in our laboratory.

Sensitization and administration

B10.S mice weighing between 17 and 20 g (SLC Japan, Shizuoka, Japan) were housed in a room with a 12/12 hr light/dark cycle (lights on from 07:00 hr to 19:00 hr) at constant temperature ($22 \pm 3^\circ$) with free access to standard chow and water throughout the experimental period, and were acclimatized to the colony room for 1 week before use in the experiments.

Effect of PS80 on sensitization. B10.S mice were immunized three times with Cry-consensus peptide (1 mg in 0.2 ml of 5% glucose with or without 10 µg of PS80 saline) at intervals of 1 week. Three weeks after the last immunization, Cry-consensus peptide (1 mg in 0.2 ml of 5% glucose) was further injected to boost the antigen-specific immune response. The splenocytes were then prepared 1 week after the boosting.

Effect of PS80 on allergic rhinitis model mice. Cry j 1 (10 µg in 0.1 ml of saline) was subcutaneously injected three times with 0.1 ml of Imject Alum (Pierce, Rockford, IL) at intervals of 1 week to sensitize the mice. Starting 3 weeks after the last sensitization, Cry-consensus peptide (1 mg in 0.2 ml of 5% glucose with or without 10 µg of PS80) or vehicle control (0.2 ml of 5% glucose) was injected subcutaneously five times at intervals of 1 week. One week after the last challenge, histamine solution (20 ng in 20 µl of saline) was administered intranasally, followed by intranasal administration of Cry j 1 solution (4 µg in 20 µl of saline) for five successive days to boost the antigen-specific immune response. The sera, nasal tissues and splenocytes were prepared 24 hr after the last boosting.

Effect of PS80 in vitro. Cry j 1 (10 µg in 0.1 ml of saline) was subcutaneously injected three times with 0.1 ml of Imject Alum at intervals of 1 week to sensitize the mice. Starting 3 weeks after the last sensitization, Cry j 1 (10 µg in 0.2 ml of saline) was injected subcutaneously to boost the antigen-specific immune response. The splenocytes were prepared 1 week after the boosting.

Cry j 1-specific IgE and IgG2a levels in the serum

Cry j 1-specific IgE antibody. Purified anti-mouse IgE monoclonal antibody (mAb) (BD Pharmingen, San

Diego, CA) was used to coat FluoroNunc plates (Nunc, Roskilde, Denmark) overnight at 4° in 10 µg/ml of 0.1 M carbonate buffer, pH 8.2. After blocking with 1% bovine serum albumin (BSA)/phosphate-buffered saline (PBS), pH 7.2, serum samples (appropriately diluted with blocking media) were added and the reaction allowed to proceed for 1 hr at room temperature. Pooled mouse serum containing Cry j 1-specific IgE prepared in our laboratory was used as a relative standard for this assay. Biotinylated-Cry j 1 (Hayashibara, Okayama, Japan) was added and the mixture was incubated at room temperature for 1 hr, followed by reaction with β -galactosidase-conjugated streptavidin for 30 min. For detection of the product, 4-methylumbelliferyl- β -D-galactopyranoside solution was added and the mixture was incubated for 2 hr at 37° , and the product was measured using Fluoroskan Ascent (Labsystems, Helsinki, Finland) at wavelengths of excitation 355 nm/emission 460 nm after the reaction had been stopped with 0.2 M glycine-NaOH, pH 10.3.

Cry j 1-specific IgG2a antibody. Immunoplate II plates (Nunc) were coated with 50 µl of Cry j 1 (10 µg/ml) in 0.5 M carbonate buffer, pH 9.5, overnight at room temperature. After blocking with 1% BSA/PBS, pH 7.2, for 1 hr at 37° , 50-µl serum samples (appropriately diluted with blocking media) were added and allowed to react for 1 hr at room temperature. Peroxidase-conjugated anti-mouse IgG2a monoclonal antibody (Roche, Indianapolis, IN) was added and the mixture was incubated for 1 hr at room temperature followed by colorization with a TMB Substrate Kit (Pierce) for 15 min. The absorbance was measured at a wavelength of 450 nm (595 nm) after the reaction had been stopped with 2 M sulphuric acid.

Eosinophil peroxidase

Eosinophil peroxidase (EPO) activity in nasal tissue was measured by the following method. The tip of the nose area was homogenized in 5 ml of ice-cold 0.5% hexadecyltrimethylammonium bromide in PBS, pH 7.2, and freeze-thawed three times. The tissue suspension was then centrifuged at 4° , and the supernatant (50 µl) was used for EPO measurement. The supernatant was mixed with 100 µl of the substrate solution (16 mM *o*-phenylenediamine/50 mM Tris-HCl, pH 8.0, including 0.01% H_2O_2) for 15 min and the reaction was terminated by adding 50 µl of 2 N sulphuric acid. Based on the absorbance at a wavelength of 490 nm, EPO activity was expressed as relative units compared to the standard prepared from guinea pig eosinophils.

Splenocyte culture

Splenocytes were prepared under ice-chilled conditions by the following method. Spleens obtained from killed mice

were gently homogenized on a stainless mesh and red blood cells were lysed in ammonium chloride haemolytic buffer. The recovered cells were washed with ice-cold medium and resuspended in culture medium. The cells were cultured at a final concentration of 10^5 cells/well in 96-well plates for 3 days at 37° , 5% CO_2 in the presence of the indicated antigen stimulation together with 0–5000 $\mu\text{g/ml}$ PS80. Control cultures were incubated with no stimulation. Culture supernatants were harvested at the indicated times and cytokine concentrations were measured by enzyme-linked immunosorbent assay (ELISA).

Proliferation assay

Splenocytes (5×10^5) cultured for 3 days were stimulated for a further 16 hr with 1 μM Cry j 1, 1 μM Cry-consensus peptide, or 0.5 $\mu\text{g/ml}$ pokeweed mitogen (PWM) in the presence of radioactive thymidine (^3H -thymidine, 0.5 $\mu\text{Ci/well}$, Perkin-Elmer, Boston, MA), and then the wells were harvested and the incorporation of ^3H -thymidine was determined in a β -counter.

Cytokine ELISA

To estimate the concentrations of IL-4, IL-5 and IFN- γ , commercial sandwich ELISA sets (BD Pharmingen) were used. Briefly, an Immunoplate II plate (Nunc) was coated with coating antibodies in 0.5 M carbonate buffer, pH 9.5, overnight at 4° . The plate was blocked with 1% FCS/PBS, pH 7.2, for 1 hr at room temperature, followed by a 2-hr reaction with 100 $\mu\text{l/well}$ of culture supernatant at room temperature. Premixed reagent consisting of biotinylated antibody and avidin-horseradish peroxidase conjugate (detection reagent) was added and the mixture was incubated for 1 hr at room temperature. Colorization was performed with an ImmunoPure TMB substrate kit (Pierce) and the absorbance was measured at a wavelength of 450 nm (595 nm) after the reaction had been stopped with 2 N sulfuric acid.

Confocal microscopy

Bone marrow-derived dendritic cells (BMDCs) were generated as described previously.²⁰ In brief, bone marrow from the femur and tibia of B10.S mice was cultured after lysing red blood cells at 10^6 cells/ml in RPMI-1640 medium containing 20 ng/ml recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) (Former Genzyme-Techne, Minneapolis, MN). The medium was replaced on days 2 and 4, and the suspended cells (immature DCs) were recovered on day 6. The BMDCs obtained were assessed by staining for CD11c and MHC class II molecules before use.

Dendritic cells at 5×10^5 cells/ml were kept on ice for 30 min, and then mixed with Cry-consensus F at a final

concentration of 50 $\mu\text{g/ml}$ in the presence or absence of PS80 (0.5–5000 $\mu\text{g/ml}$). The cells were chilled on ice for a further 30 min and unbound peptide was washed away with ice-cold medium. The cells were incubated at 37° for various times in fresh medium including the indicated concentration of PS80 but not Cry-consensus F. The cells were fixed overnight at 4° in 2% paraformaldehyde in PBS, pH 7.2, and were mounted using a ProLong Antifade Kit (Molecular Probes, Eugene, OR) and examined with a Fluoview FV1000 (Olympus, Tokyo, Japan) with a $\times 100$ oil immersion objective.

Statistical evaluation

For comparison between two groups, the data were firstly analysed for homogeneity of variance using the *F*-test. When the variances were homogeneous, results were evaluated using Student's *t*-test. When the variances were not homogeneous, the Aspin-Welch test was performed to analyse statistical differences.

For comparison among multiple groups, the data were first analysed for homogeneity of variance using Bartlett's test. When the variances were homogeneous, the parametric Scheffe multiple comparison test was performed. When the variances were not homogeneous by Bartlett's test, the non-parametric Scheffe-type multiple comparison test was performed in order to examine the significance of differences among the groups.

Results

PS80 prevents the immunotherapeutic effect in allergic rhinitis model mice

Subcutaneous injection of Cry-consensus peptide decreases the production of Th2-type cytokines from splenocytes in experimental allergic rhinitis model mice (our unpublished data). Focusing on the shift of the cytokine profiles of the model mice, we examined the immunomodulatory effect of PS80. The study design is shown in Fig. 2(a).

Firstly, the proliferation of splenocytes in response to antigen stimulation was examined. Among mice administered Cry-consensus peptide alone, Cry-consensus peptide plus PS80 or vehicle control, there was no significant difference in ^3H -thymidine uptake by Cry j 1-sensitized or PWM-stimulated splenocytes (used as a positive control) (Fig. 2b). Thus, it was considered that PS80 did not affect the antigen-stimulated cell proliferation activity. Secondly, we investigated whether Cry j 1-specific IL-5 production from splenocytes was influenced by PS80 administration. Injection of Cry-consensus peptide without PS80 decreased Cry j 1-specific IL-5 production from the cells in the model mice, but this effect was significantly diminished in the presence of 50 $\mu\text{g/ml}$ PS80 (Fig. 2c). In

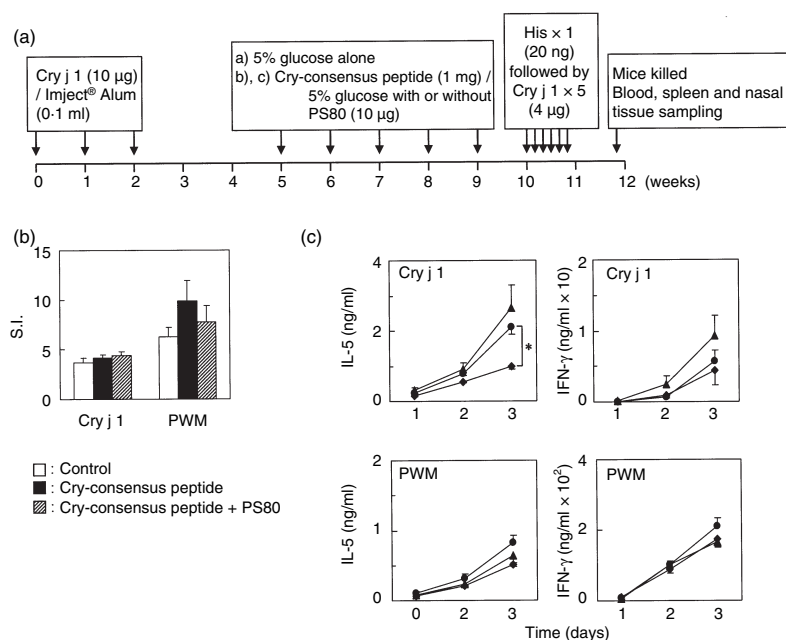


Figure 2. Effect of polysorbate 80 (PS80) on ^3H -thymidine uptake and cytokine profiles in an experimental allergic rhinitis model. (a) Experimental design used to investigate the *in vivo* effect of PS80 on an experimental allergic rhinitis model. The cells were cultured for the indicated times in the presence of $1\ \mu\text{M}$ Cry j 1 or $0.5\ \mu\text{g/ml}$ poke weed mitogen (PWM) as described in Materials and methods. (b) ^3H -thymidine uptake, (c) interleukin (IL)-5 secretion and interferon (IFN)- γ secretion of cultured splenocytes. Triangles, diamonds, and circles represent control, Cry-consensus peptide, and Cry-consensus peptide plus PS80 subcutaneous injection, respectively. Each symbol and error bar indicate the mean and standard error of seven individual determinations, respectively. An asterisk indicates a significant difference between mice challenged with Cry-consensus peptide alone and with Cry-consensus peptide plus PS80. S.I.; stimulation index.

contrast, no significant effect of PS80 on the increase of Cry j 1-specific IFN- γ production was observed at least until day 3. The IFN- γ /IL-5 ratios of the Cry-consensus peptide alone and Cry-consensus peptide plus PS80 groups were 0.15 and 0.08 at day 2 and 0.45 and 0.27 at day 3, respectively. This result indicates that PS80 changed the cytokine ratio obtained by challenge with Cry-consensus peptide alone towards Th2 dominance. The induction of IL-5 and IFN- γ secretion by PWM stimulation was equivalent among the groups (Fig. 2c).

Next, we evaluated the serum levels of Cry j 1-specific IgE and IgG2a antibodies. The relative level of anti-Cry j 1 IgE antibody was usually slightly decreased by Cry-consensus peptide challenge in the model mice; however, there was no significant difference between Cry-consensus peptide- and vehicle control-challenged mice (Fig. 3a). When PS80 was injected into the model mice with Cry-consensus peptide, the titre of IgE antibody did not change significantly in comparison with that in mice injected with Cry-consensus peptide alone. However, the level of Cry j 1-specific IgG2a antibody was increased by Cry-consensus peptide challenge, and the increase was prevented by the addition of PS80 (Fig. 3b). In our unpublished study, the Cry j 1-specific IgG2a level in serum was increased significantly by Cry-consensus peptide challenge in B10.S mice. We do not know the reason

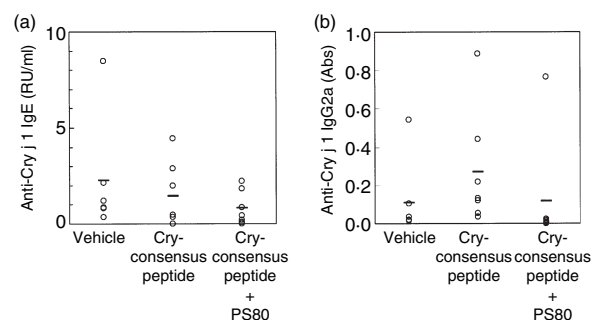


Figure 3. Effect of polysorbate 80 (PS80) on Cry j 1-specific immunoglobulin E (IgE) and IgG2a antibodies in serum. The design of the experiment is shown in detail in Figure 4(a). The sera were obtained 1 week after the last boosting, and for each mouse the Cry j 1-specific IgE and IgG2a levels were determined by enzyme-linked immunosorbent assay. Results are shown for (a) Cry j 1-specific IgE and (b) Cry j 1-specific IgG2a, and each circle and bar represent the titre of an individual mouse and the mean, respectively.

why there was no significant difference between the groups in this study, but the tendency of Cry-consensus peptide challenge to increase the level of Cry j 1-specific IgG2a was reproducible. The antigen-specific IgE/IgG2a ratio has been proposed to be a correlative parameter of the Th1/Th2 profile shift, and therefore these results

indicate that the simultaneous administration of PS80 with Cry-consensus peptide challenge may reduce the therapeutic efficacy of Cry-consensus peptide, as reflected by the immunoglobulin profile in the allergic rhinitis model mice. We also examined EPO activity as an indicator of eosinophil migration in nasal tissues, and found no significant difference in the activity among the groups (data not shown).

Th2 shift by PS80 is also induced *in vitro*

To evaluate the details of the immunomodulatory effect of PS80, the *in vitro* profiles of splenic cytokines from Cry j 1-sensitized mice were investigated. The study design is shown in Fig. 4(a). When the splenocytes were cultured with Cry j 1 in the presence or absence of PS80, the IFN- γ level was decreased by the addition of PS80 in a concentration-dependent manner, but the decrease of the IL-5 level was negligible in comparison with that of IFN- γ (Fig. 4b). A similar result was also obtained with Cry-consensus peptide stimulation (Fig. 4c). The IFN- γ /IL-5 ratio on day 3 showed a PS80 concentration-dependent decrease (Fig. 4d). Taken together, these results indicate that PS80 initially suppressed the antigen-specific secretion of IFN- γ preferentially compared with that of IL-5 at the cellular level, and induced Th2 dominance in the T helper profile *in vivo*.

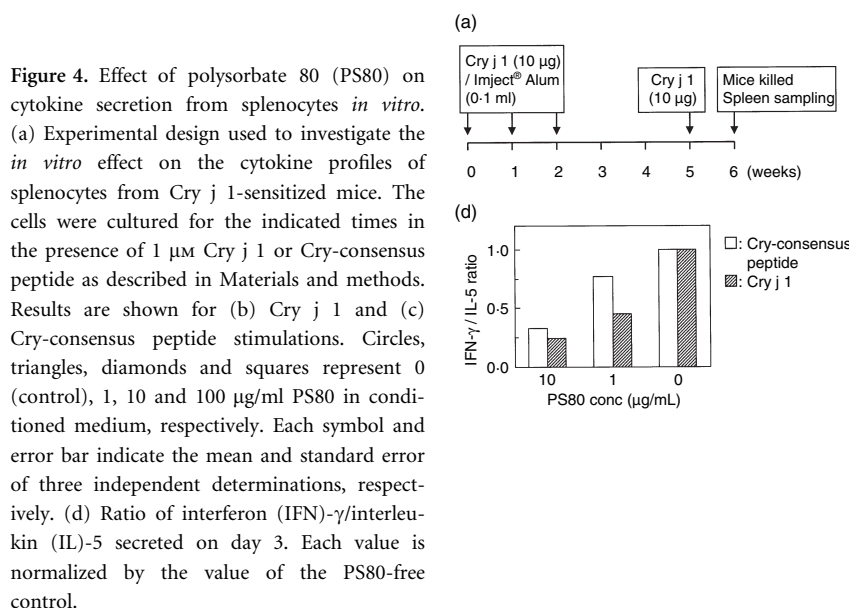
Immunomodulatory effect of PS80 on antigen recognition

Cry-consensus peptide was designed to be processed into its individual epitopes in antigen-presenting cells after endocytosis and to stimulate antigen-specific T cells

through cell–cell contact via class II MHC molecules and T-cell receptors. The *in vivo* study suggests that endocytosis might be influenced by a low concentration of PS80, and therefore we examined the effect of PS80 on antigen-specific cytokine production, which is the event that follows antigen presentation to T helper cells. Splenocytes of mice sensitized with Cry-consensus peptide alone or Cry-consensus peptide plus PS80 secreted both IL-5 and IFN- γ in response to Cry-consensus peptide or PWM stimulation (Fig. 5b). When PS80 was injected with Cry-consensus peptide into the mice, only the IL-5 level was increased significantly in comparison with the level in mice immunized with Cry-consensus peptide alone, while IFN- γ production was similar between the groups. There was no significant difference in cytokine level in response to PWM stimulation. Secretion of these cytokines was low in response to Cry j 1 stimulation (data not shown), indicating that the response of Cry j 1-specific T helper cells in terms of cytokine production is not enhanced by Cry-consensus peptide immunization.

Inhibition of endocytosis by PS80 *in vitro*

Confocal microscopy was used to examine whether the internalization and intracellular distribution of Cry-consensus F were influenced by PS80 *in vitro* (Fig. 6). When BMDCs were incubated with Cry-consensus F on ice to inhibit endocytosis, Cry-consensus F remained bound to the cell surface regardless of the PS80 concentration (0 min). When unbound peptides were washed away and the temperature was raised to 37°, the bound peptide entered the cells in a PS80-concentration-dependent manner. At the earliest time examined, immediate internalization of Cry-consensus F was observed at all



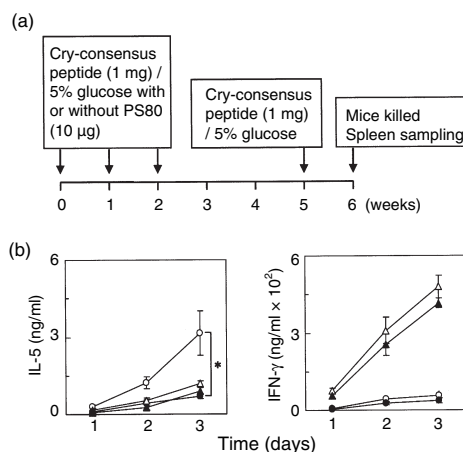


Figure 5. Polysorbate 80 (PS80) increases production of interleukin (IL)-5 but not interferon (IFN)- γ from splenocytes *in vitro*. (a) Experimental design used to investigate the effect of PS80 on antigen recognition. The cells were cultured for the indicated times in the presence of 1 μ M Cry-consensus peptide as described in Materials and methods. (b) Induction of IL-5 and IFN- γ secretion from cultured splenocytes by Cry-consensus peptide or poke weed mitogen (PWM). Circles and triangles represent Cry-consensus peptide and PWM stimulation, respectively, and closed symbols and open symbols represent Cry-consensus peptide alone and Cry-consensus peptide plus PS80 immunization, respectively. Each symbol and error bar indicate the mean and standard error of three individual determinations, respectively. An asterisk indicates significant differences between Cry-consensus peptide and Cry-consensus peptide plus PS80.

of the tested PS80 concentrations except for 5000 μ g/ml. After 15–30 min of incubation, the fluorescence had become localized throughout the cytosol at both 0.5 μ g/ml PS80 and control, whereas PS80 concentrations of 50 μ g/ml or higher delayed the intracellular spreading of the fluorescence. The results of ³H-thymidine uptake analysis showed that the proliferation and viability of the cells were fatally impaired within a few days by 5000 μ g/ml PS80, but were not affected by PS80 at concentrations of 50 μ g/ml or lower (data not shown).

Discussion

Here, we report that the pharmaceutical effect of Cry-consensus peptide (Fig. 1) was inhibited by PS80, which is used as an ingredient in dosing vehicles, and that the inhibitory mechanism may be mediated through modulation of the antigen-specific Th1/Th2 balance. In clinical studies, Cry-consensus peptide with PS80 did not show a significant effect on pharmaceutical parameters such as total symptom and medication scores, whereas a significant improvement in these parameters was achieved using Cry-consensus peptide without PS80. In the present study, the possibility that PS80 interferes with the anti-allergic effect of Cry-consensus peptide was investigated in B10.S mice. PS80 was added at a final concentration of 1% [weight/weight (w/w); 25 μ g/ml] in the pharmaceutical formulations used in the reported clinical study, and

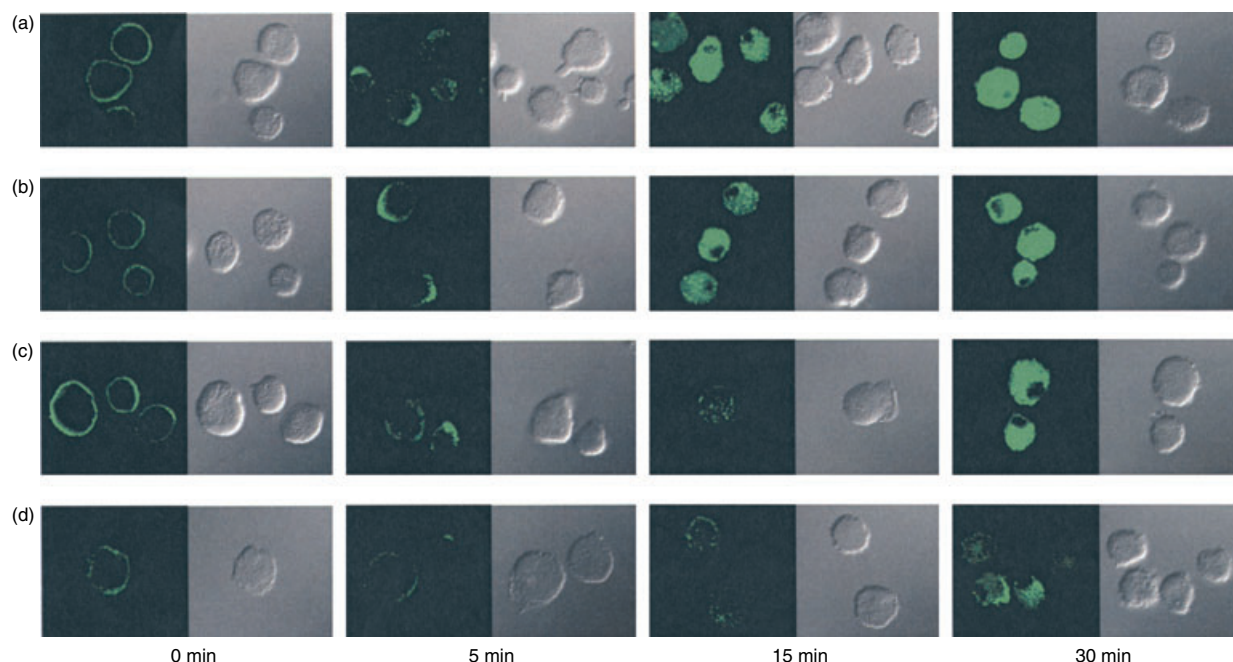


Figure 6. Inhibition of Cry-consensus peptide internalization by polysorbate 80 (PS80). Bone marrow-derived dendritic cells were incubated on ice with 50 μ g/ml Cry-consensus F (green) for 30 min with the indicated concentration of PS80: (a) 0 μ g/ml as control, (b) 0.5 μ g/ml, (c) 50 μ g/ml or (d) 5000 μ g/ml. The cells were then washed and either fixed (0 min) or incubated at 37°C in fresh medium with the above PS80 concentration without Cry-consensus F for 5, 15 or 30 min and then fixed. After fixation, the cells were examined by confocal microscopy.

we therefore also used the same PS80/Cry-consensus peptide ratio (1%) in this study. The Cry-consensus peptide concentration used in preclinical studies (5 mg/ml) was twice that used in the clinical study, and therefore 1% (50 µg/ml) was used as the standard concentration in the present study to evaluate the effect of PS80 in mice.

When mice were immunized with Cry-consensus peptide solution with PS80, the IL-5 secretion from splenocytes induced by Cry-consensus peptide stimulation increased significantly in comparison with that induced by immunization with Cry-consensus peptide alone, whereas no significant difference in IFN- γ secretion was observed between the groups (Fig. 5b). Enhancement of IL-5 but not IFN- γ secretion in response to *in vitro* stimulation indicates that PS80 induces a shift in the balance of T helper cells towards Th2 cells or the activation and migration of eosinophils, but does not suppress Th1-dominant reactions *in vivo*. PS80 challenge together with Cry-consensus peptide in allergic rhinitis model mice attenuated the inhibitory effect of Cry-consensus peptide on the regulation of IL-5 production, but did not affect the IFN- γ level (Fig. 2c). Taken together, these findings suggest that PS80 induced Th2-dominant differentiation *in vivo* through a shift in the balance of T helper cells, with augmentation of IL-5 but not IFN- γ production. The importance of IL-5 production in allergic diseases is also supported by a previous report showing that the allergen-stimulated T-cell response is associated with IL-5 but not IFN- γ expression.²¹

Next, we investigated how Th2-type responses are increased by PS80 challenge in mice. When splenocytes from Cry j 1-sensitized mice were cultured in the presence of Cry j 1, the addition of PS80 strongly inhibited IFN- γ secretion in a concentration-dependent manner; however, IL-5 secretion was only slightly inhibited at the concentrations we examined (Fig. 4b). These results together with similar results obtained with Cry-consensus peptide stimulation (Fig. 4c) indicate that the primary PS80-induced response after antigen presentation may be the suppression of antigen-specific IFN- γ secretion. For example, a slight decrease in the peptide antigen internalized into antigen-presenting cells by PS80 may first affect IFN- γ secretion, resulting in a shift of the Th1/Th2 cytokine balance towards Th2 dominance in the microenvironment, and therefore co-challenge with PS80 in mice might increase IL-5 secretion in *ex vivo* culture through an increase in Th2 populations *in vivo*.

We also determined antigen-specific IgE and IgG2a levels in serum and EPO activity in nasal tissue. Cry-consensus peptide decreased the antigen-specific IgE level (our unpublished data), but the suppressed IgE level was not restored to the control level by PS80 co-injection (Fig. 3a). In contrast, the elevation of antigen-specific IgG2a by Cry-consensus peptide challenge was prevented by PS80 co-injection, although there was no significant

difference with and without PS80 (Fig. 3b). EPO activity is thought to be an indicator of eosinophil migration based on nasal symptoms such as biphasic nasal obstruction, and is expected to be reduced by Cry-consensus peptide. However, such an effect was not found in this study and the EPO level was almost identical among the groups (data not shown). The reason why EPO levels did not increase or decrease is unknown, but EPO is definitely a valuable parameter for evaluation of eosinophil migration related to allergic rhinitis. These results indicate that the decrease of pharmaceutical efficacy produced by PS80 may be correlated with antigen-specific antibody levels but not with antigen-induced EPO activity.

When Cry-consensus peptide was internalized into BMDCs, 50 µg/ml PS80 clearly inhibited the formation of vesicles including Cry-consensus peptide at 15 min post-incubation, and its inhibitory effect was dose-dependent (Fig. 6). PS80 is a non-ionic detergent with various activities *in vivo* and *in vitro*.^{15–19} Notably, in an *in vitro* study using a Caco-2 cell monolayer, it was shown that peptide transfer between the apical side and the basolateral side is modulated by PS80,²² and another study showed that PS80 may enhance the intestinal absorption of some drugs.²³ Moreover, regarding endothelial cell function in the rabbit aorta, PS80 inhibits acetylcholine-induced endothelium-derived relaxing factor (EDRF) release by significant desquamation of the vascular endothelium.²⁴ However, these effects of PS80 were obtained when higher concentrations of PS80 were applied. At 5000 µg/ml, PS80 showed very strong inhibition of Cry-consensus peptide internalization, but measurement of ³H-thymidine uptake and morphological observation of splenocytes indicated that cell death occurred within 3 days of culture in the presence of over 1000 µg/ml PS80 (data not shown), and thus excess PS80 may cause critical impairment of cell viability under static *in vitro* conditions. As far as we know, this is the first report to demonstrate that a minimal concentration of PS80 modulates immunogenic reactions *in vivo* and *in vitro*.

Generally, subcutaneously injected drugs diffuse immediately from the injected site. Therefore, it is considered likely that Cry-consensus peptide and PS80 also do not stay in the injection site for long, at least in their soluble forms. In contrast to static conditions such as those in culture plate wells, the PS80 concentration may decrease immediately *in vivo*. Considering the immediate (within a few minutes) internalization of exogenous antigens, the initial PS80 concentration may be critical in the modulation of the internalization of exogenous antigens. This may also explain the inhibition of IFN- γ secretion by 1 or 10 µg/ml PS80, because, even at 1 µg/ml, PS80 caused a significant decrease in the IFN- γ level induced by antigen stimulation *in vitro* (Figs 4b and c).

It is known that PS80 contributes to the formation of micelles at appropriate concentrations and to solubiliza-

tion of various compounds such as hydrophobic drugs. Initially, we wondered whether a change of solubility induced by PS80 might be related to the effect of PS80 on the pharmacological activity of Cry-consensus peptide. However, taking into consideration the fact that the critical micelle concentration of PS80 in general vehicles is higher than the PS80 concentration of the Cry-consensus peptide formulation used in this study, 1% (w/w) PS80, which was equivalent to approximately 10% of the Cry-consensus peptide with respect to molar ratio, may have no effect on the solubility of Cry-consensus peptide. The interaction between Cry-consensus peptide and PS80 was also weak in our preliminary study (data not shown), and these results indicate that the molecular interaction between Cry-consensus peptide and PS80 in the pharmaceutical formulation did not affect the efficacy of Cry-consensus peptide. PS80 may act directly on the cell membrane domain, for example on lipid rafts, and decrease the capability of antigen internalization through loss of dynamism of the plasma membrane composition.^{25,26} It is also interesting that immunomodulatory effects of endogenous lipids, such as lysophosphatidylcholine and lysophosphatidic acid, which are related structurally to PS80 and are able to form micelles, have been reported.²⁷

Using rat thymocytes, Hirama *et al.*²⁸ reported that PS80 at 10 µg/ml or more decreased the cellular content of glutathione and at 30 µg/ml or more increased the intracellular Ca²⁺ concentration without affecting cell viability at concentrations of 1–100 µg/ml. We used similar concentrations of PS80 *in vitro* (Figs 4 and 6) to those in the experiments with rat thymocytes, and cell death occurred at high concentrations of more than 1000 µg/ml. The effects of PS80 on both mouse splenocytes and rat thymocytes at concentrations of 0.5–50 µg/ml might cause an adverse reaction at endoplasmic reticulum membranes without affecting cell viability.

Our findings show that PS80 may have an immunomodulatory effect, causing the induction of the type 2 response of T helper cells *in vivo* as well as *in vitro*, even at minimal concentrations. Surfactants such as PS80 are often added to various pharmaceutical formulations, but we should also note that, at low concentrations of PS80, the antiallergic response following the Th2 shift might be enhanced at the cellular level. Further studies are need to address the detailed mechanisms of this effect, especially at the level of antigen presentation/signal transduction, and such studies should allow us to improve the antiallergic efficacy of Cry-consensus peptide immunotherapy.

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