

In Vivo and In Vitro Immunomodulation Induced by the Extract of the Mycelium Fungus *Polyporus Squamosus*

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ABSTRACT

The mycelial mass of the fungus *Polyporus Squamosus* strain 64 (PS-64) was disintegrated by mechanical and ultrasound treatments. After centrifugation, the supernatant containing the disintegrate was dialyzed and lyophilized. The resultant PS-64 extract was subsequently investigated as an immunomodulator of IgE and IgG responses to ovalbumin (OA) in (CBAxCS7BL/6)F1 mice using passive cutaneous anaphylaxis (PCA) and enzyme-linked immunosorbent assay (ELISA), respectively. Multiple injections of PS-64 extract in doses of 1.5, 15, and 150 mg/kg administered before the primary or secondary immunization of mice with OA resulted in a dose-dependent inhibition of both IgE and IgG antibody responses to OA. In contrast to the inhibition of the anti-OA IgE response noted during the entire 3-week observation period, the anti-OA IgG response was restored to control level by the third week of secondary immunization. The glass microfiber-based whole blood histamine release assay demonstrated that various concentrations of the PS-64 extract did not influence histamine release induced either by anti-IgE or by specific aller-

gens from basophils derived from whole blood of allergen-sensitized patients. Using the hemolytic plaque assay, significant suppression of IgM-secreting cell formation was noted in (CBAxCS7BL/6)F1 mice administered various doses of the PS-64 extract before immunization. The PS-64 extract inhibited the in vitro proliferation of human mononuclear cells upon stimulation with phytohemagglutinin (PHA). In a dose-dependent manner, the PS-64 extract also inhibited delayed-type hypersensitivity reaction and skin graft rejection, similar to the effect noted with usage of Cyclosporin A (CsA) in (CBAxCS7BL/6)F1 mice. Our investigation suggests that the immunomodulatory effects of PS-64 should be studied further for potential clinical therapeutic utility. (Allergy and Asthma Proc 18:301-310, 1997)

Immunomodulators include any agent or substance that has an effect on the host immune system including stimulatory, suppressive, or regulatory effects. Many drugs, chemicals, and microbial products have been identified as having specific as well as nonspecific immunomodulatory properties.¹ In recent years, the investigation of immunomodulators has grown significantly in two distinct areas: 1) the study of new immunomodulating properties of previously identified agents; and 2) the search for new preparations with immunomodulating activities. Among the important immunomodulators in which dysregulation of the immune system occurs are biological agents, including cytokines, and chemical agents, both naturally occurring and artificially synthesized. Immunomodulation continues to have a major role in advancing and improving the quality of

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treatment of human as well as animal diseases. During the last decades, the immunomodulatory activities of some fungi, especially those used in oriental medicine, have been investigated.

In earlier studies using mice models, the immunomodulatory activity of crude mycelial mass of the fungus *Polyporus Squamosus* strain, 64(PS-64), on IgE and IgG responses to allergens was investigated. Results from these investigations indicate that multiple injections of the PS-64 crude mycelial mass before primary immunization of the mice resulted in a significantly decreased immune response to subsequent immunization with non-related antigens.² The objectives of this study were to investigate extensively the modulatory activity of the sonicated extract from PS-64 using several immunological in vivo and in vitro assays, and to thereby project the possible therapeutic role of PS-64 extract in clinical medicine.

MATERIALS AND METHODS

Antigens: Preparation of Extract

The mycelial mass of *Polyporus Squamosus* (PS-64) obtained by microbiological synthesis² was disintegrated by both mechanical and ultrasound treatment. After centrifugation, the supernatant was dialyzed and lyophilized to form the PS-64 extract.

Immunization and Treatment of Mice with PS-64 Extract

Male (CBAxC57BL/6)F1 mice, 8–12 weeks of age, were used in all experiments. Male Wistar rats, 9–12 weeks of age, were used for passive cutaneous anaphylaxis (PCA) to detect mouse anti-OA IgE. Ovalbumin (OA) (Sigma Chemical Co., St. Louis, Mo.) was used as the model allergen in mouse immunization experiments. In order to detect the influence of PS-64 extract on IgE and IgG anti-OA response, OA was injected intraperitoneally (IP) into mice (10 µg/mouse) two times at 3-week intervals. Five days before the primary or secondary immunizations, mice received multiple IP injections of the PS-64 extract in total doses of 150 mg/kg; 15 mg/kg, and 1.5 mg/kg. On day 12 of the primary immunization and after weeks 1 and 3 of the secondary immunization, 200 µL of blood was obtained from the retro-orbital sinus. Sera samples from the immunized mice were stored at –70°C before analysis.

Detection of IgE and IgG anti-OA Antibodies in Sera Samples

Anti-OA allergen-specific IgE levels in pooled mice sera were detected by PCA.³ Anti-OA IgG antibodies in individual sera samples were determined by ELISA method.⁴ Statistical differences among treatment groups in anti-OA IgE antibody levels from pooled sera were calculated using Fox criteria.⁵ Comparisons of IgG anti-OA antibody levels in individual sera from treatment groups were performed using Student's *t* test.

Hemolytic Plaque Assay

To detect plaque forming cells (PFC) that indicate IgM antibody production, the hemolytic plaque assay was performed according to the method described previously.⁶ Five days before the immunization of (CBAxC57BL/6)F1 mice with sheep red blood cells (SRBC), the PS-64 extract was injected IP in doses of 1.5, 15, and 150 mg/kg. PFC were counted per spleen of each mouse and the mean value was calculated.

Delayed-Type Hypersensitivity (DTH)

Both CBA and (CBAxC57BL/6)F1 mice were immunized with 5×10^5 cells of SRBC intravenously. Four days after the immunization, the mice were challenged with 10^8 SRBC cells in 0.05 mL normal saline injected intracutaneously into the left hind footpads of the mice. Control animals for both experiments were not immunized, but were challenged with SRBC. Measurement of footpad weight was carried out 24 hours after challenge with the antigen. Subsequently, animals were killed by cervical dislocation, both the challenged (left) and the unchallenged (right) footpads being removed at the tarsus and each footpad weighed on an electronic balance. Increase in footpad weight was calculated as the difference between values of challenged and unchallenged footpad weights from the same animal.⁷ The PS-64 extract was injected intraperitoneally (5 injections) into mice in total doses of 150; 15, and 1.5 mg/kg before immunization with SRBC.

Skin Graft Survival Evaluation

The influence of the PS-64 extract (150 mg/kg) on skin graft survival was estimated using the method previously described.⁸ Skin from the tail of donor mice R 101 (0.5–1.0 cm² size) was transplanted to the posterior of the recipient mice (CBAxC57BL/6)F1. The bandage was removed in 7–8 days with the transplant site examined daily. Treatment of mice with CsA (40 mg/kg) was used as a positive control.

Glass Fiber-Based Histamine Release Assay

The whole blood leukocyte histamine release assay was performed as described by Nolte et al.⁹ Heparinized whole blood (5 mL) was drawn from normal donors and from patients with Timothy grass and birch pollen sensitivities previously confirmed by case history, skin test, and anti-allergen IgE antibodies detected by RAST. Blood was incubated with various concentrations of the PS-64 extract (0.002, 0.02, 0.2, 2, 20, 200, and 2000 µg/mL) for 1, 2, and 4 hours. Assay samples were then incubated at 37°C for 60 minutes with anti-IgE or specific allergens in microtiter plates containing 96 wells coated with a fiber glass matrix that selectively binds histamine. After rinsing with distilled water and subsequent incubation with a solution of Triton and Renafix (Bie & Berntsen, Denmark) for 30 minutes at 37°C, *o*-phthaldialdehyde was added to couple histamine

bound by the fiberglass matrix. The reaction was terminated after 10 minutes by addition of perchloric acid (0.59% concentration), and histamine content was determined by spectrophotofluorometry. Results were reported as percentage of the corresponding control level of histamine released.

Human Mononuclear Cells (MNC) Proliferation Assay

The proliferative activity of human MNC in response to PHA (Sigma Chemical Co., St. Louis, Mo.) stimulation was detected using standard methodology.¹⁰ Peripheral blood of healthy donors was mixed with Ficoll-Paque, and after centrifugation, MNC were collected and cultured in RPMI 1640 supplemented with 1 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin and 10% fetal calf serum. The PS-64 extract was added to the MNC culture in several concentrations including 1, 5, 10, and 50 μ g/mL, MNC being subsequently cultivated with several concentrations of PHA. The control level of proliferation was determined as proliferation occurring in cultures containing no extract of PS-64.

RESULTS

In order to assess the ability of PS-64 extract to modulate the production of IgE and IgG anti-OA antibodies, a saline solution of the PS-64 extract was injected IP before and after the standard immunization with OA (10 μ g/mouse). It was found that multiple injections of the PS-64 extract before the primary immunization of mice with OA produced a dose-dependent inhibition of both IgE and IgG anti-OA antibody responses (Figs. 1A and B). A marked suppression was noted in IgE immune response 1 week after secondary immunization (Fig. 1A). IgG anti-OA antibody response was also significantly suppressed by the PS-64 extract when administered in doses of 150 and 15 mg/kg (Fig. 1B).

Injections of PS-64 extract before the secondary immunization also produced significant inhibition of IgE and IgG anti-OA immune responses. Although anti-OA IgE response was suppressed throughout the entire observation period, anti-OA IgG response was restored to the control level by the third week of secondary immunization (Fig. 2A and B).

Using the hemolytic plaque assay, significant suppression of antibody-producing cells in the spleen of mice was found when mice were treated with various doses of the PS-64 extract before immunization with SRBC (Fig. 3), the most effective doses of the PS-64 extract being 15 and 1.5 mg/kg. This finding suggests that the PS-64 extract can also down-regulate IgM antibody production.

Substantial reduction of the in vivo DTH reaction in mice was demonstrated when CBA mice were treated with the PS-64 extract before immunization and subsequent challenge with SRBC. Significant inhibition of the DTH reaction occurred at all doses of PS-64 extract. Multiple treatment of (CBAXC57BL/6)F1 mice with different doses of

PS-64 extract before immunization with SRBC did not change the intensity of the DTH reaction in comparison with the control level, with the exception of a dose of 150 mg/kg of PS-64 extract (Fig. 4).

Using the skin graft transplant model in mice, it was found that the PS-64 extract injected either before or after transplantation produced statistically significant ($p < 0.05$) prolonged skin graft survival in comparison with the control. The effect was comparable to that of CsA used in a dose that was effective in suppressing graft rejection (Fig. 5).

The glass fiber-based histamine assay did not demonstrate significant histamine release from basophils in whole blood treated in vitro with different concentrations of the PS-64 extract before incubation with anti-IgE or specific allergens in comparison with histamine release induced by anti-IgE or specific allergens from basophils not treated by the extract of PS-64 (Fig. 6). Treatment of whole blood basophils with a high dose of PS-64 (2000 μ g/mL) led to nonspecific basophil histamine release due to destruction of the basophils.

The PS-64 extract demonstrated a concentration-dependent suppression of in vitro proliferation of human MNC stimulated with several concentrations of PHA, the most significant suppression of proliferation being observed at 50 μ g/mL PS-64 extract concentration (Fig. 7).

DISCUSSION

Immunomodulation as treatment of diseases having altered immune regulation has a significant role in clinical practice. Selective manipulation of the immune response to specific antigens may be an effective therapeutic strategy for these immune disorders. Many substances are currently being investigated as potential immunomodulators, including mycelial fungi. Among these fungi is *Ganoderma lucidum* (GL) which has been used in traditional medicine in China, Japan, and Korea for treatment of bronchitis, bronchial asthma, hepatitis, dermatitis, and other diseases. It was found that an aqueous extract of the fruit body of GL prevented the development of experimentally-induced bronchial asthma and contact dermatitis in mice, as well as autoimmune nephritis in rats with a concomitant decrease of immune complexes in the blood.¹¹ Further investigations of GL demonstrated its capacity to inhibit histamine release from mast cells of rats.¹²

This investigation has shown that administration of varying doses of the PS-64 extract into mice before primary as well as secondary immunization with OA suppressed both antigen-specific IgE and IgG immune responses, the amount of suppression being dose-dependent. In mice treated with the PS-64 extract before the secondary immunization, IgE anti-OA response was suppressed during the entire observation period, whereas IgG anti-OA response was restored during the third week after immunization. Possible explanations are that the PS-64 extract possesses a selective capacity to influence different classes of immuno-

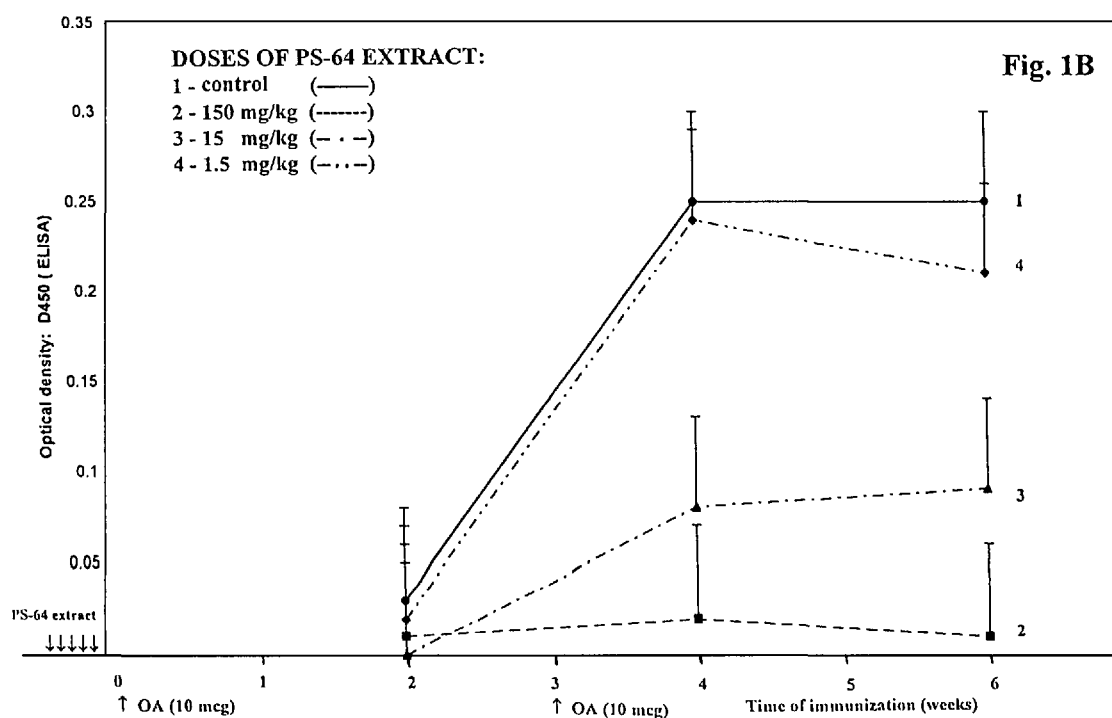
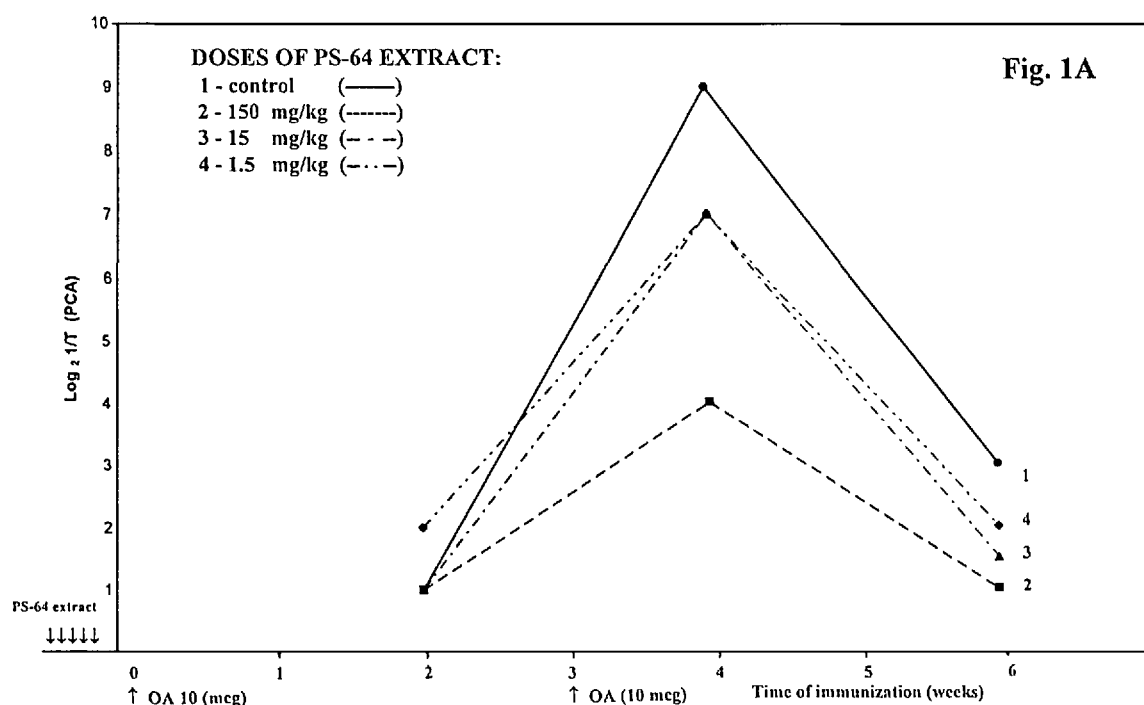


Figure 1. Effect of PS-64 extract upon IgE (A) and IgG (B) anti-OA antibody responses of mice administered PS-64 extract in doses 150, 15, and 1.5 mg/kg before primary immunization with OA.

globulins and that the PS-64 extract may downregulate isotype IgE immune response. Recent studies of orally administered novel immunomodulators such as Hochu-ekki-to extract (a traditional Chinese herbal medicinal) or synthetic muramyl dipeptide derivative have demonstrated the efficacy of these agents in inhibiting an antigen-specific IgE response without affecting the induced or constitutive levels of other classes of immunoglobulins.^{13,14}

Similar observations were noted in studies of irradiated mice that were treated with Lobenzarit Disodium (LD). It was found that LD selectively inhibited IgE antibody response enhanced by irradiation through activation by LD of IgE class-specific suppressor T-lymphocytes.¹⁵

Mice inoculated with the PS-64 extract before immunization with SRBC demonstrated suppressed IgM antibody production as determined by direct plaque-forming cell

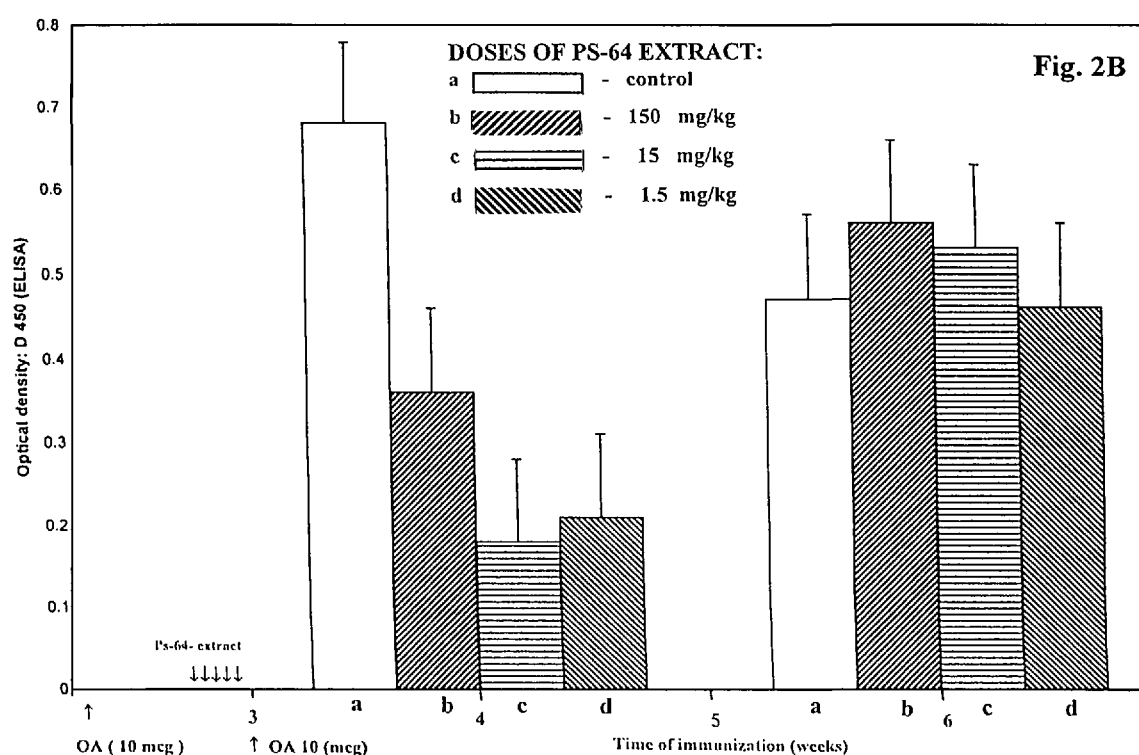
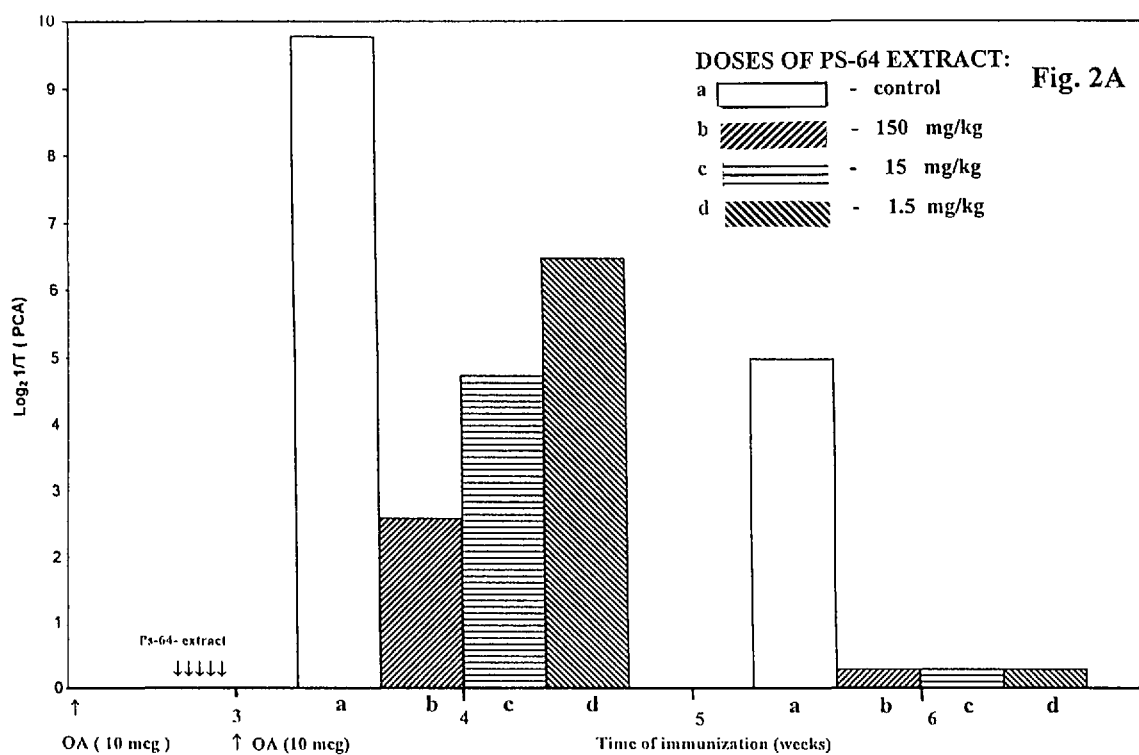


Figure 2. Effect of PS-64 extract upon IgE (A) and IgG (B) anti-OA antibody responses of mice treated with PS-64 extract in doses 150, 15, and 1.5 mg/kg before secondary immunization with OA.

assay. These results are similar to those of Kino et al.¹⁶ who found significant immunosuppressive activity of LZ-8, the protein fraction from the fungus GL, resulting in the blockade of antigen-specific antibody production.

The PS-64 extract did not have an inhibitory effect upon histamine release, nor did use of the PS-64 extract prevent systemic anaphylaxis or PCA reaction in rats. GL extract, on the other hand, strongly inhibited histamine release in-

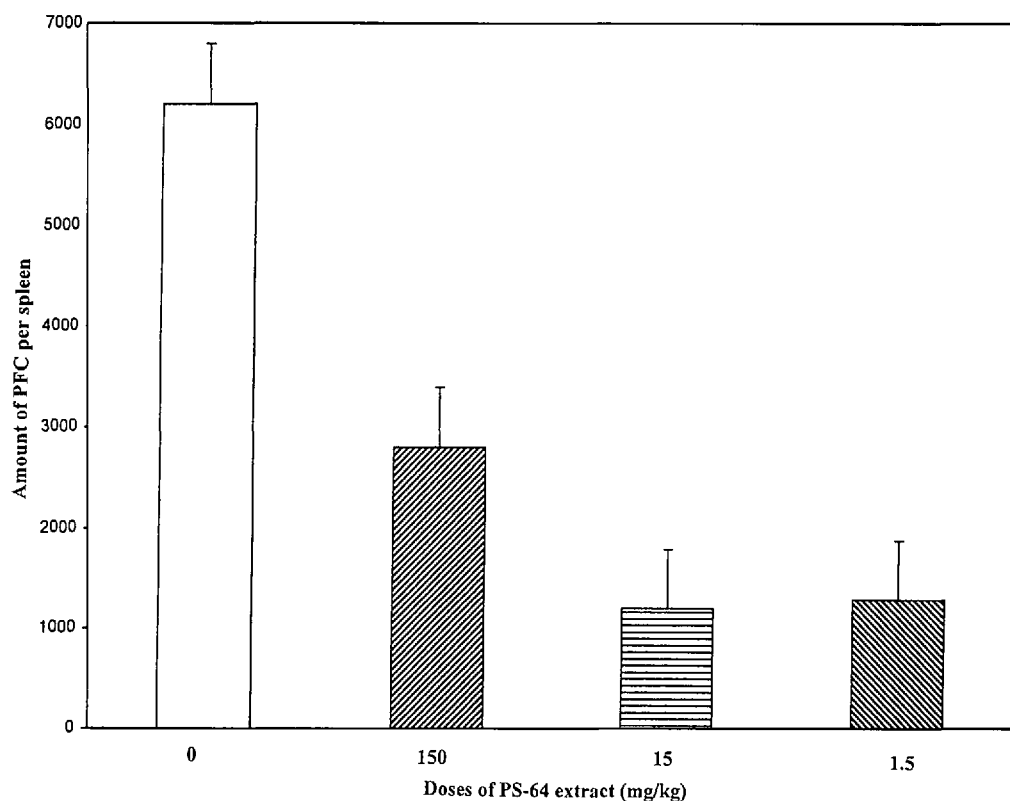


Figure 3. Suppression of antibody-producing cells (plaque forming cells, PFC) in spleens of (CBAx57BL/6)F1 mice induced by treatment with PS-64 extract five days before immunization with sheep red blood cells (SRBC).

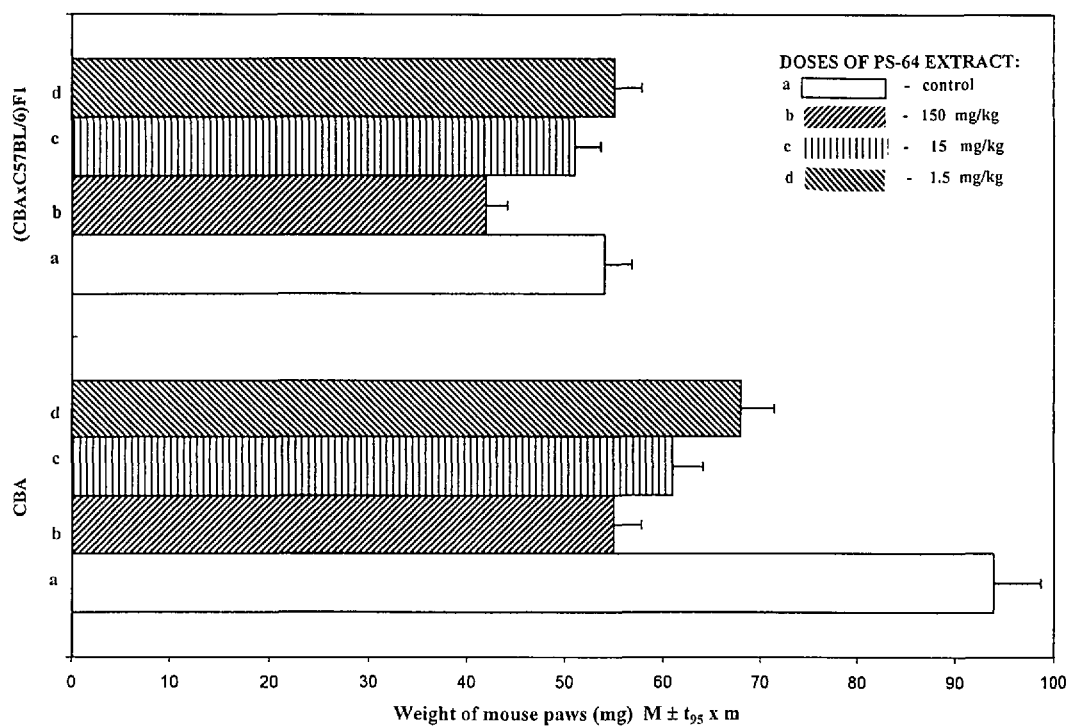


Figure 4. Effect of PS-64 extract on in vivo delayed type hypersensitivity (DTH) induced by immunization of mice with sheep red blood cells (SRBC); weight of mouse paws, $M + t_{95} \times m$: CBA and (CBAx57BL/6)F1 mice treated with five injections of PS-64 extract before immunization with SRBC.

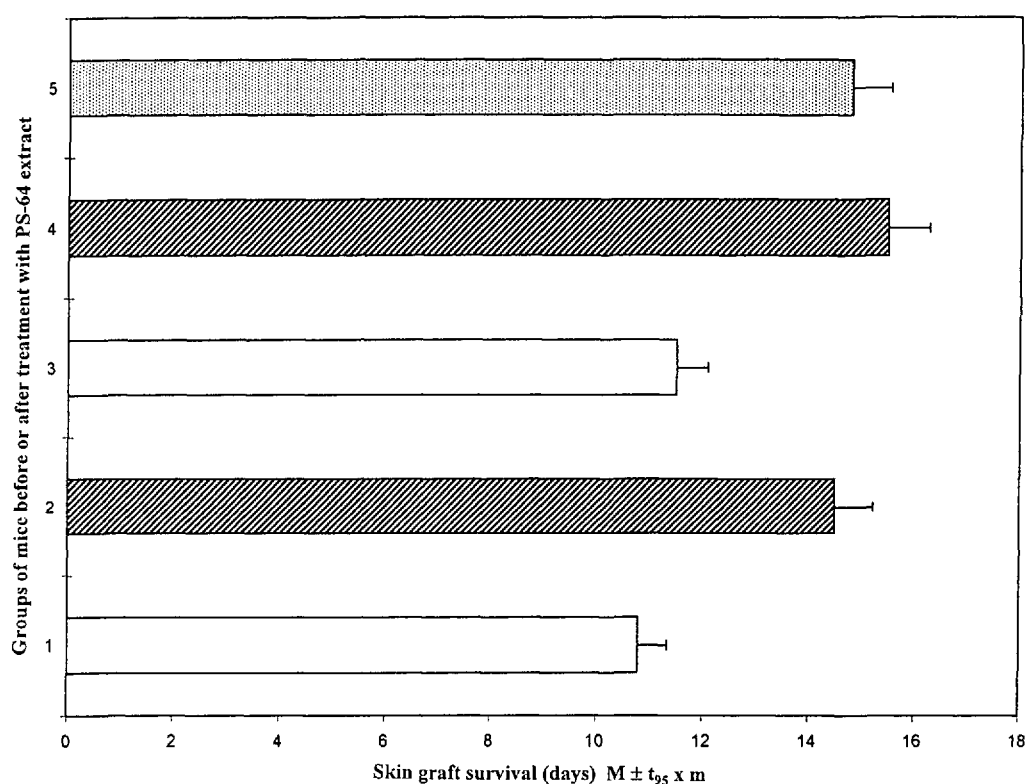


Figure 5. Influence of PS-64 extract injections on skin graft survival (in days, $M + t_{95} \times m$): 1) Control, Hanks' solution before transplantation; 2) PS-64 extract, 150 mg/kg before transplantation; 3) Control, Hanks' solution after transplantation; 4) PS-64 extract, 150 mg/kg after transplantation; 5) CsA, 40 mg/kg after transplantation.

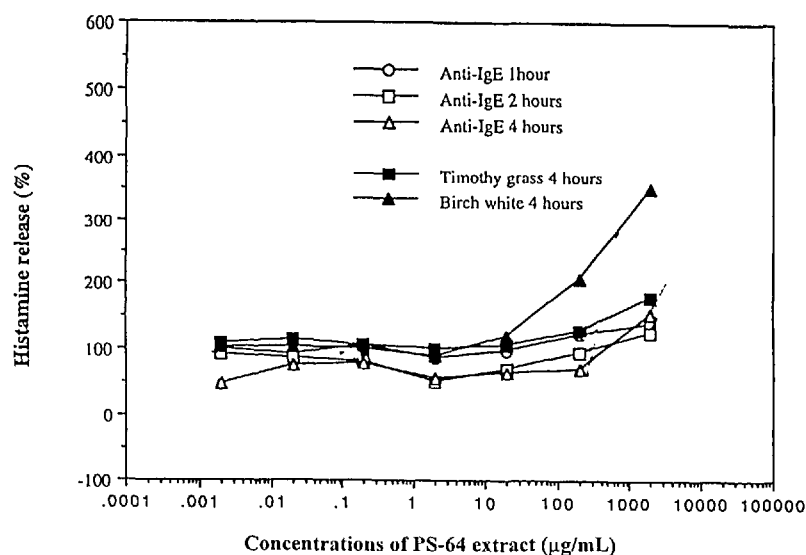


Figure 6. Histamine release from whole blood basophils induced by anti-IgE or specific allergens (Timothy grass and birch pollens) after incubation with PS-64 extract at different concentrations.

duced by compound 48/80 or ionophor A-23187 in rat mast cells, and also prevented systemic anaphylaxis.¹⁷

In further investigations of LZ-8,¹⁸ it was noted that LZ-8 appeared to be immunosuppressive in vitro using human purified T-cells. A significant effect of LZ-8 on cell immu-

nity also occurred in an allografted mouse skin model substantiated by an increased graft survival time.

Another new fungal immunomodulatory protein isolated from the edible golden needle mushroom, *Flammulina velutipes* (FIP-five), suppressed systemic anaphylaxis in mice

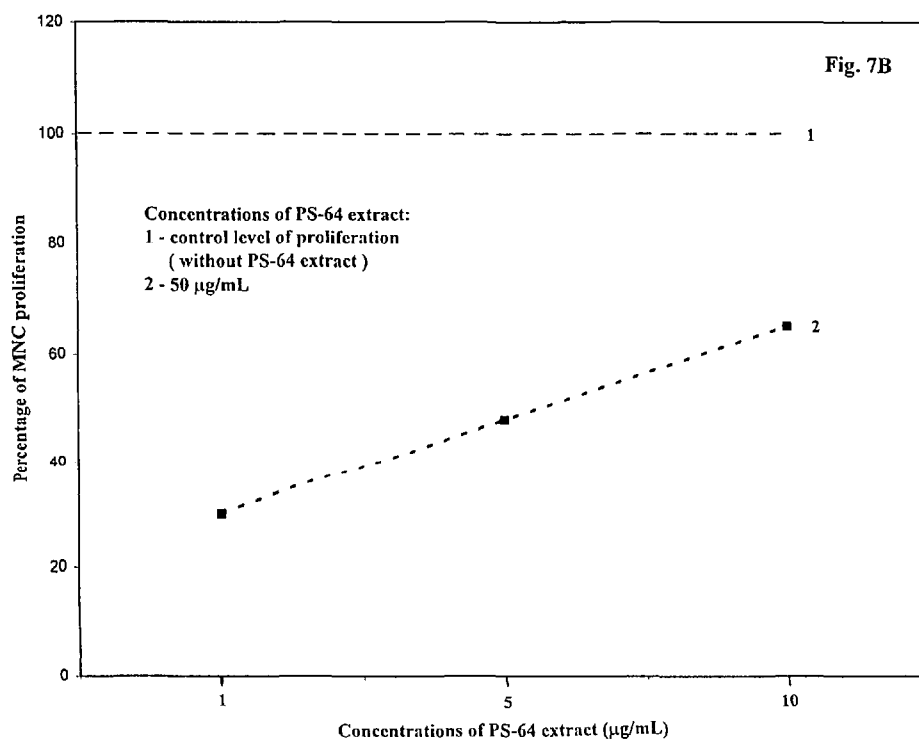
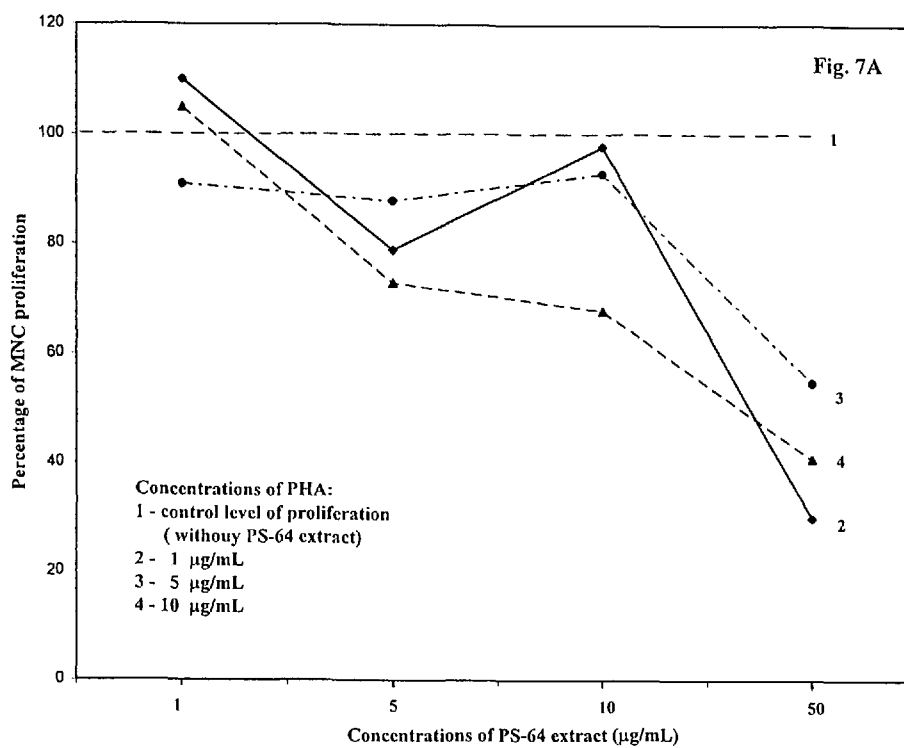


Figure 7. Suppression of *in vitro* human mononuclear cells (MNC) proliferation by PS-64 extract: Levels of MNC proliferation in presence of PS-64 extract at different concentrations (A) and at a single concentration. (B).

and local swelling of mouse footpads. FIP-five was found to enhance the transcriptional expression of interleukin-2 and interferon-gamma.¹⁹ Results of this study suggest that the

administration of the PS-64 extract suppressed not only antibody production, but also cellular immunity. The PS-64 extract inhibited in dose-dependent manner the delayed-

type hypersensitivity reaction and skin graft rejection as previously had been noted with administration of LZ-8. These *in vivo* observations seem to support strongly the theory that PS-64 extract may have immunosuppressant activity similar to that of CsA in transplant rejection.

The studies investigating the possible effect of PS-64 extract in mice with experimentally-induced influenza virus have demonstrated no significant difference in survival rates, nor were any differences noted in titers of virus in the lungs of infected mice or in control groups (unpublished data).

Recently some new immunomodulators, for example, Leflunomide, have been shown to have immunosuppressive effect similar to that of CsA or FK506.²⁰ In addition, concurrent administration of suboptimal doses of specific immunomodulators such as Tepoxalin and CsA can lead to synergistic immunosuppressive effects.²¹ Therefore, concurrent use of antigen-specific and antigen-nonspecific immunomodulators may possibly allow doses of both substances to be decreased, thus producing immunosuppression similar to a high dosage of only one of the immunomodulating agents.

Combined usage of immunomodulators obtained from different sources may also be useful in the treatment of immunological diseases. Treatment of antigen-induced arthritis in rats with either immunomodulatory thymic or splenic peptides combined with CsA inhibits specific immune response, concomitant with decreasing serum levels of antibodies against the specific antigen. Symptoms associated with clinical arthritis, including increase in joint swelling and exacerbation in the severity of macroscopically and histologically evaluated synovitis were also alleviated.²²

Combined administration of specific allergen immunotherapy and suppressive immunomodulators such as CsA may also lead to significant and stable downregulation of the IgE immune response, suggesting that this novel approach may be useful in allergen-specific immunotherapy.²³

For their future development, biological agents that are potent immunomodulators must be better purified and their molecular structures defined and synthesized, an example being the muramyl dipeptides that are products of BCG.²⁴

In recent years, considerable advances in our knowledge of cellular and molecular events following administration of immunomodulators have occurred.²⁵ Based on the current theory of the roles T helper cell subsets, including Th1 and Th2,²⁵ it has been suggested that successful results in immunomodulation may be associated with microenvironmental conditions that appear after administration of immunomodulators, including the presence of selected cytokines. Furthermore, the structure of the peptide antigen strongly influences differentiation of CD4⁺ helper cells into functionally distinct subsets.²⁷ Further investigation of the PS-64 extract on T cell subsets differentiation could provide additional information concerning the mechanism of its

action, thus allowing for a more precise clinical utilization of the PS-64 extract.

This study suggests that PS-64 extract is a novel immunomodulator that may have clinical relevance in modulation of immune response of patients with autoimmune diseases, transplant recipients, and patients with inflammatory diseases. It is also possible that use of the PS-64 extract, either combined with other immunomodulatory agents obtained from different sources or combined with allergen-specific immunotherapy, may be a novel approach in treatment of allergic diseases. The *in vivo* inhibition of cell-mediated immune reactions and IgE-responses concomitant with restoration of IgG-response noted in these studies suggest that the immunosuppressive action of PS-64 extract will not be accompanied by a lowered resistance to infectious diseases induced by commonly occurring microorganisms.

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