Activation of Murine Peritoneal Macrophages by Water-Soluble Extracts of *Bursaphelenchus xylophilus*, a Pine Wood Nematode

Hiroaki KAJI,1 Akihiro TAI,1 Kazufumi MATSUBUHIITA,1 Hiroshi KANZAKI,2 and Itaru YAMAMOTO1,4

1Department of Immunochemistry, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-naka 1-1-1, Okayama 700-8530, Japan
2Department of Bioresources Chemistry, Faculty of Agriculture, Okayama University, Tsushima-naka 1-1-1, Okayama 700-8530, Japan

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In our previous study, water-soluble extracts from *Bursaphelenchus xylophilus* (*B. xylophilus*), a pine wood nematode, were shown to enhance interleukin (IL)-4 plus lipopolysaccharide-induced polyclonal immunoglobulin (Ig) E production *in vitro* in mice and to increase serum levels of an antigen-nonspecific IgE *in vivo*. Here we examined whether the nematode extracts stimulate immunofunctions of murine peritoneal macrophages. In both resident and inflammatory macrophages, Fcγ receptor-mediated phagocytosis was markedly activated by *B. xylophilus* extracts, while non-specific phagocytosis was not. The enhancement of specific phagocytosis was accompanied by an increase in the formation of IgG-Fcγ receptor rosettes. *B. xylophilus* extracts also stimulated IL-1β production in both types of macrophages, and enhanced NO production and mRNA expression of inflammatory cytokines in inflammatory macrophages. These results indicate that the extracts of *B. xylophilus* contain an activating substance(s) for immunofunctions in macrophages, besides an enhancing factor for polyclonal IgE production.

Key words: *Bursaphelenchus xylophilus*; peritoneal macrophage; phagocytosis; immunomodulator

It is well-known that infection with animal parasitic nematodes or administration of extracts of them can cause intense antigen-specific and nonspecific polyclonal immunoglobulin E (IgE) production,1–3 which is dependent on interleukin (IL)-4 production linked with the development of the Th2 response.4–6 The increase in parasitic antigen-specific IgE is associated with elimination of parasites from infected hosts,7 while the increase in antigen-nonspecific polyclonal IgE can be involved in the survival of invading parasites.2,4,8 Recently, Lynch et al. have reported a parallel relationship between the decline of parasitosis and increase in allergic disease.9,10 These findings suggest that the allergen-specific IgE produced is diluted by abundant nonspecific polyclonal IgE and that the resulting polyclonal IgE accumulation might be responsible for inhibiting allergy reaction with a saturation of Fcγ receptors on mast cells.

In our previous study, we found that extracts of an animal non-parasitic nematode, as well as animal parasitic nematodes, show polyclonal IgE-inducing activities.11 The animal non-parasitic nematode is a pine wood nematode, *Bursaphelenchus xylophilus* (*B. xylophilus*), transmitted by the pine sawyer, *Monochamus alternatus*. It is considered that this plant parasite causes pine wilt disease.12 *B. xylophilus* extracts not only augmented IL-4 plus lipopolysaccharide (LPS)-induced polyclonal IgE production in murine splenocytes *in vitro*, but also increased the serum level of polyclonal IgE in immunized mice.11 These results indicate that *B. xylophilus* extracts contain an enhancing substance(s) for polyclonal IgE production by humoral immunity. But, whether the extracts activate cell-mediated immunity is not yet clear.

Macrophages are one of the immunocompetent cells and are associated with both cell-mediated and humoral immunity. They play a role in tumor killing, protection of microbial infections, and antigen presenting.13,14 In addition, the importance of macrophage activation in host immune responses against nematode infection has been documented. For example, mononuclear phagocytes increase or are activated during infection by nematodes,15,16 macrophages kill infective larvae of *Nippostrongylus brasiliensis* *in vitro* in the presence of complement and enhance IgG-dependent eosinophil-mediated killing of nematodes,17 and nitric oxide (NO) production by macrophages is useful for parasite killing.18 On the other hand, a secreted product of the filarial nematode *Acanthocheilonema vitaeae* suppresses production of IL-6, IL-12, and tumor necrosis factor (TNF)-α from murine macrophages activated by interferon (IFN)-γ plus LPS.19

In this study, we examined whether *B. xylophilus* extracts promote macrophage functions such as Fcγ...
receptor-mediated phagocytosis and IL-1β production in both resident and inflammatory macrophages. We also investigated whether the nematode extracts enhance NO production and mRNA expression of inflammatory cytokines such as IL-1β, IL-6, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in inflammatory macrophages.

Materials and Methods

Animals. Female BALB/c mice, 7 weeks old, were purchased from Charles River Japan (Yokohama, Japan). The care and use of the animals in this study followed the guidelines of Okayama University. Mice were kept in our animal facility for 1 week before use. All mice were used at 8 to 12 weeks of age. They were housed in a room controlled as to temperature (23–25 °C), humidity (50–60%), and a preset light–dark cycle (12 h:12 h), bred with sterile wood-chip bedding, and provided with food and water ad libitum under specific pathogen-free conditions.

Reagents. RPMI-1640 medium was purchased from ICN Biomedicals Japan (Tokyo). Eagle’s MEM was from Nissui Pharmaceutical (Tokyo). FBS was from Gibco BRL (Gaithersburg, MD). Thioglycollate medium was from Difco Laboratories (Detroit, MI). Heparin sodium salt was from Wako Pure Chemical Industries (Osaka, Japan). Penicillin G potassium was from Banyu Pharmaceutical (Tokyo). Streptomycin sulfate was from Zymed Laboratories (San Francisco, CA). Sulfanilamide and 1-naphthylethylenediamine dihydrochloride were from Nacalai Tesque (Kyoto, Japan). Sheep red blood cell (SRBC) was from Japan Ramdrock International (Hiroshima, Japan). Rabbit anti-SRBC IgG fraction was kindly provided by K. Kawazu, Emeritus Professor of Okayama University. The culture of *B. xylophilus* was as described previously. Briefly, *B. cinerea*, whose mycelia were ingested by *B. xylophilus*, was cultured on a Czapek-Dox modification agar plate (0.2% NaNO₃, 0.1% K₂HPO₄, 0.005% KCl, 0.005% MgSO₄, 0.0001% FeSO₄, 3.6% glucose, and 1.5% agar) in a plastic dish (φ 100 mm) for 1 week at 26 °C. *B. xylophilus* was added to the plate and cultured for 5–7 d at 26 °C in the dark. After cultivation, the propagated nematodes were collected from the agar plates in a clean bench for 12 h by the Baermann funnel method. The collected nematodes were washed five times with sterile water, homogenized with an ultrasonic generator, and centrifuged twice at 10,500 × g for 20 min. The supernatant was lyophilized and store at 4 °C until use. The content of protein in the extracts comprised about 28% of the total weight. The extracts were analyzed for the presence of LPS by the Limulus amebocyte lysate test (BioWhittaker, Walkersville, MD), and were found to contain 3.1 ng of LPS/mg of the extracts.

Ingestion and rosette formation assays. The mice were intraperitoneally injected with 2.0 ml of 3% (w/v) thioglycollate medium. Exudate cells were harvested by peritoneal lavage with 7.5 ml phosphate-buffered saline (PBS) containing 20 U/ml heparin sodium salt 4 d after injection. After washing, the cells were resuspended in RPMI-1640 medium containing 100 U/ml penicillin G potassium and 0.1 mg/ml streptomycin sulfate and enumerated. The desired number (1–2 × 10⁶ cells/ml) of peritoneal exudate cells was determined. Aliquots of the cells (0.5 ml) were layered onto 15-mm cover glass (Matsunami Glass, Osaka, Japan) which had been placed in the 16-mm diameter wells of tissue culture plates (Nalge Nunc International, Rochester, NY) and incubated at 37 °C in a 5% CO₂ incubator for 1 h to allow thioglycollate-elicited macrophage adherence to the cover glasses. Resident macrophages were obtained from untreated mice in a similar manner to that described above. Nonadherent cells were removed, 0.5 ml of RPMI-1640 containing various concentrations of reagents was added, and the cells were cultured for 3 h. Ingestion of SRBC coated with immunoglobulin G (SRBC-IgG) was determined according to Yamamoto et al. Briefly, washed erythrocytes were coated with subagglutinating dilutions of purified rabbit anti-SRBC IgG fraction for 15 min at 37 °C. For Fcγ receptor-mediated ingestion assay, 0.5 ml of 0.5% suspension of the SRBC-IgG in RPMI-1640 medium without PBS was overlayed on each macrophage-coated (monolayer) cover glass and cultured at 37 °C in a humidified 5% CO₂ incubator for 90 min. Noninternalized erythrocytes were lysed by immersing the cover glass in a hypotonic solution (1:5 PBS) for 5–10 s. In the case of rosette formation, the macrophage monolayer and SRBC-IgG were cultured at 4 °C for 60 min. Nonbinding erythrocytes were washed five times with PBS. The macrophages were fixed and stained with Giemsa’s solution. Ingestion and rosetting formation were quantified microscopically. The data were expressed as ingestion and rosetting indexes according to Bianco et al. and Fadok.
et al.\textsuperscript{22}} respectively. Ingestion (rosetting) index = % macrophages with ingested (rosetted) erythrocytes x average number of erythrocytes ingested (rosetted) per macrophage.

**Determination of IL-1β level.** Peritoneal cells (1 × 10^6 cells) were added to 96-well flat-bottom tissue culture plates (Nunc, Roskilde, Denmark) and incubated at 37 °C in a 5% CO_2 incubator for 1 h to allow macrophage adherence. Nonadherent cells were removed, and then cultured for 24, 48, and 72 h in RPMI-1640 medium containing 10% FBS with various reagents. Supernatants were collected and the IL-1β level was quantified by sandwich ELISA.\textsuperscript{23} ELISA plates (Maxisorp, Nunc) were coated with 50 μl of anti-mouse IL-1β (4 μg/ml) overnight at 4 °C. After washing and blocking (1% BSA in 7.5 mM PBS), the wells were seeded with 50 μl of culture medium and incubated for 2 h at room temperature. After washing, 50 μl of biotin-conjugated anti-mouse IL-1β (100 ng/ml) was added. After 2 h, the IL-1β level was determined using HRP-conjugated streptavidin, a substrate solution that consisted of 0.1 mg TMB per ml in 0.1 M acetate buffer (pH 5.5), and 0.004% H_2O_2. After a stop solution (2 N H_2SO_4) was added, the plates were read at a wavelength of 450 nm with an ELISA reader (Model 680 Microplate Reader, BIO-RAD, Hercules, CA).

**Measurement of NO production.** NO release was quantified by the accumulation of nitrite in the supernatants of peritoneal macrophage cultures, using the standard Griess method.\textsuperscript{24} Briefly, peritoneal cells (2 × 10^5 cells) were added to 96-well flat-bottom tissue culture plates (Nunc), and the plates were incubated at 37 °C in a 5% CO_2 incubator for 1 h to allow macrophage adherence. Nonadherent cells were removed and 0.2 ml of RPMI-1640 containing various concentrations of reagents was added. After 24 h, 100 μl of the supernatant was harvested and added to the same volume of Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride, and 2.5% H_2SO_4) freshly prepared prior to use. Standards were prepared using sodium nitrite solutions (3.125–100 μM), and added to each assay plate. The plates were read at 550 nm with an ELISA reader.

**Determination of inflammatory cytokine mRNA levels.** Peritoneal macrophages (1 × 10^6 cells) were cultured with 300 μg/ml of *B. xylophilus* extract or 10 μg/ml of LPS in 100-mm diameter tissue culture dishes (BD Biosciences, Franklin Lakes, NJ). After 3 h, the cells were harvested and washed twice by MEM. The total cellular RNA was isolated using the RNAzol B reagent, and then reverse-transcribed using M-MLV reverse transcriptase and oligo (dT)_12-18 primer. Briefly, 0.5 μg oligo (dT)_12-18 primer and 10 μg RNA were mixed and incubated at 72 °C for 15 min. The resulting mixture was added to 50 mM Tris–HCl, 75 mM KCl, 3 mM MgCl_2, 10 mM DTT, 40 units RNase inhibitor, 5 mM dNTP, and 200 units reverse transcriptase, and the mixture incubated at 42 °C for 1 h. The reaction mixture was then heated at 70 °C for 15 min and treated with RNase H at 37 °C for 30 min. The mRNA levels of macrophage inflammatory cytokines (IL-1β, IL-6, TNF-α, TGF-β, and GM-CSF) were determined with a Quantitative PCR Detection Kit. Aliquots of cDNA synthesized as above were used as a template for polymerase chain reaction (PCR). The PCR reaction mixture consisted of 0.25 mM dNTP, the primer mixture, a cDNA sample, and 0.25 U Ex Taq. The conditions for PCR were 94 °C (denaturation) for 60 s and annealing and extension at 60 °C for 150 s in a thermal cycler (Model TP-240, Takara Bio). For each message PCR was carried out for 30 cycles. After cycling, 10 μl of PCR products was analyzed by agarose gel electrophoresis and ethidium bromide (EtBr) staining, and quantified with a densitometer. Differences in gene expression were determined by normalizing expression against housekeeping GAPDH expression.

**Results**

*Effect of B. xylophilus extracts on Fcγ receptor-mediated phagocytosis and rosette formation in peritoneal macrophages*

Phagocytosis by macrophages is one of the most important roles in the immune system when foreign bodies invade. Especially, antigen-antibody (IgG) complex can easily be treated through the Fc receptor for IgG (the Fcγ receptor) on the macrophage membrane.\textsuperscript{25} We examined whether *B. xylophilus* extracts promote Fcγ receptor-mediated phagocytosis in peritoneal macrophages elicited by thioglycollate medium. As shown in Fig. 1A, Fcγ receptor-mediated phagocytosis was increased by *B. xylophilus* extracts at concentrations from 50 μg/ml to 1,000 μg/ml. A maximal response was obtained at a concentration of 300 μg/ml. In addition, LPS did not affect Fcγ receptor-mediated phagocytosis in thioglycollate-elicited macrophages (data not shown). The effect of *B. xylophilus* extracts on rosette formation was also investigated (Fig. 1B). Rosette formation in which Fcγ receptor combines with antigen-IgG complex is the first step in phagocytosis. Rosette formation was increased by *B. xylophilus* extracts at concentrations from 10 μg/ml to 1,000 μg/ml. A maximal response was obtained at a concentration of 100 μg/ml. The profile of rosette formation enhanced by the extracts agreed fairly well with that of phagocytosis, although a slight difference in the optimum concentration was observed between the two experiments.

In a similar manner to that above, we examined whether *B. xylophilus* extracts activate resident macrophages. Fcγ receptor-mediated phagocytosis was increased by the extracts at concentrations from 0.1 μg/ml to 1 μg/ml (Fig. 1C). At concentrations of 100 μg/ml or greater, phagocytosis was suppressed as compared with
the control. Rosette formation also increased at concentrations from 0.01 μg/ml to 0.1 μg/ml (Fig. 1D). In resident macrophages, as well as thioglycollate-elicited macrophages, there was agreement in the profiles of phagocytosis and rosette formation enhanced by the extracts. On the other hand, *B. xylophilus* extracts did not affect non-specific phagocytosis by resident and thioglycollate-elicited macrophages (data not shown).

**Effect of *B. xylophilus* extracts on IL-1β production in peritoneal macrophages**

IL-1β, one of various cytokines produced by macrophages, is the essential cytokine triggering primary immune responses.26) Hence, we examined whether *B. xylophilus* extracts modulate IL-1β production in resident and thioglycollate-elicited macrophages. The levels of IL-1β were estimated by ELISA after 24 h incubation. *B. xylophilus* extracts enhanced IL-1β production in the thioglycollate-elicited macrophages at concentrations ranging from 1 μg/ml to 1,000 μg/ml (Fig. 2A). In the resident macrophages, the extracts also increased IL-1β secretion at concentrations ranging from 1 μg/ml to 100 μg/ml (Fig. 2B). The IL-1β production of the resident macrophages was higher than that of the thioglycollate-elicited macrophages by the addition of the extracts at concentrations of 1–100 μg/ml. Moreover, the enhancement of IL-1β production was maintained until 72 h in both macrophages (data not shown). These findings suggest that *B. xylophilus* extracts can contribute to the enhancement of the cell-mediated immune response after phagocytosis of foreign bodies.

**Effect of *B. xylophilus* extracts on nitrite production in peritoneal macrophages elicited by thioglycollate medium**

Nitric oxide (NO), which is released mainly by activated macrophages, is well-known as an effector molecule for killing infectious and tumor cells.27) Furthermore, it is well-known that IL-1β leads the enhancement of NO production.28) Hence, we measured the amount of NO production in peritoneal macrophages elicited by thioglycollate medium (Fig. 3A). NO production was dose-dependently enhanced by the extracts, reaching a maximal level at concentrations of 100 μg/ml or greater. Additionally, NO production was also investigated in thioglycollate-elicited macrophages cultured with LPS and *B. xylophilus* extracts (Fig. 3B). A suboptimal concentration (500 ng/ml) of LPS was
employed for evaluation of an additive or synergistic effect with *B. xylophilus* extracts. The extracts did not effect NO production by LPS.

**Effect of B. xylophilus extracts on mRNA expression of inflammatory cytokines in peritoneal macrophages elicited by thioglycollate medium**

We investigated whether *B. xylophilus* extracts induce mRNA expression of various cytokines in the thioglycollate-elicited macrophages, because the foregoing results make it clear that the extracts activate macrophage functions. The mRNA expression of IL-1β, IL-6, TNF-α, and GM-CSF as inflammatory cytokines and TGF-β was analyzed by a RT-PCR technique after 3 h incubation with 300 μg/ml of *B. xylophilus* extract. Each induction level was compared with that of LPS (10 μg/ml). As shown Fig. 4, the IL-1β mRNA level was increased by the extracts. This result agrees well with the enhancement of the IL-1β protein level (Fig. 2A). The IL-6, TNF-α, and GM-CSF mRNA levels were also increased by *B. xylophilus* extracts. In particular, the mRNA expression of IL-6 was markedly enhanced. TGF-β, a cytokine that suppress the inflammatory response, was not induced by the extracts. LPS showed the same induction profile in the mRNA expression of inflammatory cytokines as did *B. xylophilus* extracts. These results indicate that *B. xylophilus* extracts activate macrophage function extensively and contribute to immunopotentiation.

**Discussion**

In a previous paper, we reported that *B. xylophilus* extracts augmented the polyclonal IgE production induced by LPS and IL-4 in murine splenocytes and purified B cells as determined by ELISA and ELISpot assays. We also observed that the antigen-nonspecific IgE levels were increased in the sera of mice treated...
The level of mRNA expression was investigated against nematodes. In this study, we found that macrophages are well-known as effector cells because they are stimulated by IFN-\(\gamma\) from resident macrophages. The extracts also induced NO production at lower concentrations of the extracts than inflammatory macrophages did (Fig. 2). It has been reported that galactose/N-acetylgalactosamine specific lectin was expressed on the membrane of inflammatory macrophages but not of resident macrophages. It is also known that the number of receptors changes according to the macrophage-activating state. The C5 component, which is produced from the inflammatory macrophages, upregulates the number of complement receptors and Fc\(\gamma\) receptors. The lectins or receptors on the macrophage membrane lead to activation of macrophages per se. These reports indicate that a characteristic of macrophages is dependent on the macrophage-activating state. Therefore, our different results between resident and inflammatory macrophages might be caused by the expression change of the number and variety of receptors.

NO production is one of the roles of inflammatory macrophages. NO is a major effector molecule of macrophage cytotoxicity against a variety of microbial targets, including protozoan and helminth parasites. Hibbs et al. reported that macrophage cytotoxicity for tumor or infected cells is \(L-\)arginine dependent and that the active molecule of this new pathway is NO. As shown in Fig. 3, NO production from inflammatory macrophages was markedly increased by B. xylophilus extracts dose-dependently. It is known that IL-1\(\beta\) has the ability to activate Fc\(\gamma\) receptor-mediated phagocytosis. Phagocytosis falls into two types, non-specific and specific. Specific phagocytosis includes Fc\(\gamma\) receptor-mediated phagocytosis, known as the IgG-dependent pathway, Fc\(\epsilon\) receptor-mediated phagocytosis, known as the IgE-dependent pathway, and C3 receptor-mediated phagocytosis, known as the complement 3-dependent pathway. These pathways play a major role in eliminating germs and so on effectively. B. xylophilus extracts enhanced Fc\(\gamma\) receptor-mediated phagocytosis in both resident and inflammatory macrophages (Fig. 1A and C). The enhancement of Fc\(\gamma\) receptor-mediated phagocytosis by B. xylophilus extracts was accompanied by an increase in rosette formation (Fig. 1B and D). Fc\(\gamma\) receptor-mediated phagocytosis consists of two steps in the mechanism. In the first step, antigen-antibody complex binds to the Fc\(\gamma\) receptor on the macrophage membrane (rosette formation). In the second step, antigen-antibody-receptor complex is incorporated by endocytosis (phagocytosis). Therefore, these results suggest that B. xylophilus extracts induce augmentation of the number of Fc\(\gamma\) receptors on the macrophage membrane and enhance Fc\(\gamma\) receptor-mediated phagocytosis. In addition, it is interesting that the resident macrophages activated the Fc\(\gamma\) receptor-mediated phagocytosis at lower concentrations of B. xylophilus extracts than inflammatory macrophages did. The optimal concentrations in resident and inflammatory macrophages were 1 \(\mu\)g/ml and 300 \(\mu\)g/ml respectively (Fig. 1A and C). The resident macrophages also enhanced IL-1\(\beta\) production at lower concentrations of the extracts than inflammatory macrophages did.
function of inducible NO synthase (iNOS) inducer of thioglycollate-elicited macrophages, and that TNF-α augments NO-dependent macrophage cytotoxicity against *Entamoeba histolytica*.\(^3\)\(^2\)\(^3\)\(^6\) \(B. \) *xylophilus* extracts induced production of IL-1β and mRNA expression of IL-1β and TNF-α in thioglycollate-elicited macrophages (Figs. 2A and 4). Thus it seems that *B. xylophilus* extracts directly induced NO production and that the extracts increased IL-1β and/or TNF-α levels, and then induced NO production indirectly. Furthermore, we observed that mRNA expression of GM-CSF and IL-6, as well as of IL-1β and TNF-α, was increased by the addition of *B. xylophilus* extracts (Fig. 4). The expression of TGF-β, known as a suppressive factor of macrophages, had no influence. These results are similar to the response of macrophages that are stimulated by LPS, but LPS did not enhance Fcγ receptor-mediated phagocytosis (data not shown), and *B. xylophilus* extracts did not affect the NO production induced by LPS at a suboptimal concentration (Fig. 3B). Therefore, it is not thought that LPS acts as a macrophage-activating factor in *B. xylophilus* extracts. We also examined the activities of *Botrytis cinerea* (a bait for *B. xylophilus*), *Ascaris suum* (an animal-parasitic nematode), and *Caenorhabditis elegans* (a non-parasitic soil nematode) extracts. These extracts had no influence or little activation on macrophage function (data not shown), and *B. xylophilus* extracts directly induced NO production indirectly. Furthermore, we tested the effect of *B. xylophilus* extracts treated at 100°C for 10 min on Fcγ receptor-mediated phagocytosis in macrophages. The activation of macrophages was not affected by the heat treatment, although polyclonal IgE induction was abrogated (data not shown). These results suggest that *B. xylophilus* extracts include a macrophage-activating substance(s) other than LPS and a polyclonal IgE-inducing factor(s), and that the extracts might serve as an immunomodulator through macrophage activation against cancer or infectious disease.

In recent years, type I allergic diseases such as atopic dermatitis, bronchial asthma, and pollinosis have been increasing with the expulsion of parasites, especially in modern countries.\(^3\)\(^7\) An allergy-suppressive effect of parasite extracts by enhancing polyclonal IgE production has also been reported.\(^3\)\(^8\) Additionally, we showed that *B. xylophilus* extracts induced polyclonal IgE in our previous report.\(^1\)\(^1\) These facts suggest that *B. xylophilus* extracts have an allergy-suppressive effect. But, since certain parasite extracts enhance the Th2-type response markedly,\(^3\)\(^9\) parasite extracts appear to show suppression of the Th1-type response and the consequent high risk of infectious disease or cancer. In this study, *B. xylophilus* extracts promoted Fcγ receptor-mediated phagocytosis and inflammatory cytokine production. These results suggest that *B. xylophilus* extracts can suppress the allergic reaction by polyclonal IgE induction without inhibition of the Th1 response. These data on the immunobiological features of *B. xylophilus* extracts should facilitate the development of vaccination therapy for allergy. Further studies are in progress to identify the components of *B. xylophilus* extracts for macrophage activation as well as polyclonal IgE production, and to determine the mechanism of action of these compounds.

**References**

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