

Short Communication

Augmentation by *Bursaphelenchus xylophilus*, a Pine Wood Nematode, of Polyclonal IgE Production Induced by Lipopolysaccharide Plus Interleukin-4 in Murine Splenocytes

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Abstract. *Bursaphelenchus xylophilus* (*B. xylophilus*) is a pine wood nematode that is known to cause pine wilt disease. We report here that *B. xylophilus* extracts augmented the polyclonal immunoglobulin E (IgE) production induced by lipopolysaccharide (LPS) plus interleukin-4 (IL-4) both in murine splenocytes and purified B cells as determined by ELISA and ELISPOT assays, but they did not cause such a promotion in the absence of either LPS or IL-4. We also observed that the antigen-nonspecific IgE levels were increased in sera of mice treated with *B. xylophilus* extracts, which were comparable to those of *Ascaris suum* extracts. These findings suggest that administration of *B. xylophilus* extracts could suppress allergic diseases via a saturation of mast cell Fcε receptors or/and an inhibition of antigen-specific IgE synthesis to the allergen by a polyclonal response.

Keywords: *Bursaphelenchus xylophilus*, *Ascaris suum*, polyclonal IgE

It is well known that infection with nematodes or a helminth such as *Ascaris suum* and *Nippostrongylus brasiliensis* or administration of those extracts in humans or other animals can cause intense productions of antigen-specific and nonspecific polyclonal immunoglobulin E (IgE), which is dependent on interleukin-4 (IL-4) production linked with the development of Th2 response (1 – 5). This augmented synthesis of IgE can, under appropriate circumstances, increase allergic reactivity (4, 5), but when excessive, it has been suggested to actually be suppressive (6, 7). Parasitic antigen-specific IgE is associated with elimination of parasites from infected hosts, while antigen-nonspecific polyclonal IgE may be involved in the survival of invading parasites (8, 9).

Allergic diseases such as atopic dermatitis, bronchial asthma, and pollinosis have been increasing during this half-century with the expulsion of parasites especially in modern countries. Furthermore, studies by Lynch et al. (7) on certain aspects of the allergen reactivity in

children treated with anthelmintics suggest that there is a parallel relationship between the decline of parasitosis and increase of allergic disease (4, 6, 10, 11). These results indicate that this allergy inhibition due to nematode infection could be via a saturation of Fcε receptors on mast cells by polyclonal IgE and/or an inhibition of specific IgE antibody synthesis to environmental allergens by the polyclonal response (7).

Bursaphelenchus xylophilus (*B. xylophilus*) is a pine wood nematode, which is transmitted by the pine sawyer, *Monochamus alternatus*, and it is said that this parasite could cause pine wilt disease (12). In this study, we have shown that *B. xylophilus* extracts augmented the polyclonal IgE production induced by lipopolysaccharide (LPS) plus IL-4 in both murine splenocytes and purified B cells as determined by enzyme-linked immunosorbent assay (ELISA) and ELISPOT assays, but they did not cause such a promotion in the absence of either LPS or IL-4. These nematode extracts also increased serum levels of non-specific IgE, but, in contrast, that of antigen-specific IgE was rather decreased in mice.

Both *B. xylophilus* and the bait, *Botrytis cinerea*

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(*B. cinerea*), were kindly provided by K. Kawazu, Professor Emeritus of Okayama University. The culture of *B. xylophilus* was described previously (12). 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 1.3% agar) in a Petri dish (ϕ 90 mm) for 1 week at 26°C. *B. xylophilus* was added on the plate and cultured for 5–7 days at 26°C in the dark. After cultivation, the propagated nematodes were collected from agar plates in a clean bench for 12 h by the *Baermann* funnel method. The collected nematodes were washed 5 times with sterile water, homogenized by an ultrasonic generator, and centrifuged twice at 10,500 $\times g$ for 20 min. The supernatant was lyophilized and stored at 4°C until use. The content of protein in the extract comprised about 28% of the total weight. *Ascaris suum* extract was purchased from LSL (Tokyo).

To determine the polyclonal IgE level in vitro, BALB/c mice (female, 8–12 weeks of age; Charles River Japan, Yokohama) were sacrificed and then their spleens were removed. Spleens were minced and a single cell suspension was obtained. Cells were cultured in RPMI-1640 medium (ICN Biomedicals Japan, Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (FBS) containing 100 U/ml penicillin G potassium and 0.1 mg/ml streptomycin sulfate. Splenocytes (1 $\times$ 10⁵ cells/well), in which red blood cells had been lysed by adding ammonium chloride solution (144 mM NH₄Cl, 16.5 mM Tris, pH 7.2), were cultured with 10 μ g/ml of LPS (Sigma, St. Louis, MO, USA), IL-4 at 100 U/ml (Genzyme, Cambridge, MA, USA) and indicated concentrations of nematode extract in a 96-well round-bottom microplate (Nunc, Rochester, NY, USA) for 7 days. In the case of B cell purification, erythrocytes were lysed and adherent cells were removed by incubating the splenocytes on plastic Petri dishes (ϕ 60 mm) in 10 mM HEPES (pH 7.2) buffered minimum essential medium (MEM) with 10% heat-inactivated FBS at 37°C for 90 min, and lastly T-cells were removed by treatments with 1/500 diluted anti-Thy 1.2 Ab (Dainipponseiyaku, Osaka) at 4°C for 30 min and then with 1/15 diluted low toxicity rabbit complement (Cederlane, Ontario, Canada) at 37°C for 40 min. The purified B-cells (5 $\times$ 10⁴ cells/well) were cultured with nematode extract, IL-4 and LPS for 7 days. The culture medium was collected and frozen at –30°C for IgE ELISA. Briefly, ELISA plates (96-well flat bottom plate, MAXISOAP; Nunc) were coated with 50 μ l of anti-mouse IgE (Technopharm Biotechnology, Paris, France) (4 μ g/ml) overnight at 4°C. After washing and blocking (1% BSA in 7.5 mM PBS), the wells

were seeded with 50 μ l of culture medium or diluted sera and incubated for 2 h at room temperature. After washing, 50 μ l of biotin-conjugated anti-mouse IgE (Technopharm Biotechnology) (2.5 μ g/ml) were added. After 2 h, IgE level was detected using HRP-conjugated streptavidin (Zymed, San Francisco, CA, USA) in substrate solution that consisted of 1.37 mg ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) (Sigma) per ml in 0.1 M citrate buffer (pH 4.0) and 50 mM H₂O₂. Then the plates were read at a wavelength of 405 nm with an ELISA reader (Model 680 Microplate Reader; BIO-RAD, Hercules, CA, USA). The IgE secreting cells were measured by ELISPOT assay. Briefly, the cells cultured with LPS, IL-4, and *B. xylophilus* extract were seeded with 100 μ l of culture medium on the BSA-coated ELISA plate. After 18 h, 50 μ l of biotin-conjugated anti-mouse IgE (2.5 μ g/ml) were added and incubated for 2 h. The IgE secreting cells were detected using HRP-conjugated streptavidin in substrate agar (1%) that consisted of 2 mg 3,3'-diaminobenzidine (Sigma) per ml in 0.05 M phosphate buffer (pH 7.4), 0.53 mM H₂O₂, 0.076 mM CoCl₂ $\cdot$ 6H₂O, and 0.076 mM NiCl₂ $\cdot$ 6H₂O. The spots of IgE-secreting cells were quantified microscopically. To determine the polyclonal IgE levels in vivo, mice were immunized intraperitoneally with 500 μ g of *B. xylophilus* extract or 200 μ g of *Ascaris suum* extract in 3 mg of aluminum hydroxide gel as adjuvant. The animals were boosted 2 weeks after immunization with the same dose of the nematode extract. Mice were periodically bled and the serum IgE levels were determined by ELISA. All data are presented as means with S.D. or S.E.M. Statistical analyses were performed by an unpaired Student's *t*-test.

To examine the ability of the extracts of *B. xylophilus* to stimulate antigen-nonspecific polyclonal IgE production, LPS- and IL-4-stimulated murine splenocytes and purified B cells were incubated in the presence of varying concentrations of the extracts, and compared with that of *Ascaris suum* extract. The levels of IgE were determined by ELISA or ELISPOT assay. As shown in Fig. 1 (A and B), the polyclonal IgE production as assessed by ELISA was significantly enhanced by addition of *B. xylophilus* extracts in a dose-dependent manner both in splenocytes and purified B cells, and the optimal stimulation was obtained at a concentration of 50–300 μ g/ml. This promotion of polyclonal IgE by the nematode was not observed in the absence of either LPS or IL-4. Although it is known that *Ascaris suum* extracts markedly induce polyclonal IgE production in animal models, this extract had no effect on the IgE production in murine splenocytes stimulated with LPS and IL-4.

We next assessed IgE responses of *B. xylophilus* by

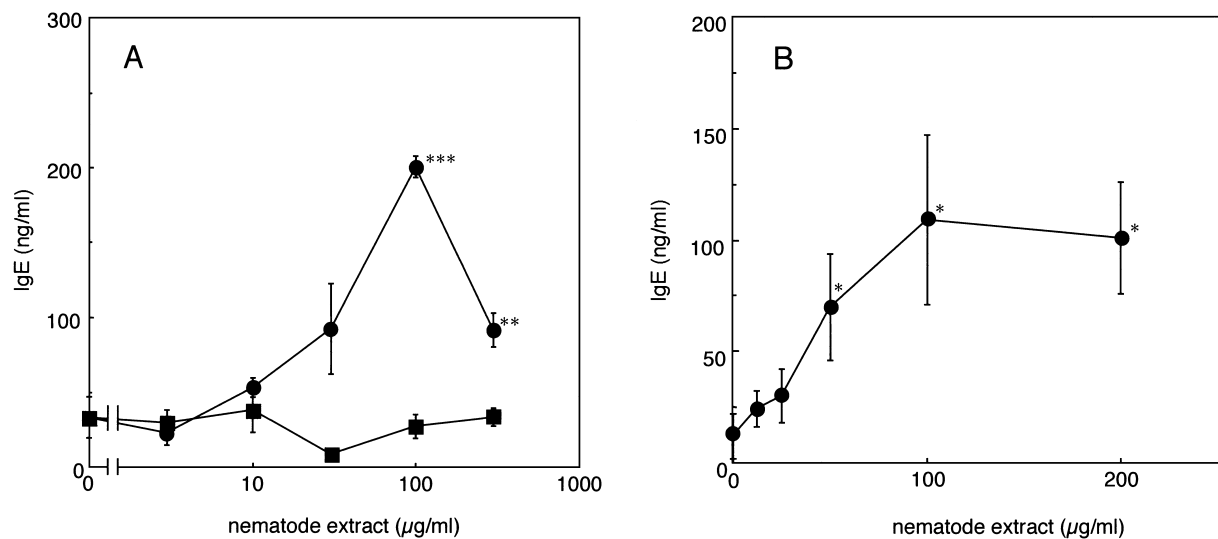


Fig. 1. Effects of *B. xylophilus* and *Ascaris suum* extracts on IgE production in cultured murine splenocytes in vitro. Splenocytes (1×10^5 cells in $200 \mu\text{l}$ medium/well) (A) or purified B cells (5×10^4 cells in $200 \mu\text{l}$ medium/well) (B) were cultured with LPS ($10 \mu\text{g/ml}$), IL-4 (100 U/ml), and various concentrations of *B. xylophilus* extract (circle) or *Ascaris suum* extract (square) in 96-well culture plates under a 95% air – 5% CO₂ atmosphere for 7 days. IgE levels were measured by ELISA. Values are the mean $\pm$ S.D. for triplicate cultures. Statistical significance was determined by a Student's *t*-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as compared with the control.

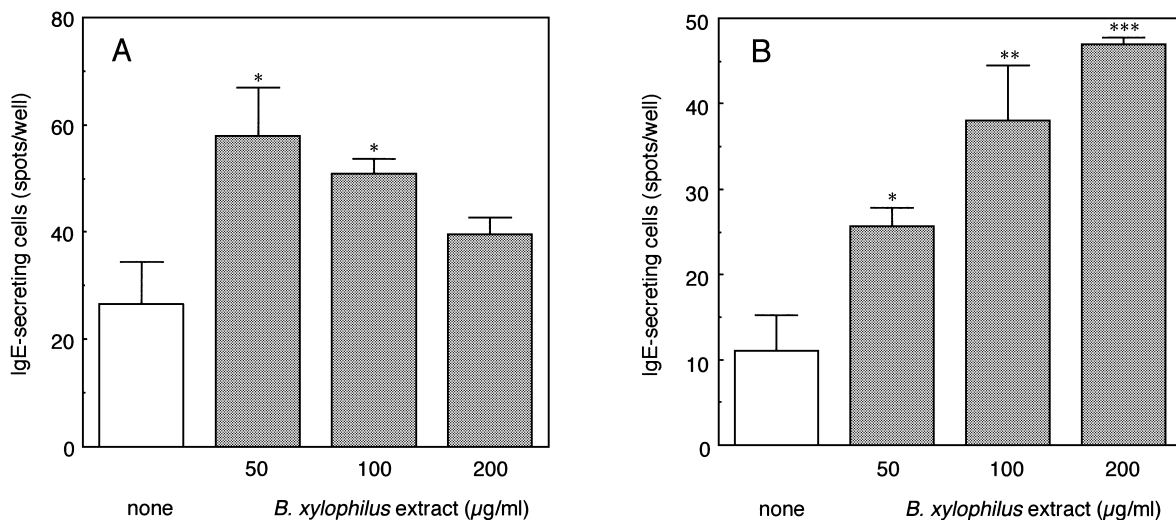


Fig. 2. Effects of *B. xylophilus* extract on the increase of IgE-secreting cells in cultured murine splenocytes in vitro. Splenocytes (1×10^5 cells in $200 \mu\text{l}$ medium/well) (A) or purified B cells (5×10^4 cells in $200 \mu\text{l}$ medium/well) (B) were cultured with LPS ($10 \mu\text{g/ml}$), IL-4 (100 U/ml), and various concentrations of *B. xylophilus* in 96-well culture plates under 95% air – 5% CO₂ atmosphere for 7 days. After incubation, IgE-secreting cells were detected by ELISpot assay. Values are the mean $\pm$ S.D. for triplicate cultures. Statistical significance was determined by a Student's *t*-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as compared with the control.

counting the number of IgE-secreting cells (ELISpot assay). IgE secreting cell development in LPS- and IL-4-induced splenocytes and purified B cells were also significantly increased by addition of *B. xylophilus* extracts at the concentration of 50 – 200 $\mu\text{g/ml}$ (Fig. 2: A and B). Enhancement of the IgE response by this

nematode was also found to be LPS- and IL-4-dependent. Augmentation of the polyclonal IgE production by *B. xylophilus* implies that it would act directly on the B cells and then enlarge the number of effector B cells, via the LPS- and IL-4-dependent class switch, which are able to produce polyclonal IgE antibodies.

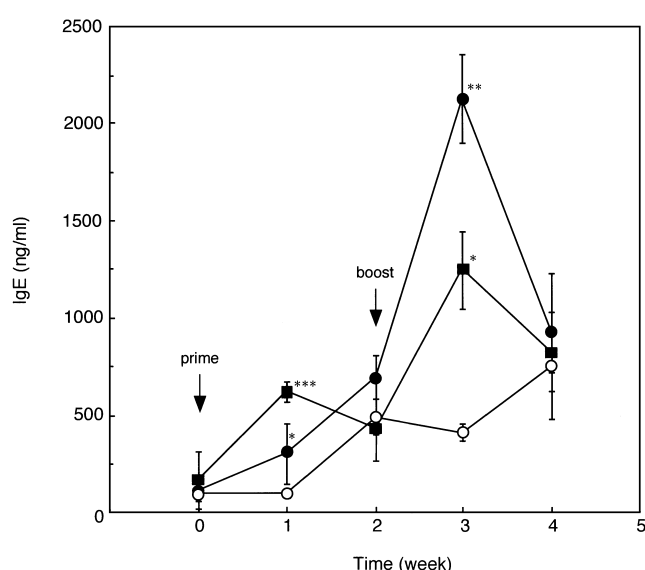


Fig. 3. Effects of *B. xylophilus* and *Ascaris suum* extracts by intraperitoneal injection on serum IgE levels in mice. *B. xylophilus* extract (500 µg/body) (closed circle), *Ascaris suum* extract (150 µg/body) (closed square), or saline (open circle) was injected intraperitoneally with aluminum hydroxide gel (3 mg/body) at 0 week (prime) and 2 weeks (boost). Serum IgE levels were determined by ELISA. Values are the mean $\pm$ S.E.M., $n = 5$. Statistical significance was determined by a Student's t -test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as compared with vehicle.

Next, we examined the serum levels of polyclonal IgE in mice intraperitoneally injected with *B. xylophilus* extracts and compared them with that of mice injected with *Ascaris suum*. As shown in Fig. 3, the polyclonal IgE levels in serum were increased by the sensitization with *Ascaris suum* extract at 1 week after immunization, and the maximal induction (1300 ng/ml) was obtained at 3 weeks. *B. xylophilus* extracts also profoundly induced the polyclonal IgE antibody, and the maximal level (2200 ng/ml) was reached at 3 weeks after sensitization.

In recent years, evidence has begun to accumulate that the Th2 response is induced (1, 3, 13) and IgE production is increased (4, 6, 7) by nematode extract in animal models. However, the mechanisms of the polyclonal IgE production by nematode extracts have not yet been clarified. For example, it is reported that *Ascaris suum* extracts work directly on the B cells and shows its effect as a mitogen (3). In the present report we have demonstrated that *Ascaris suum*, unlike *B. xylophilus* extract, could not enhance IgE production in vitro. We have conceived the notion that *Ascaris suum* extract is unable to induce the class switch to IgE due to the absence of the factors that synergistically induce the class switch to IgE from IgM together with IL-4, while *B. xylophilus* extract might have these

factors. Therefore, the metabolized or secondary factors might be involved in in vivo augmentation by *Ascaris suum* immunization of the polyclonal IgE production. Conclusively, these findings indicate that the use of *B. xylophilus* is a suitable tool for investigating the detailed immune mechanisms underlying cytokine and antibody isotype productions in culture cells.

Ascaris suum and other animal parasitic nematodes are reported to enhance excessive polyclonal IgE productions that might cause inhibition of allergic diseases, while immunosuppressive effects by these nematodes have also been reported. Protease inhibitors secreted and/or immunosuppressive cytokines generated are involved in the immunosuppression induced by these nematodes (11, 13, 14), where Th2-type cytokines productions such as IL-4 and IL-10 and their responses are predominantly stimulated by *Ascaris suum* (15) and oppositely, Th1-type cytokines productions such as IL-2 and interferon- γ (IFN- γ) and their responses induced by allergens are suppressed.

Our preliminary data have indicated that extracts from a plant-parasitic nematode such as *B. xylophilus* do not stimulate Th2-type cytokine generations, but Th1-type cytokine generations such as IFN- γ are promoted (data not shown). Although the ability to induce antigen-specific IgE production against *B. xylophilus* remain elusive, we have performed the PCA reaction against OVA by using the sera of mice sensitized with *B. xylophilus* extract and OVA. As a result, the PCA reaction elicited with OVA was inhibited by sera of mice sensitized with OVA and *B. xylophilus* extract (data not shown). This result suggests that *B. xylophilus* extract may suppress the hypersensitivity to allergens being accompanied by decreasing antigen-specific IgE productions.

Our present studies on the immunobiological features of *B. xylophilus* extracts would facilitate the elucidation of molecular mechanisms underlying the enhancement of polyclonal IgE productions by nematodes and the development of vaccination therapy for allergy. Purification and identification of the effective molecules are now in progress in our laboratory.

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