Extract of *Nippostrongylus brasiliensis* Stimulates Polyclonal Type-2 Immunoglobulin Response by Inducing De Novo Class Switch

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Infection with the nematode parasite *Nippostrongylus brasiliensis* induces a pronounced type-2 T-cell response that is associated with marked polyclonal immunoglobulin E (IgE) and IgG1 production in mice. To examine the differential roles of the infection and products produced by nematodes, we investigated a soluble extract of *N. brasiliensis* for the ability to mediate this type-2 response. We found that the extract induced a marked increase in IgE and IgG1 levels, similar to that induced by the infection. The extract did not affect the level of IgG2a in serum, showing that the effect was specific to IgE and IgG1 (type-2-associated immunoglobulin) rather than inducing a nonspecific increase in all immunoglobulin isotypes. This response was also associated with increased interleukin-4 production in vitro. These results confirm that the extract, like infection, is a strong inducer of polyclonal type-2 responses and a reliable model for investigating the regulation of nematode-induced responses. The extract induced the production of IgG1 when added to in vitro cultures of lipopolysaccharide-stimulated B cells. This provides evidence for the induction of class switch. It did not induce upregulation of IgG1 in naive (unstimulated) B cells or expand B cells in in vitro cultures. Analysis of DNA from the spleens of mice treated with the extract by digestion-circularization PCR demonstrated a marked increase in the occurrence of γ1 switch region gene recombination in the cells in vivo. These results provide strong evidence that soluble worm products are able to mediate the marked polyclonal γ1/e response and that infection is not required to mediate this response. Furthermore, these data provide evidence that the soluble nematode extract induces this effect by causing de novo class switch of B cells and not by an expansion of IgG1 B cells or an increase in antibody production by IgG1 plasma cells.

*Nippostrongylus brasiliensis* infection in mice has been widely used to address the mechanisms involved in the regulation of the type-2 responses associated with nematodes. One significant aspect of the type-2 shift induced by this worm in mice is the bias of the immunoglobulin (Ig) response towards IgE and IgG1 (29, 72, 75). A compelling characteristic of this response is the fact that most of this antibody is not directed against the parasite; only a small fraction is specific to nematode antigens (20, 21). This clearly indicates that nematode infection induces a polyclonal activation of reaginic antibodies. Further to this, there is strong evidence that nematode infection will bias the developing immune response to unrelated antigens towards IgE and reaginic IgG. For example, we (31) have shown dramatic effects on the developing antibody response to third-party antigens by treatment with nematodes and nematode extracts. This observation is significant in that it suggests that the influence of nematodes on immune responses is far reaching and may have profound effects on developing immune responses to unrelated antigenic challenge. This could have significant effects on the outcome of vaccination strategies in areas where nematode infection is endemic.

Currently, the prevailing evidence supports important roles for both interleukin-4 (IL-4) and IL-13 in IgE and IgG1 production, including such antibody production following nematode infection. This has been confirmed by cytokine blocking experiments (1, 2, 10, 27, 72) and gene knockout experiments (25, 26, 40, 41, 51). Several in vitro culture studies have shown that production of both IgE and IgG1 is dependent on IL-4, as the addition of antibodies to either IL-4 or the IL-4 receptor completely inhibits IgE and IgG1 production (12–14).

This cytokine dependence is likely due to the role of these cytokines in mediating class switch. For example, it has been demonstrated that the addition of IL-4 to cultures of either lipopolysaccharide (LPS)- or anti-CD40 antibody-stimulated murine B cells induces high levels of germline e and γ1 transcripts that correlate with the amount of IgE and IgG1 induction, respectively (5–7, 16, 23, 37, 57). Similar observations have been reported in a number of in vivo model systems (73, 74). The production of both IgE and reaginic IgG has common regulatory elements (15, 38, 52, 59, 62); there is now convincing evidence in mice of a sequential switch from μ to γ1 and then to e (38, 59, 74). Such a sequential switch has also been documented in other species (43).

It is clear that IL-4 and IL-13 are involved in reaginic antibody production in response to nematodes. What remains to be addressed, however, is the relationship between the inflammatory parameters of infection, the products produced by the nematode, and the immunoglobulin response that follows. It is unclear whether the large polyclonal γ1/e response seen after nematode infection is due to infection per se or to the elaboration of factors which either directly or indirectly mediate class switch by nematodes. The evidence that cytokine-like molecules can be produced by nematodes (17, 47) is suggestive of a direct role for nematode factors in the mediation of the γ1/e response. This is of significant interest since it could form the foundation for nematode-based therapeutics.
In this study, we examined the effects of a soluble *Nippostrongylus* protein extract on IgE and IgG1 responses in mice. We confirmed that soluble worm products are able to mediate the heightened polyclonal y1/2 response and that infection is not required to mediate this response. Furthermore, we demonstrated that the soluble nematode extract induces the effects by causing de novo class switch of B cells.

**MATERIALS AND METHODS**

**Animals.** Female nude (nu/nu) BALB/cByJ mice and control littermates (nu/+ mice) were purchased from Jackson Laboratories (Bar Harbor, Maine). All mice were used at 8 to 12 weeks of age. Male Sprague Dawley (SD) rats (220 to 250 g) used in the maintenance of *N. brasilensis* and in the preparation of adult worm homogenate (AWH) were purchased from Harlan Sprague Dawley (Indianapolis, Ind.). All animals were maintained in compliance with the Canadian Council on Animal Care guidelines, with food and water provided ad libitum.

**Parasites.** Third-stage (infective) larvae of *N. brasilensis* were obtained from Dean Befus (University of Alberta, Edmonton, Canada). The life cycle of the worms was maintained regularly by passage in Sprague Dawley rats as previously described (30).

*Nippostrongylus* adult worm extract preparation. A whole adult worm extract (AWH) was prepared essentially as previously described by Nawa and coworkers (46). Briefly, SD rats were infected by subcutaneous injection of 5,000 third-stage larvae in 0.5 ml of phosphate-buffered saline (PBS) containing 100 U of penicillin and 100 μg of streptomycin per ml (PBS-PS). Rats were sacrificed by carbon dioxide asphyxiation at 8 to 10 days postinfection. Adult worms were recovered by injecting a small amount of PBS into the peritoneal cavity and aspirating the fluid with a Pasteur pipette. The worms were collected and then centrifuged at 1,000 × g for 15 min at 4°C. The supernatant was discarded, and the pellet was resuspended in PBS containing 100 μg of penicillin and 100 μg of streptomycin per ml, and the suspension was centrifuged at 15,000 × g for 30 min at 4°C. The homogenate was sterilized by syringe filters (0.22 μm; Millipore Corp.), aliquoted into 1.5-ml microcentrifuge tubes (Fisher Scientific, Nepean, Ontario, Canada), and stored at −20°C. On the day of use, 1 ml of homogenate was centrifuged at 15,000 × g for 30 min at 4°C. The supernatant, which contained the antigenic extract, was transferred into a glass test tube, and 1 ml of double-distilled water was added to a final volume of 2 ml. Protein content of the supernatant was determined with the bicinchoninic acid protein assay kit (Pierce; Rockford, Ill.) according to the manufacturer’s instructions.

**In vivo treatment.** For in vivo experimentation, BALB/c mice were injected subcutaneously with 200 μl (200 μg of protein) of either AWH or killed mycobacteria (Sigma-Aldrich Co., Oakville, Ontario, Canada). Control animals were either untreated (naïve) or injected with either PBS or Freund’s incomplete adjuvant (Sigma-Aldrich Co.). Prior to infection, the wheat germ extract was added to the adult worm extract and adult worms were recovered from the small intestine using a modified Baermann apparatus. The adult worms were washed at least 10 times with sterile PBS-P5. The last two washes were done in PBS alone. After washing, the worms were counted, transferred into a glass tube, and homogenized in 1 to 2 ml of PBS with a glass tissue homogenizer. The homogenate, in addition to 3 to 5 ml of PBS used to rinse the homogenizer, was transferred into 15-ml polypropylene tubes. To eliminate large particles, the homogenate was centrifuged at 1,000 × g for 15 min at 4°C. The supernatant was collected and then further clarified to eliminate fine particles by centrifugation at 15,000 × g for 30 min at 4°C. The homogenate was sterilized by syringe filters (0.22 μm; Millipore Corp.), aliquoted into 1.5-ml microcentrifuge tubes (Fisher Scientific, Nepean, Ontario, Canada), and stored at −20°C. No protease inhibitors were added at any stage of the procedure. Protein content of the extract was determined with the biocinchoninic acid protein assay kit (Pierce Laboratory, Rockford, Ill.) according to the manufacturer’s instructions.

**Cytokine ELISA.** Supernatants from spleen cell cultures were analyzed for the levels of IL-4 by a sandwich ELISA as described previously (33). Briefly, flat-bottomed 96-well ELISA plates were coated with anti-mouse IL-4 (1 μg/ml; Pharmingen); in 0.1 M carbonate buffer (pH 9.0) at 100 μl per well and incubated overnight at 4°C. After blocking, test supernatants and recombinant IL-4 (10 ng/ml; Pharmingen) were added to the wells and were incubated overnight at 4°C. Following this, biotinylated anti-IL-4 antibody (Pharmingen) was added to the wells and incubated for 1 h at room temperature. The plates were then washed, and extravidin-peroxidase (Sigma-Aldrich Co.), with the detection substrate being 3,3′-5′-tetramethylbenzidine plus H2O2, was added. The substrate solution that consisted of 0.4 μg of o-phenylenediamine substrate (Sigma-Aldrich Co.) per ml in citrate buffer (pH 5.0) and hydrogen peroxide (0.04%; Sigma-Aldrich Co.). Absorbance was read at a wavelength of 490 nm using a Titertek plate reader (ICN). Absorbance at 490 nm was calculated by subtracting the absorbance of the blank (containing the substrate alone) from the absorbance at 490 nm of the sample. The IL-4 concentration was determined using the standard curve obtained for the 10 ng/ml of IL-4 standard (Pharmingen).

**Proliferation assay.** Mice were infected with 5,000 third-stage larvae of *N. brasiliensis* (AWH) prepared essentially as previously described by Nawa and coworkers (30). Briefly, third-stage (infective) larvae of *N. brasiliensis* were obtained from Dean Befus (University of Alberta, Edmonton, Canada). The life cycle of the worms was maintained regularly by passage in Sprague Dawley rats as previously described (30).

**Reverse transcription (RT) reaction.** To perform a reverse transcription reaction, 1 μg of total RNA was added to a reaction mixture containing 1 mM each of the primer sets, and 2.5 U of reverse transcriptase enzyme (200 U), and RNA solution (1 μl). The RT reaction was performed for 1 h at 37°C. The reactions were terminated by heating at 95°C for 5 min. The cDNA obtained was amplified by PCR. One microliter of the PCR product was further amplified by the manufacturer’s instructions.

**PCR.** Total cellular RNA was isolated from spleen cells of mice injected with AWH or worm or naive (untreated) mice using TRIzol reagent (Life Technologies). RNA was isolated from 10 7 spleen cells as previously described (30). Reverse transcription (RT) reaction of each RNA sample was performed on 1 μg of total RNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies). Each reaction mixture (total volume, 20 μl) contained first-strand buffer (1×), dithiothreitol (0.01 M), deoxyribonucleoside triphosphates (dNTPs; 0.5 mM), random hexamer primers (1 μg), M-MLV reverse transcriptase enzyme (200 U), and RNA solution (1 μl). The RT reaction was performed for 1 h at 37°C. The reactions were terminated by heating at 95°C for 5 min. The cDNA obtained was amplified by PCR. One microliter of the PCR product was further amplified by the manufacturer’s instructions.

**DC-PCR.** For digestion-circularization-PCR (DC-PCR), genomic DNA was isolated from spleen cells of naive (untreated) mice or treated mice by standard methods as previously described (18, 64) or with DNAeasy reagent as recommended by the manufacturer (Life Technologies). Digestion of each DNA sample was performed in 1.5-microliter microcentrifuge tubes with 5 to 10 μg of DNA in a 100-μl volume using EcoRI as the restriction endonuclease. Each reaction mixture contained 10 μl of 10× EcoRI buffer (1× final; Life Technologies), EcoRI (2 μg/ml of DNA; Life Technologies), DNA solution, and the appropriate volume of double-distilled water required to adjust the volume to 100 μl. The reaction mixtures were then incubated overnight in a 37°C waterbath, following which the enzyme was inactivated by incubation at 70°C for 20 min. For ligation (circularization), 10 to 20 μl of digested DNA samples was placed in 1.5-microliter microcentrifuge tubes to which 20 μl of 5× T4 DNA ligase buffer (1× final; Life Technologies), 2 μl (20 U) of T4 DNA ligase (Life Technologies), and double-distilled water was added to a final volume of 100 μl. The reaction mixtures were incubated overnight in a 16°C waterbath. Ligated DNA samples were then amplified by PCR. PCR was performed on ligated DNA products for the detection of *S.* *Sy1* recombinant and nictinic acetylcholine receptor (nAChR) β subunit gene with appropriate primers in clean 0.5-microliter microcentrifuge tubes. Portions (5 to 20 μl of DNA) digested with EcoRI and placed into 100-μl PCR tubes with the reaction mixture containing 5 μl of 10× GeneAmp PCR buffer II (100 mM Tris-HCl [pH 8.3], 500 mM KCl), 2 μl (1.0 mM) of MgCl2, 0.2 mM each of the dNTPs, 0.5 μM

**In vitro stimulation of B cells for immunoglobulin production.** Purified B cells (2 × 106/ml) were stimulated with 10 μg of LPS per ml alone or LPS in combination with AWH (20 μg/ml) or IL-4 (5 ng/ml; Genzyme, Cambridge, Mass.). The cells were incubated in 1 ml of supplemented RPMI in 24-well plates (Nalge Nunc Inc.) at 37°C. Culture supernatants were harvested 7 days later, centrifuged to eliminate cells, and frozen at −20°C until analyzed for immunoglobulin levels using antibody capture ELISA.

**Antibody ELISA.** The level of IgE, IgG1, and IgG2a in mouse serum and IgG1 and IgM levels in B-cell culture supernatants were determined by standard capture ELISA. Flat-bottomed 96-well maxisorb immuno-plates (Nalge Nunc Inc.) were coated with 2 μg of the respective capture anti-immunoglobulin antibody (goat anti-mouse IgG1, goat anti-mouse IgG2a, or goat anti-mouse IgM; Cedarlane Laboratories, Hornby, Ontario, Canada) or anti-mouse IgE monoclonal antibody (Pharmingen, Mississauga, Ontario, Canada) overnight at 4°C. After blocking, the wells were seeded with 100 μl of either serum or B-cell culture supernatants and appropriate standards and incubated overnight at room temperature. Immunoglobulins were detected using secondary antibodies conjugated to horseradish peroxidase (Cedarlane Laboratories; a substrate solution that consisted of 0.4 μg of o-phenylenediamine substrate (Sigma-Aldrich Co.) per ml in citrate buffer (pH 5.0) and hydrogen peroxide (0.04%; Sigma-Aldrich Co.). Absorbance was read at a wavelength of 490 nm using a Titertek plate reader (ICN).
RESULTS

*N. brasiliensis* extract induces IgE and IgG1 production. Increased production of circulating IgE and IgG1 in mice infected with nematodes has been widely reported (15, 29, 75), but the mechanism involved in this response is unclear. We adopted a reductive approach using an extract (AWH) of *N. brasiliensis* to test the hypothesis that it could induce type-2-associated immune responses. We found that, like infection, injection of mice with AWH (200 μg of protein, which is equivalent to about 200 worms) resulted in a marked increase in the level of total IgE in the serum (Fig. 1A). This was about five- to sixfold higher than the control level at 3 weeks post-AWH injection. In mice injected with the vehicle alone, no increase in IgE level was detected. In fact, in these mice the baseline level at day 0 was maintained at all time points examined. In addition, a marked increase in the level of IgG1 was observed in mice injected with AWH (Fig. 1B). The pattern of the increase in IgE and IgG1 levels in the serum of mice injected with AWH is very similar to the pattern observed in mice infected with the worm (75). However, the final absolute levels were always higher in nematode-infected (IgE, 30 μg/ml; IgG1, 2 mg/ml) than in AWH-treated mice.

*N. brasiliensis* is a strong inducer of type-2-associated immune response (15, 28, 30, 33). To verify whether the induction of increased IgE and IgG1 levels by AWH is specific to these type-2 immunoglobulins, the level of IgG2a, a type-1 immunoglobulin isotype, was also examined. The level of IgG2a in the serum of the AWH-injected mice was similar to that observed in the mice that were injected with the vehicle control (Fig. 1C). These observations show that AWH induces an immune response that is biased toward type-2 immunoglobulins, similar to our observations with *N. brasiliensis* infection (data not shown).

To confirm that the response induced by AWH is not simply a response to a complex antigen, the immune response induced by AWH was compared to the immune response induced in mice injected with killed mycobacteria in the same vehicle as AWH. As shown in Fig. 2A, IgE was detected in significant amounts in mice injected with AWH (reaching a level of 20 μg/ml) but was not detected in the serum of mice injected with mycobacteria. The level of IgG1 in mycobacteria-treated mice was not greater than that observed in mice injected with the vehicle control or in naïve (untreated) mice (Fig. 2B). In contrast, mice injected with mycobacteria showed much higher levels of IgG2a in the serum than control animals, from 150 to 200 μg/ml. The control group was injected with the vehicle alone (FIA). Each group of mice was bled for serum each week for 3 weeks, and the sera within the groups were pooled. The pooled serum samples were assayed for IgE (A), IgG1 (B), and IgG2a (C) levels by capture ELISA. The data are from one experiment and are representative of three (IgE), five (IgG1), and four (IgG2a) independent experiments.
A antigen mixture, specifically induced the increased type-2-associated immunoglobulin response observed. AWH induces IL-4 and IL-13 production. Increased IgG1 and IgE levels are associated with increased IL-4 and IL-13 production (1, 15, 41, 69). In nematode infections, the levels of mRNA and protein for these cytokines have been demonstrated to be increased in both spleen and mesenteric lymph node cells (19, 28, 39, 67, 69). If our hypothesis regarding the increase in IgE and IgG1 activity induced by AWH is correct, we would expect that the increased levels of these immunoglobulins would likewise be associated with an increase in the levels of these cytokines. To investigate this possibility, spleen cells from mice treated with AWH were analyzed for increased IL-4 production. Figure 3A demonstrates IL-4 mRNA expression in spleen cells from AWH-treated mice. A similar increase in IL-13 mRNA was also observed in the spleen cells from AWH-treated mice (Fig. 3B). In agreement with the data on levels of IgE and IgG1 in serum, the cytokine mRNA level was higher in worm-infected animals than in AWH-treated mice. Amplicons for IL-4 and IL-13 could not be detected by RT-PCR of spleen cells from naive (untreated) mice. Since an increase in mRNA levels does not always reflect increased protein production, IL-4 protein levels were also assessed. Spleen cells isolated from mice 2 and 3 weeks post-treatment, were stimulated with ConA in vitro; supernatants were collected and analyzed for IL-4 levels by ELISA. IL-4 was detected in the supernatants of ConA-stimulated spleen cells from both worm- and AWH-treated mice (Fig. 4A), but not in supernatants of cells from naive control mice. The level of IL-4 was significantly higher (P < 0.001) in worm-infected (620 pg/ml) than in AWH-injected (110 pg/ml) mice. This increase in IL-4 levels is due specifically to the injection of AWH and not to a nonspecific effect of treatment with a complex antigen mixture. As shown in Fig. 4B, substantial levels of IL-4 (>120 pg/ml) were observed in the culture supernatants of ConA-stimulated spleen cells from AWH-injected mice, whereas <20 pg/ml (limit of detection) of IL-4 was present in the supernatants from mycobacterium-treated mice. When these data were normalized for the vehicle, the cytokine pattern correlated well with the observed immunoglobulin subclass levels shown in Fig. 2. These data confirm that the IL-4 induced in the spleen cells of mice treated with AWH is mediated specifically by the extract and not simply a “response to antigen” effect (i.e., that the observed responses are not simply due to the injection of a complex mixture of antigens but specifically due to factors in the extract).

AWH induces IgG1 production in in vitro B-cell culture. The observation that AWH induces an increase in IgE and IgG1 levels in mouse serum suggests an effect on B-cell activ-

FIG. 2. Increased IgE and IgG1 response is specific to AWH treatment. BALB/c mice in groups of three were injected with killed mycobacteria (Myc) or AWH emulsified in FIA (vehicle). The third group received vehicle alone (FIA). Mice in each group were bled for serum at 14 and 21 days posttreatment. Serum samples were pooled within each group. Serum samples were assayed for IgE, IgG1, and IgG2a levels by capture ELISA. Data shown are the IgE (A), IgG1 (B), and IgG2a (C) levels at 21 days posttreatment. Results are expressed as mean ± SD of triplicate wells and are representative of three experiments (A) ***, P < 0.0001; two-tailed, unpaired Student’s t test; (B and C) ***, P < 0.001; NS, not significant (P > 0.05), one-way ANOVA.

FIG. 3. IL-4 and IL-13 mRNA induction by AWH. Spleen cells were isolated from mice 21 days after treatment with AWH or N. brasiliensis (Nb). For assessment of IL-4 (A) and IL-13 (B) mRNA expression, total cellular RNA was isolated with TRIZOL and reverse-transcribed into single-stranded cDNA using M-MLV reverse transcriptase and random hexamers as described in Materials and Methods. The resulting cDNA template was used in a PCR with primers specific for either IL-4 or IL-13. β-Actin mRNA levels were also determined by RT-PCR to control for equal RNA loading. PCR amplicons were resolved on a 1.5% agarose gel with ethidium bromide staining. Results are representative of three experiments. Nv, naive (untreated). The negative controls in panel B were no enzyme in RT reaction, no RNA in RT reaction, no cDNA in PCR, and no primers in PCR (left to right, respectively).

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ity. To examine this, we determined whether AWH could induce B cells in culture to produce IgG1.

Purified B cells were stimulated with LPS and then incubated in the presence of AWH for 7 days. Supernatants from these cultures were assayed for IgG1 by capture ELISA. As a positive control, LPS-stimulated B-cell cultures were incubated with IL-4. B cells stimulated with LPS secrete significant amounts of IgM, but in the presence of IL-4, the cells undergo class switch to become IgG1- (or IgE)-secreting B cells. The data shown in Fig. 5A demonstrate that IL-4 induced an increase in IgG1 levels in the B-cell culture supernatants. In the same experiment, AWH also induced an increase in IgG1 levels in cultures of LPS-stimulated B cells. This increase was not observed in cultures of naive B cells stimulated with AWH alone, nor did LPS alone induce IgG1 production in B cells in the absence of AWH. In addition, the induction of increased IgG1 levels in the B-cell culture was associated with a decrease in the levels of IgM in the culture supernatants (Fig. 5B). These data are very suggestive that the soluble nematode extract (AWH) induces LPS-stimulated B cells to undergo class switch from μ to γ1.

Increased IgG1 level in AWH-treated mice associated with de novo class switch. The data above suggest that the induction of increased immunoglobulin levels is mediated by class switch.

However, the data could be explained by (i) an expansion of existing IgG1-committed B cells, (ii) an increase in antibody production by individual cells, or (iii) an increase in de novo class switch. To discriminate between these possibilities, we first investigated whether the increase in IgG1 levels was mediated by an expansion of existing IgG1-committed B cells in vitro in the presence of AWH. B cells from control (naive) mice were stimulated with LPS and cultured in the presence of AWH. An expansion of an existing IgG1-committed population would yield a significant increase in proliferation. Since higher levels of IgG1 are found both in vivo and under these in vitro conditions, this experiment appropriately addresses the issue of expansion of memory cells. As expected, LPS itself induced significant proliferation, but this proliferative response was significantly inhibited \( P < 0.001 \) when the cells were cultured in the presence of AWH (Fig. 6). Furthermore, AWH alone did not demonstrate stimulatory activity when added to B cells. This inability of AWH to either stimulate naive B-cell proliferation or enhance LPS-induced B-cell proliferation effectively rules out the possibility of expansion of B cells.

**FIG. 4.** Increased IL-4 response in spleen cell cultures is specific to AWH treatment. For assessment of IL-4 protein levels, spleen cells isolated from naive (untreated) mice or mice treated with AWH or worms (Nb) (A), killed mycobacteria (Myc), or AWH emulsified in FIA (B) 21 days posttreatment were stimulated with ConA (5 μg/ml) for 24 or 48 h. Culture supernatants were then analyzed by ELISA for IL-4 levels as described in Materials and Methods. Results are expressed as the mean concentration of IL-4 ± SD of three replicate wells. **...**, \( P < 0.001 \), one-way ANOVA. Data in panel B were normalized for vehicle. ND, below detection limit (15 pg/ml). Results are representative of three experiments.

**FIG. 5.** Influence of AWH on immunoglobulin production in in vitro B-cell culture. Highly purified naive B cells from uninfected mice (8 to 12 weeks old) isolated as described in Materials and Methods were not stimulated (NS) or stimulated in culture containing AWH alone (20 μg/ml), LPS alone (10 μg/ml), or LPS in combination with AWH (20 μg/ml) or IL-4 (5 ng/ml). Culture supernatants were harvested 7 days later and then analyzed for IgG1 (A) and IgM (B) levels using antibody capture ELISA as described in Materials and Methods. Results are expressed as the mean concentration ± SD of three replicate wells and are representative of six separate experiments. **...**, \( P < 0.001 \), one-way ANOVA.
of IgG1 cells as a mechanism of action of AWH. AWH also inhibited the proliferation of B cells obtained from mice infected with *N. brasiliensis*. Since infection of mice with this nematode has been shown to cause significant expansion of IgG1+ B cells in the spleen (22, 70, 71), the B-cell population tested would have increased IgG1+ B cells present compared to the control. The fact that this population did not proliferate in response to AWH confirms that AWH does not cause selective expansion of IgG1+ B cells.

To distinguish between upregulation of antibody secretion by switched cells and an increase in class switch (de novo class switch), we adopted the molecular approach, DC-PCR, which allows the semiquantitative assessment of switched DNA (6). The specificity of this technique for IgG1 class switch has been demonstrated in a number of previous studies (45, 52, 58, 76) and was confirmed in this study by assessing genomic DNA from an IgG1-producing hybridoma (TSI-18; positive control) and an IgG2a-producing hybridoma (IB4; negative control) for the presence of γ1 rearrangement (hybridomas were a generous gift from A. Issekutz, Dalhousie University, Halifax, Nova Scotia, Canada). The TSI-18 hybridoma is homogenous for switched γ1. In contrast, the IB4 hybridoma does not have γ1 rearrangement but has undergone γ2a rearrangement. Using previously published primers (6, 7) the presence of the characteristic 219-bp switched γ1 DNA amplicon was amplified by DC-PCR from TSI-18 DNA. This amplicon was not present after DC-PCR of IB4 DNA (Fig. 7A). This observation confirms the specificity with which DC-PCR can assess switch DNA recombination, allowing the discrimination of the two remaining possible mechanisms of action of AWH (increased type-2 antibody secretion from individual cells versus increased de novo class switch).

To assess class switch after AWH treatment, genomic DNA was isolated from the spleen cells of mice injected with AWH or infected with worms. Control mice were injected with the vehicle alone. Amplicons indicative of γ1 switch (219 bp) were found after DC-PCR was performed on DNA from spleen cells from either AWH-treated or worm-infected animals (Fig. 7B).

FIG. 6. AWH does not expand B cells. B cells from BALB/c mice were stimulated in culture containing LPS alone (5 μg/ml) or LPS in combination with AWH (10 or 50 μg/ml). After 72 h of incubation at 37°C, the cultures were pulsed with [3H]thymidine, and the cells were assayed for incorporation 18 h later. Data shown are expressed as mean disintegrations per minute (dpm) of triplicate wells ± SD and are representative of seven experiments. ***, *P < 0.001, one-way ANOVA.

This indicates significant γ1 switch in the spleens of these animals. As expected, γ1 switch could also be seen, but to a lesser extent, after DC-PCR on DNA from control mice. The presence of an amplicon in these mice was not unexpected, since normal mice constitutively produce IgG1 in response to environmental challenge. nAChRe levels were also determined by DC-PCR to control for equal template loading and allow semiquantitation of the Sµ-, Sγ1 products. These data provide strong evidence that the increase in total IgG1 levels observed in the serum of mice infected with worms or injected with AWH is associated with increased γ1 gene recombination, resulting in increased switched γ1-specific B cells.

DISCUSSION

Infection of mammals with parasitic nematodes induces elevated levels of reaginic antibody. In humans, these are IgE and IgG4. In mice, they are IgE and IgG1. Of significant interest is that much of this reaginic antibody is not specific to worm antigens. For example, it has been estimated that less than 20% of the IgE induced in the serum of *N. brasiliensis*-infected animals is specific to worm antigen (20, 21). This increase in immunoglobulin level demonstrates that *N. brasiliensis* has the ability to modulate B-cell activities in vivo. However, the mechanisms involved in the development of this unique polyclonal IgG1 and IgE response to nematode infection are unclear. We have used a reductive approach involving the use of a nematode extract to address this question. This approach provides the opportunity to assess the modulatory effect of the worms in both in vivo and in vitro systems. The results presented here show that injection of mice with the whole-worm homogenate, AWH, induced very dramatic increases in the levels of total IgE and IgG1 in the serum. Previous attempts to induce IgE responses by the injection of rodents with extracts of adult *N. brasiliensis* worms have been unsuccessful (24, 47). The difference in the outcome of these experiments and the observation reported in our study could be attributed to the difference in the preparation of the extracts and/or the concentration of the extracts injected into the rodents and the mode of administration. Furthermore, unlike our study, earlier extracts were administered in PBS, which would be rapidly absorbed. In our study the extract was released slowly from the injected vehicle. Soluble extracts of *Brugia* (49, 50), *Toxocara* (8), and *Ascaris* (31, 65) organisms are known to induce type-2 modulation. Uchikawa and coworkers (68) have reported IgE induction in rats using excretory/secretory (ES) products of *N. brasiliensis*, but in our view, the results of experimentation with ES products must always be viewed carefully because of the significant potential for contamination during what is effectively a nonsterile culture procedure to obtain the ES. The data reported here are the first evidence that a fresh, sterile extract of *N. brasiliensis* induces dramatic IgE and IgG1 production in vivo under controlled conditions. This provides the basis for experimentation into the mechanisms by which this response is mediated.

The fact that AWH induces marked increases in total (polyclonal) IgE and IgG1 levels but does not affect the level of IgG2a confirms that the effect of AWH is restricted to these type-2-associated immunoglobulins. In addition, the response induced by AWH is not merely a response to the injection of a complex antigen. Mice injected with killed mycobacteria produced IgG2a but not IgE or IgG1. These data provide evidence that the complex parameters of infection are not required for the reaginic response to the worm and strongly suggest the presence of a nematode factor(s) which mediates this re-
response. However, these data do not elucidate the mechanisms behind this important response.

It is well established that the production of IgE and IgG1 immunoglobulin isotypes in both in vivo and in vitro systems requires the presence of the cytokines IL-4 and IL-13 (2, 3, 25, 27, 40, 41, 45, 51, 72). These cytokines are clearly associated with increases in the level of these immunoglobulins in the serum of mice infected with nematodes (15, 39, 51). A number of investigators have demonstrated mRNA and protein for these type-2 cytokines from spleen and mesenteric lymph node cells of mice infected with different nematode parasites (28, 39, 67, 69). Their importance has been conclusively demonstrated in studies with IL-4 and IL-13 single and double knockout mice (2, 3, 25, 41, 51, 72). A remaining question, however, has been the manner in which the nematode infection activates these cytokines. The multiple parameters associated with infection make it difficult to address this question. In our study, the presence of IL-4 and IL-13 was adequately demonstrated in spleen cells isolated from mice treated with the soluble protein extract (AWH). The pattern of the production of these cytokines correlated well with the immunoglobulin production pattern in the different treatment groups. Furthermore, the increase in both mRNA and protein indicates that AWH, like live worms, stimulates both transcription and translation of the cytokines. The detection of IL-13 mRNA in the spleen cell cultures from mice injected with AWH was of significant interest. It demonstrates the full spectrum of type-2 cytokines that regulate IgE and IgG1 production. This observation is of importance in light of the increasing evidence detailing the significant role of IL-13 in the regulation of type-2-associated immunoglobulin responses (2, 40, 41, 51, 72). It is important to note that, as observed with the immunoglobulin response, the increase in IL-4 production by spleen cells did not occur in response to injection of mice with killed mycobacteria, a control complex antigenic mixture, confirming the specificity of the response. Although the role of IL-4 in the regulation of IgE and IgG1 production is well established, there is some evidence to support the presence of regulatory mechanisms independent of IL-4 for type-2-associated immune responses (11, 44). This observation suggests that nematodes (or nematode factors) could modify host immune responses directly, to promote their survival.

An extract of the intestinal nematode parasite Heligmosomoides polygyrus has recently been reported to stimulate the production of IgG1 in naive spleen cell culture in vitro (56). The data in that study showed that the extract mediated its effect by stimulating T cells to produce a factor (presumably IL-4) that induced IgG1 production in the culture. Our data showing the induction of IgG1 in cultures of LPS-stimulated B cells by AWH, however, suggest a more direct effect of AWH on B-cell differentiation that is T-cell independent. The purity of the B cells used in the in vitro cultures in this study was greater than 95%, as shown by fluorescence-activated cell sorting analysis, and similar results were observed when B cells from T-cell-deficient mice were used in the in vitro cultures. No residual T-cell activity was detected in these B-cell-activated cultures (assessed with ConA). Although the level of IgE was not assessed in these B-cell-activated cultures, the level of the alter-
nate reaginic antibody, IgG1, is a sufficient marker of activation of the reaginic response, especially since class switch to \( \varepsilon \) in mice is preceded by a \( \gamma 1 \) switch (38).

The in vitro activity of AWH reported here suggests strongly that AWH contains a switch factor or that it mediates the production of a switch factor by the B cells themselves (or by the small number of accessory cells present to ensure adequate LPS stimulation of B cells). This activity of AWH does not appear to be IL-4 mediated because IL-4 was not present in the culture supernatant of LPS- and AWH-activated B cells when analyzed by ELISA (detection limit, 15 pg/ml; data not shown). Nonetheless, evidence exists that some nematodes contain cytokine-like molecules (17, 36, 48, 53). For example, a gamma interferon homologue has been reported in the intestinal nematode parasite *Trichurus muris* (17). Similarly, Pastmana and coworkers have also reported the secretion of a homologue of human macrophage migration inhibitory factor by the filarial nematode parasites *Brugia malayi*, *Wuchereria bancrofti*, and *Onchocerca volvulus* (48). These findings demonstrate that nematodes have the capacity to produce cytokine-like factors. These cytokine homologues may have the potential to modify host immune responses to promote parasite survival.

This observation that AWH may induce class switch in vitro suggests a possible mechanism for the in vivo observations made in this study. However, there are alternate mechanisms by which AWH could exert its effect to increase the levels of IgE and IgG1 in vivo. The increase in immunoglobulin levels in vivo could be as a result of (i) an expansion of IgG1 B cells or (ii) an increase in antibody production by IgG1 plasma cells.

Robinson and coworkers (54, 55) and Lee and Xie (32) demonstrated B- and T-cell mitogenic activity in nematode extracts, suggesting a capability of inducing an expansion of IgG1 B cells (the first alternative above). However, this possibility is not supported by the data presented in the study reported here. Data obtained from the in vitro proliferation assay showed that AWH does not expand B cells from naive, AWH, or worm-treated mice. In fact, AWH induced a significant inhibition of B-cell proliferation. This observation rules out expansion of existing IgG1 B cells as the mechanism by which AWH induced the production of IgG1. The observations that AWH has the ability to induce IgG1 production in LPS-stimulated B cells and is also able to inhibit the B-cell proliferative response appear contradictory. These observations are even more interesting since it has been suggested that DNA synthesis is necessary for isotype switching (42, 61, 63). However, other reports suggest that immunoglobulin class switch is not directly linked to cell proliferation (9, 34, 35, 60). This was most clearly proven in the report by Snapper and coworkers (60), which demonstrated that B cells lacking RelB, which exhibit fourfold less proliferation, undergo normal levels of immunoglobulin class switching. At this point it is reasonable to assume, from available evidence, that certain factors involved in class switch require proliferation, while others do not.

A more convincing hypothesis is that the increase in IgG1 in response to AWH treatment is the result of an increase in the percentage of B cells exhibiting class switch to IgG1, i.e., an increase in de novo class switch of naive B cells. Based on the increased production of IgG1 in LPS-stimulated B cells exposed to AWH observed in our in vitro study and the fact that it did not stimulate IgG1 production when added alone, a hypothesis that immunoglobulin class switch is the main mechanism by which AWH mediates the marked increase in total IgG1 levels in vivo is more tenable.

To evaluate whether the increase in total IgG1 by AWH in vivo was, in fact, due to wholesale class switch, we adopted the DC-PCR technique, first described by Chu and colleagues (6, 7). This technique is specifically designed to examine immunoglobulin class switch, and its sensitivity and reliability in assessing the extent of immunoglobulin heavy-chain gene recombination events have been well reported (37, 45, 52, 58, 76). With this technique, it is important to note that only DNA segments in which switch recombination has occurred will generate PCR products. In the absence of switch, the switch regions, which are several kilobases apart, exist on different fragments. These fragments lack the appropriate sequences to which the primer anneals during amplification.

Using this system, we were able to amplify \( \gamma 1 \)-switched DNA (S\( \mu \)-Sy1; 219 bp) at significant levels from DNA preparations of spleen cells from both worm- and AWH-treated mice at 3 weeks posttreatment. A control positive signal for the IgG1 class switch was provided by the use of DNA from an IgG1-producing hybridoma (TSI-18). This signal also served as a basis for comparison of the level of immunoglobulin class switch detected in the different treatment groups in this study. Because of the fact that both IgG1 and IgE are upregulated by *N. brasiliensis* infection and other Th2-activating treatments (4, 13, 29, 66) and the evidence of sequential switch from \( \mu \) to \( \gamma 1 \) and then to \( \varepsilon \) (38, 43, 66, 74), we concentrated on IgG1 as a marker for induction of immunoglobulin class switch by this nematode. Marked levels of \( \gamma 1 \) switch recombination were seen in both the worm- and AWH-treated mice. Only a very low background S\( \mu \)-Sy1 switch was detected in control animals.

This study provides strong evidence that the induction of increased IgG1 and IgE levels in worm-infected and AWH-treated mice is primarily due to stimulation of de novo class switch to these type-2-associated immunoglobulins by the nematode extract. The data are supported by the work of Yoshida and colleagues (74), who detected switch region recombination indicative of sequential \( \mu \) to \( \gamma 1 \) and then to \( \varepsilon \) switch in *N. brasiliensis*-infected animals.

We have confirmed in this study that nematode infection is not required for polyclonal reaginic antibody production; soluble nematode products also have this effect. In addition, these products do not induce this response by amplifying an existing \( \gamma 1 \) B-cell response but by inducing a \( \mu \) to \( \gamma 1 \) class switch. These data are of significant interest because of their implications for vaccine strategies in areas where nematode infection is endemic.

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