

IgE regulation by nematodes: The body fluid of *Ascaris* contains a B-cell mitogen

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*Nematode infection of human beings or laboratory animals leads to markedly increased levels of circulating IgE, most of which is not specific to worm antigens. This phenomenon is known to be interleukin-4-dependent, but little is known about the mechanism of activation of the response. In an attempt to elucidate this mechanism, we have used a reductive approach with worm products rather than infections. In a previous article we showed that injection of the body fluid of the nematode *Ascaris* yields a marked increase in circulating IgE. In this study we demonstrate that the body fluid contains a B-cell mitogen. Incubation with purified splenic B cells with 50 µg/ml body fluid yields marked proliferation of B cells, as measured by tritiated thymidine uptake. Similarly, *Ascaris* body fluid stimulates G₀ B cells to enter the cell cycle. T cells are unaffected by the mitogen, and the response is dependent on viable accessory cells. Contamination of *Ascaris* body fluid or reagents by bacterial lipopolysaccharide has been ruled out as a source of artefactual data. A model is proposed, which suggests that the B-cell mitogen in *Ascaris* body fluid stimulates polyclonal B-cell activity and that other nematode factors either stimulate the release of interleukin-4 or act in an interleukin-4-like manner to cause class switch to IgE. (J ALLERGY CLIN IMMUNOL 1995; 95:1246-54.)*

Key words: Nematode, IgE, *Ascaris*, mitogen

Nematode infections are characterized by mast cell hyperplasia, eosinophilia, and markedly elevated levels of circulating IgE. Initially, all of these responses were considered to be part of the immune effector mechanisms responsible for worm expulsion or protection against re-infection.¹ More recently, it has been suggested that these responses may be either irrelevant to host immunity² or, indeed, part of an immune evasion mechanism.³ In the latter case, it has been argued that inflammatory cytokines, such as those produced by the T_{H1} subset of T helper cells, (interferon- γ , tumor necrosis factor- α , interleukin (IL)-2)⁴ activate path-

Abbreviations used

ABF:	<i>Ascaris</i> body fluid
FCS:	Fetal calf serum
IL:	Interleukin
LPS:	Lipopolysaccharide
PBS:	Phosphate-buffered saline

ways that are deleterious to helminth survival. Cytokines secreted by the T_{H2} subset (IL-4, IL-5, IL-10), on the other hand, would activate immunologic pathways such as IgE production,⁵ which are irrelevant to or even enhance worm survival. Because these subsets are known to be cross-regulatory,⁶ direct stimulation of the T_{H2} subset by nematodes, with the corresponding IgE production, would be a means to escape T_{H1} responses. The finding that most of the increased circulating IgE resulting from nematode infection is not reactive with parasite antigens⁷ supports this hypothesis. Little is known about the manner in which nematodes stimulate IgE production, except that

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the process is dependent on the activities of IL-4,⁸⁻¹⁰ presumably produced by stimulated T_{H2} cells (although other IL-4-producing cells cannot be completely ruled out).

We have taken a reductive approach to address the early stages of the stimulation of this response. We have used soluble nematode products, rather than infections, to establish a role for direct nematode stimulation of IgE responses. In a previous article¹¹ we showed that the pseudocoelomic fluid of the nematode *Ascaris* (*Ascaris* body fluid [ABF]) is capable of prolonging IgE responses to third-party antigens and increasing total circulating IgE levels in BALB/c mice after a single subcutaneous injection. In this article we address the mechanisms by which this IgE stimulation occurs and describe the mitogenic activity of ABF on murine B cells.

METHODS

Animals

Female BALB/c mice were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). Female C3H/HEJ mice were purchased from Jackson Laboratories (Bar Harbour, Maine). All mice were housed in the Animal Care Centre at Dalhousie University and fed and watered ad libitum. All mice were 8 to 10 weeks old at the time of use, and all were rested for a week after arrival at the facility.

Preparation of ABF

The preparation of ABF was as described previously.¹² Briefly, after extensive washing in sterile phosphate-buffered saline, (PBS), adult *Ascaris suum* were severed at the posterior end, and pseudocoelomic fluid was collected in a sterile environment. The fluid was spun at 15,000 g for 20 minutes (10° C) to remove coarse particulates and then at 100,000 g for 1 hour (4° C) to remove fine particulates. The supernatant was resuspended to 10× volume with sterile PBS and concentrated back to the original volume with a Centricon 10 centrifugal concentrator (Amicon Inc., Beverly, Mass.; molecular weight cutoff, 10 kd). This latter step was performed twice to remove the nonspecific mast cell-degranulating peptide (<5 kd) and to osmotically equilibrate the ABF preparation with PBS. After this process, the ABF was filter-sterilized, aliquoted, and frozen (−20° C) until use.

Cell isolation and purification

Murine spleen cells were isolated according to standard protocols.¹³ The cells were purged of red blood cells by rapid hypotonic NaCl lysis (0.2% NaCl) to produce a population referred to as "whole spleen cells." Spleen lymphocytes were obtained by centrifugation of cells over Lympholyte (Cedarlane Laboratories, Hornby, Ontario, Canada) according to the manufacturer's protocol. Contaminating macrophages were removed by three cycles,

each of 1 hour, of plastic adherence in 25 mm² tissue culture flasks (Falcon IBD, Lincoln Park, N.J.). Cells were prepared, maintained, and used during the experiment in RPMI 1640 (Flow Laboratories, McLean, Va.) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco/BRL, Burlington, Ontario, Canada), 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid and 2 mmol/L L-glutamine (all from Sigma Chemical Company, St. Louis, Mo.). Enriched T cells were obtained by glass wool non-adherence and nylon wool nonadherence as described previously.¹⁴ T-cell populations were purged of B cells by treatment with anti-CD45R (Immuno Select, Gibco/BRL) and Low-Tox rabbit complement (1:10 concentration at 1 × 10⁷ cells/ml; Cedarlane Laboratories). B cells were obtained by glass wool nonadherence and nylon wool adherence as described previously.¹⁴ B-cell populations were purged of T cells by treatment with anti-Thy 1.2 (1:10 dilution of stock supplied by manufacturer; Cedarlane Laboratories) and complement (as described above). Purified B cells were consistently greater than 90% B cells (93% ± 3% B cells by flow cytometry). G₀ B cells were obtained by layering T-cell-depleted B cells on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient composed of 50%, 60%, 66%, and 70% ice-cold Percoll. B cells were spun (1900 g, 4° C) through this gradient for 15 minutes, and the 66%/70% interface was collected. This interface represents small resting B cells,¹³ which were consistently 1% or less in S or M phase of the cell cycle as determined by flow cytometry. Accessory cell populations were obtained by incubating peritoneal lavage cells in individual wells of 96-well plates (at 1 × 10⁵/well) for 1 hour. After this time, the wells were washed vigorously three times with RPMI to remove nonadherent cells. The lymphocytes were then added to the cells already within the wells. Trypsinization of the wells and cytocentrifuge smears of the resulting adherent populations showed that the cells were consistently greater than 90% macrophages.

Stimulation assay

Cells (4 × 10⁵) were incubated in a total volume of 250 µl RPMI in 96-well flat-bottom plates (Flow Laboratories). ABF (50 µg/ml) or lipopolysaccharide (LPS) (5 µg/ml; Sigma) was added at the time of plating, and plates were pulsed after 48, 72, or 96 hours for 12 hours with 1 µCi/well tritiated thymidine (ICN Radiochemicals, Costa Mesa, Calif.). Wells were harvested on filter strips with a Skatron harvester (Skatron Instruments, Inc., Sterling, Va.), and radioactivity was detected on a scintillation counter (Wallac 1410; Wallac, Inc., Gaithersburg, Md.). Data are expressed as disintegrations per minute and shown as means ± SD of triplicate wells. Depending on the study, *n* = 3 or 5.

Cell cycle analysis

Cells were prepared for cell cycle analysis as described in *Current Protocols in Immunology*.¹³ Briefly, cells were plated in 24-well plates (Flow Laboratories; 1 ml/well;

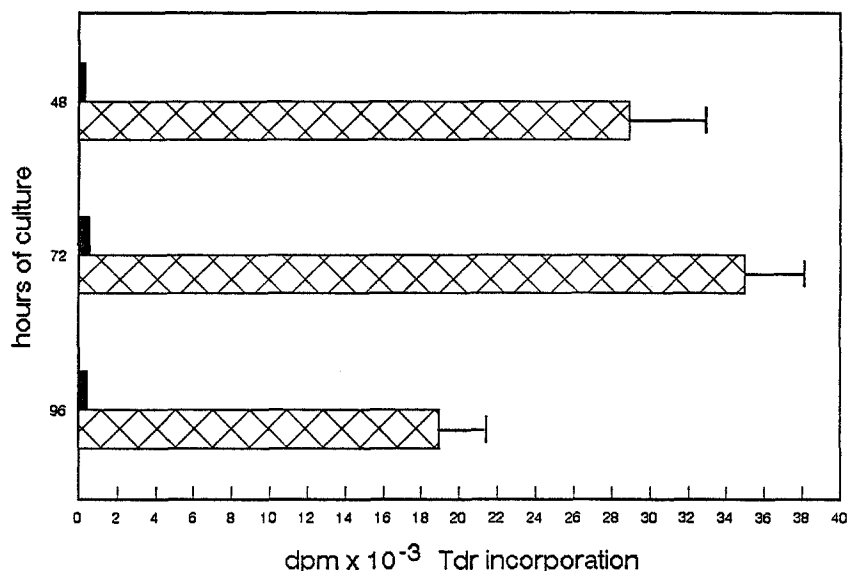


FIG. 1. Response of isolated spleen cells to ABF. Spleen cells, depleted of only red blood cells, were incubated in the presence (hatched bars) or absence (black bars) of 50 $\mu\text{g/ml}$ ABF for 48, 72, or 96 hours. Data (means $\pm$ SD) are expressed as disintegrations per minute of tritiated thymidine (Tdr) incorporation as a measure of proliferative activity.

2×10^6 cells/well). After incubation, cells were removed from wells, spun at 200 g to pellet, and fixed on resuspension with ice-cold 70% ethanol. Cells were fixed for 48 hours at 4° C. After fixation, the cells were stained with a solution of propidium iodide (50 $\mu\text{g/ml}$, Sigma) and RNase A (1000 Kunitz U/ml, Sigma) in PBS (with 0.1% glucose). Samples were allowed to incubate at room temperature for 30 minutes before flow cytometry was performed on a Becton Dickinson FACScan (Becton Dickinson and Company, Franklin Lakes, N.J.). Cell cycle data analysis was performed with the Becton Dickinson DNA Cell Cycle Analysis Program (Version C-Polynomial model). Data are shown as means $\pm$ SD of four repetitions.

RESULTS

Response of spleen lymphocytes to ABF

When used at a concentration of 50 $\mu\text{g/ml}$, ABF caused a significant proliferative response in spleen cells isolated from BALB/c mice and purged only of red blood cells (Fig. 1). The response was evident by 48 hours, increased only slightly by 72 hours, and had begun to wane by 96 hours. The timing of the response is characteristic of a mitogenic rather than an antigenic response.¹⁵ The latter might result from cross-reaction with bacterial antigens to which the mice had been previously exposed.

Because the proliferative response appeared maximal at 72 hours, this time point was chosen to test the effect of varying the concentration of ABF

on the stimulation of cell proliferation. The results, shown in Fig. 2, demonstrate that a proliferative response could not be identified with 20 $\mu\text{g/ml}$ ABF but that a significant response was evident at 100 $\mu\text{g/ml}$. Peak responsiveness appears at 200 $\mu\text{g/ml}$, with no significant increase when 300 $\mu\text{g/ml}$ is used and a decrease from the peak when 400 $\mu\text{g/ml}$ is used. Because ABF represents a heterogeneous mixture of proteins, it is not surprising that a significant amount needs to be used to initiate a response and that other factors in the mixture could interfere with cell proliferation at higher concentrations.

Because of the postulated role of nematodes in immunomodulation by affecting T-cell function, we first investigated the effect of ABF on isolated spleen T lymphocytes. After preparation of the lymphocyte fraction by centrifugation over Lympholyte and several cycles of adherence depletion, an enriched nylon wool nonadherent population of T cells was tested for proliferative activity in the presence of 50 $\mu\text{g/ml}$ ABF in the presence or absence of adherent accessory cells (Fig. 3). No proliferative activity was seen in the absence of accessory cells. Addition of accessory cells restored activity, but the activity seen in the enriched T-cell population was limited when compared with the whole lymphocyte population. These isolated T cells, however, were always found to be responsive to concanavalin A under the same conditions

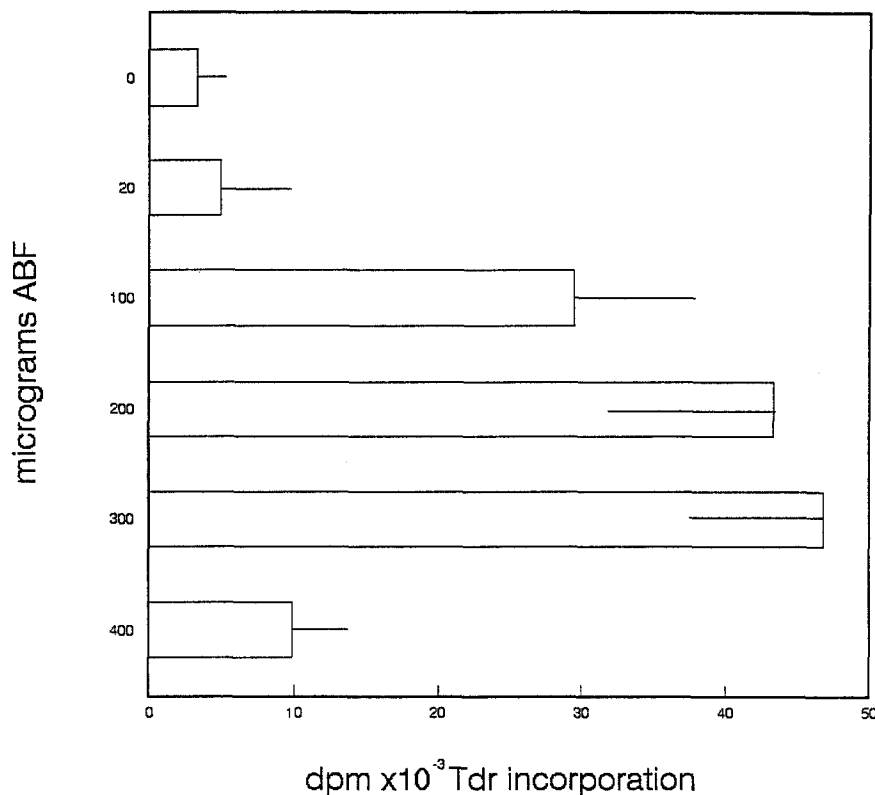


FIG. 2. Dose response of ABF. Dose response of isolated whole spleen cell population to stimulation by concentrations of ABF ranging from 0 to 400 $\mu\text{g/ml}$. *Tdr*, Tritiated thymidine.

(>100,000 dpm, in triplicate). The reduction in activity suggested that as the percentage of T cells in the population rose and the percentage of B cells fell, the proliferative activity decreased. This was confirmed by the depletion of B cells from this population with antibody and complement. Such depletion of B cells resulted in a total reduction in the proliferative response in this population when incubated with ABF (Fig. 3).

These data suggested that B cells, rather than T cells, were responding to ABF. This was confirmed by incubation of the nylon wool-adherent fraction of the purified splenic lymphocytes (Fig. 4). B cells in the absence of accessory cells showed no response to 50 $\mu\text{g/ml}$ ABF. Addition of accessory cells, however, produced a dramatic increase in proliferative response, which was consistently two to three times the response seen in mixed lymphocyte populations. Depletion of T cells by antibody treatment plus complement (percent B cells, $93\% \pm 3\%$, as confirmed by flow cytometry, $n = 5$) increased, rather than decreased, this response, confirming that B cells rather than T cells were responding to ABF.

The possibility that the adherent accessory cells,

rather than B cells, were responding in the assay can be ruled out for three reasons. First, no proliferation was seen when identical numbers of accessory cells were incubated with the purified T cells (Fig. 3). Second, there were identical numbers of accessory cells in the cell population only enriched for B cells and in the population of B cells depleted of T cells by antibody and complement, yet the response was increased in the latter cell population (Fig. 4). Third, reduction of the accessory cells by a factor of 5 had no effect on the proliferative response of the purified B cells, but reduction of the B-cell population by as little as a factor of 2 dramatically reduced the proliferative response (data not shown).

Confirmation of the B-cell mitogenic activity of ABF

The data above suggest that ABF contains a strong B-cell mitogen. Artefactual effects of FCS were controlled for by the use of heat-inactivated fresh mouse serum drawn from individual BALB/c mice at the time of sacrifice to obtain splenocytes for stimulation. In the presence of 1% heat-inactivated fresh mouse serum, the proliferative re-

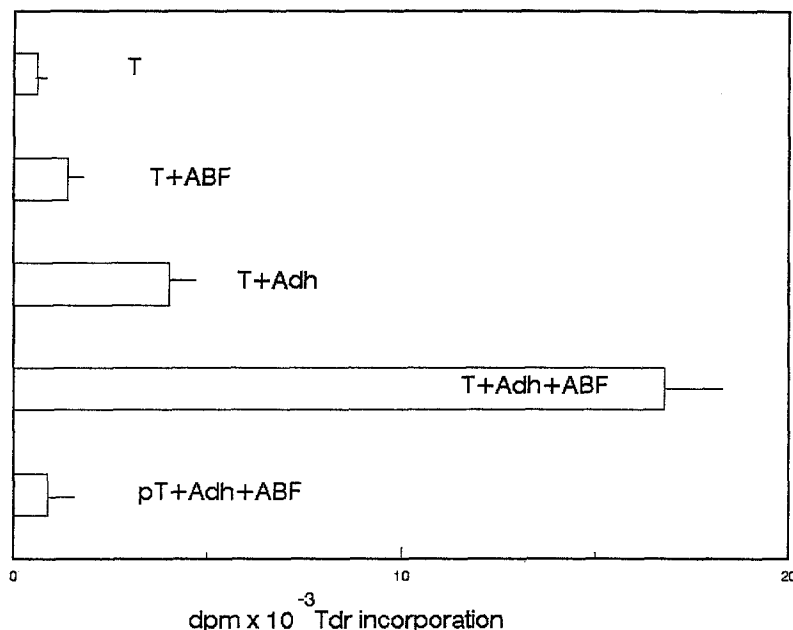


FIG. 3. Response of T cells to ABF. All cells were incubated for 48 hours. Disintegrations per minute of tritiated thymidine (*Tdr*) are expressed as means $\pm$ SD. *T* + ABF, Nylon wool-purified T cells incubated with 50 μ g/ml ABF for 48 hours; *T* + Adh, T cells as above incubated with adherent accessory cells; *T* + Adh + ABF, as T + Adh but with 50 μ g/ml ABF; *pT* + Adh + ABF, as T + Adh + ABF but T cells were treated with antibody to deplete contaminating B cells; *T*, response of T cells in medium alone.

TABLE I. Response of purified B cells from LPS-nonreactive (C3H/HEJ) mice to ABF

Treatment	Tritium incorporation (dpm)
None	2,047 $\pm$ 66
LPS	2,061 $\pm$ 590
ABF	18,214 $\pm$ 113

sponse of T cells and B cells showed a pattern identical to that seen in the cultures containing FCS, with a stimulation index of 15 for the B cells and no stimulation seen in the T-cell population. Although lower in intensity than the response in the presence of FCS, the stimulation index was nearly identical to that in the cultures in which FCS was used (15 vs 12). Bacterial lipopolysaccharide (LPS) contamination of ABF or reagents was ruled out conceptually by the observation that LPS but not ABF was capable of stimulating proliferation in the nylon wool-enriched B-cell population in the absence of additional accessory cells. This is probably due to the presence of very small numbers of accessory cells in this population, sufficient for LPS activity but not for ABF activity. Experimentally, LPS was ruled out by the use of purified B cells from C3H/HEJ mice, which are nonrespon-

sive to LPS. B cells from these mice responded well to stimulation with ABF but did not respond to LPS stimulation (Table I).

By definition, a B-cell mitogen will induce mitosis, in that it will induce B cells to leave G_0 and enter the cell cycle. To test the ability of ABF to stimulate this shift, G_0 B cells were purified from the spleens of mice and incubated with 50 μ g/ml ABF for 48 hours. The data shown in Fig. 5 represent results of four experiments and show that G_0 B cells, which are 1% or less in S or M phase of the cell cycle, can be stimulated by both LPS (12% in S + M) and ABF (9% in S + M) to enter the cell cycle. Thus ABF fulfills the basic requirement of a mitogen in that it induces mitosis.

DISCUSSION

The upregulation of IgE responses by nematodes is an area of considerable interest to researchers in diverse fields, especially in immunoparasitology and allergy. The phenomenon has been well documented¹⁶ and has been shown to be dependent on IL-4^{8,17} and possibly other T-cell cytokines,¹⁸ but there is still little information available as to how it is initiated. It has been speculated that the stimulation of antibody responses of the IgE class is somehow directed by the

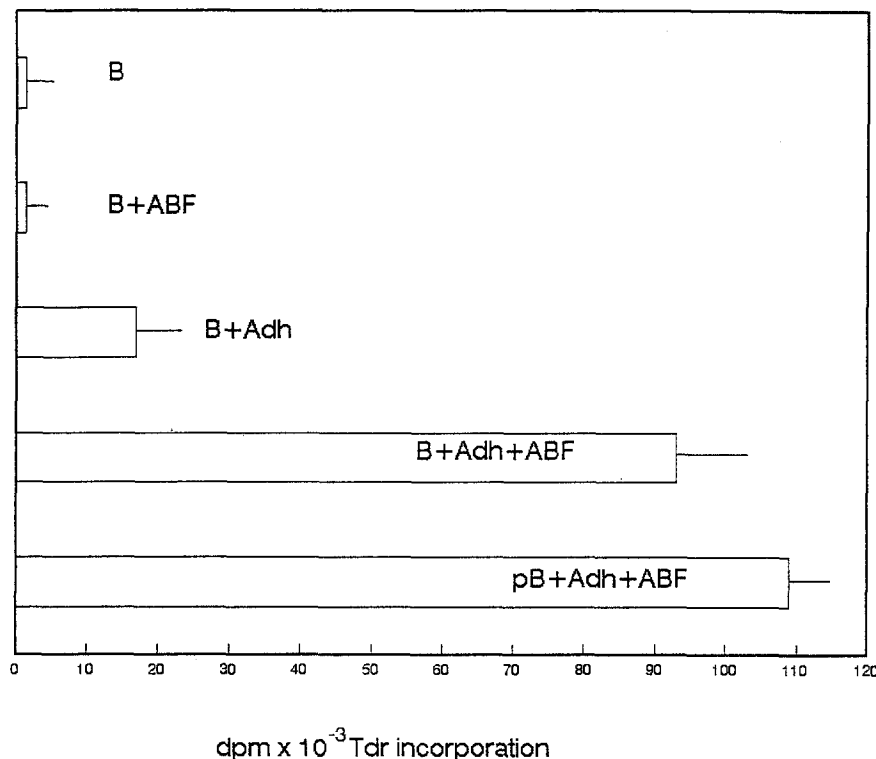


FIG. 4. Response of B cells to ABF. Disintegrations per minute of tritiated thymidine (*Tdr*) incorporation are expressed as means $\pm$ SD. *B + ABF*, Enriched B cells incubated with 50 μ g/ml ABF for 48 hours; *B + Adh*, B cells as above incubated with adherent accessory cells; *B + Adh + ABF*, as *B + Adh* but with 50 μ g/ml ABF; *pB + Adh + ABF*, as *B + Adh + ABF* but B cells were treated with antibody to deplete T cells; *B*, response of B cells in medium alone.

biophysical characteristics of the allergen molecule itself¹⁹⁻²¹ or by the way such molecules are handled during presentation.²²⁻²⁴ Although this could also be true for nematode allergens, such as those isolated from *Nippostrongylus brasiliensis*²⁵ and *Ascaris* species,^{12,26} it does not encompass the more interesting phenomenon of massive upregulation of IgE responses, which are not related to worm antigens. *Nippostrongylus* infection of BALB/c mice, for example, will increase circulating IgE from less than 1 μ g/ml up to 20 μ g/ml or greater.⁸ The worm-specific component of the IgE response, however, has been shown to be minor.⁷ Although we know that this response is IL-4-dependent,^{8,9} the mechanism by which this non-specific IgE response is stimulated remains to be elucidated.

In this study we provide in vitro evidence that ABF contains a B-cell mitogen. Our data show that the body fluid (ABF) isolated from the pseudo-coelom of adult *Ascaris* is capable of stimulating B-cell proliferation as measured both by thymidine uptake and by examination of its ability to shift cells from a G₀ state into the cell cycle. ABF has no

such effect on T cells. Our data also show that ABF stimulation of B cells requires the assistance of adherent accessory cells isolated from the peritoneal cavity, as described by Corbel and Melchers.²⁷ This requirement for accessory cell assistance has been well documented for other B-cell mitogens.²⁷⁻²⁹ LPS contamination in our preparations was ruled out for two reasons. First, there was no response to ABF in cell populations, such as the purified B cells without exogenous accessory cells, which showed responsiveness to LPS. There is a very small number of adherent cells in this population, enough to support the mitogenic activity of LPS but not of ABF. Second, C3H/HEJ mice, which are nonresponsive to LPS,³⁰ responded normally to ABF but not to LPS. The temporal characteristics of the response with a substantial increase by 48 hours and little change at 72 hours, suggest a mitogenic rather than an antigenic response. Responses of 6- to 8-week-old BALB/c mice to bacterial antigens (the most likely cross-reacting antigens) are not significant by 48 hours and become significant only after 72 hours, peaking at 120 hours.¹⁵ In addition, in these 8-week-old

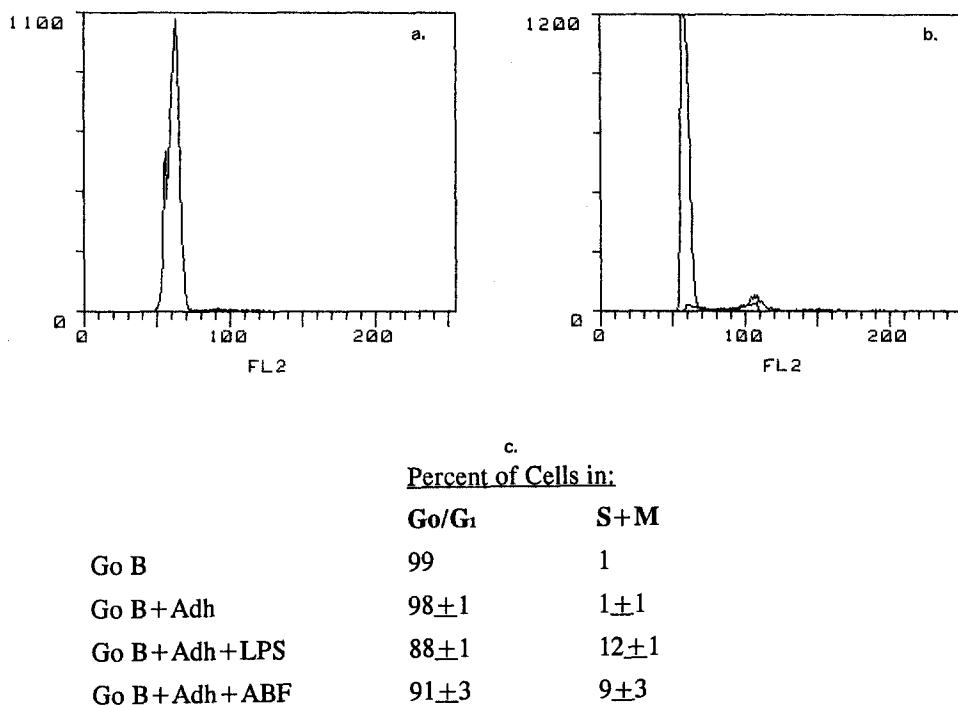


FIG. 5. Response of G₀ B cells to ABF. **a.** G₀ B cells plus adherent accessory cells incubated for 48 hours without stimulus and stained for DNA content. **b.** G₀ B cells incubated with adherent accessory cells and ABF. The second peak at 4N represents cells in M phase. **c.** Cell cycle analysis of the G₀ B cells incubated in the presence or absence of ABF.

parasite-free mice, no serum antibody response to ABF could be demonstrated by Western blotting, whereas antibodies to bacterial antigens could be easily demonstrated (data not shown).

These data provide very strong evidence that the B-cell stimulation seen in these experiments is indeed due to the presence of a polyclonal B-cell mitogen in ABF. Further evidence to support this comes from the level of shift of G₀ cells into the cell cycle. Under our conditions, only slightly fewer cells become committed to the cell cycle after stimulation with ABF than after stimulation with the polyclonal B-cell mitogen LPS. Interestingly, we tested ABA-1, the major allergen of *Ascaris* species, which represents more than 60% of the total protein content of the body fluid, and found it to have no mitogenic activity (data not shown).

The finding of B-cell mitogen activity in ABF suggests a hypothesis that the marked nonspecific IgE production, which accompanies nematode infection, is due to the elaboration of a polyclonal B-cell stimulatory molecule or molecules, much in the same way that bacterial LPS polyclonally stimulates B cells in vivo and in vitro.^{31, 32} Polyclonal stimulation of murine B cells with LPS does not, however, lead to class switch in vitro,³³ but in the presence of exogenous IL-4, most stimulated cells

will switch to IgG₁ or IgE.^{33, 34} There is now evidence that this IL-4-induced switch may be sequential, involving first a switch to IgG₁ and then to IgE.³⁵ However, the in vivo evidence in nematode-infected mice, which have elevated levels of both of these isotypes, does not strongly support this hypothesis.⁸ We have suggested a model on the basis of our findings (Fig. 6). It suggests that there is polyclonal stimulation of B cells, induced by a nematode mitogen, which is converted into a polyclonal IgE response when these stimulated B cells come under the influence of IL-4 or an IL-4-like molecule. Worm products could either stimulate this IL-4 production by T cells in an as yet undefined manner, or they may indeed elaborate IL-4-like molecules that act as IL-4 agonists; either way the end result is the induction of class switch to IgE B cells. The development of such an IL-4 stimulatory mechanism or the elaboration of an IL-4-like protein would obviously be of evolutionary advantage to the parasite if it "tricks" the immune system into an irrelevant or even beneficial pathway. There is now evidence that IL-4 plays a critical role in IgE induction in vivo but that it acts in concert with other T-cell-derived cytokines to produce this effect.^{18, 36} Thus the former, rather than the latter, activity of nematode products is

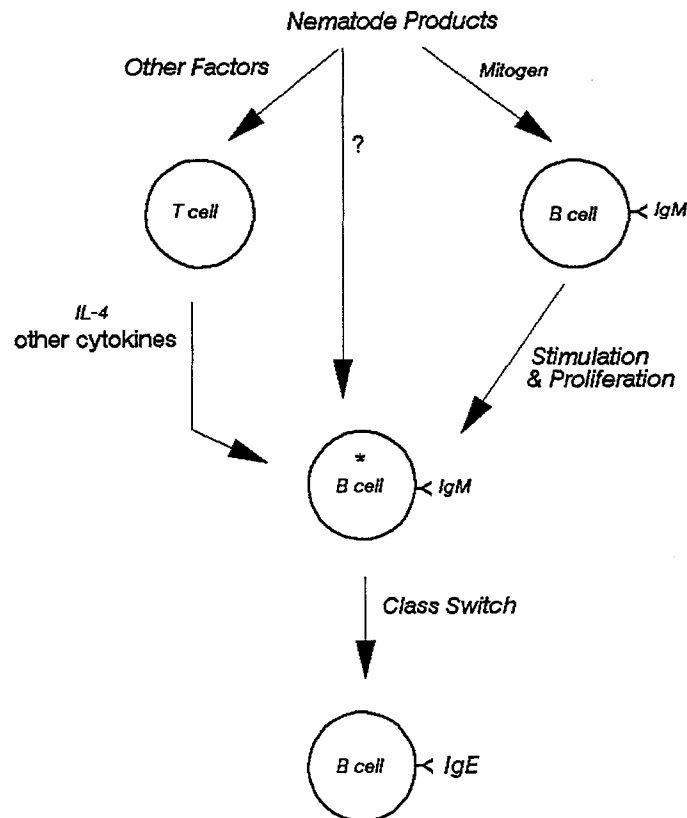


FIG. 6. Model of IgE induction by nematodes. Nematode products contain a B-cell mitogen that polyclonally activates B cells. This is accompanied by other factors, which either activate T cells to produce IL-4 and related cytokines or act directly on activated B cells. The end result of either pathway is the class switch of polyclonally activated B cells to produce IgE, most of which is not specific to worm antigens.

most likely; however, we cannot as yet rule out direct switching effects of nematode products on B cells.

The concept of the production of T_H2 -mediated effects as an escape mechanism to avoid rejection is controversial. Recently, a considerable body of work by Finkelman and coworkers and Grecis and coworkers has suggested that the immune responses mediated by IL-4 are indeed involved in parasite rejection,^{37,38} but there are still unanswered questions as to why some specific effects, such as the IgE response, seem unrelated to worm survival and why nematodes would induce such strong nonspecific responses of the type that could be damaging to them.³⁹

In a previous publication,¹¹ we demonstrated that both ABF and the major allergen purified from it, ABA-1,¹² are able to prolong ongoing IgE responses to third-party antigens. In that model system we injected BALB/c mice intraperitoneally with ovalbumin plus alum and, on the same day, injected them subcutaneously at a distant site with ABF, ABA-1, or the vehicle in which they were suspended. Both ABF and ABA-1 were able to

dramatically prolong the ongoing IgE response to ovalbumin. As noted above, ABF was also capable of markedly increasing the level of total circulating IgE in this system. The purified allergen, ABA-1, however, was not capable of inducing this marked increase. The fact that ABA-1 can prolong ongoing IgE responses but cannot markedly increase total IgE suggests that ABA-1 is not the mitogenic factor in ABF but that it may be the molecule capable of stimulating the release of IL-4 by acting in an IL-4-like manner. We are currently investigating the potential of ABA-1 to act in this manner and conducting experiments to isolate the B-cell mitogen from ABF.

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