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Extracts of *Ascaris suum* egg and adult worm share similar immunosuppressive properties

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Abstract

Adult *Ascaris suum* body extract (Asc) prepared from male and female worms (with stored eggs) down-regulates the specific immune response of DBA/2 mice to ovalbumin (OA) and preferentially stimulates a Th2 response to its own components, which is responsible for the suppression of the OA-specific Th1 response. Here, we investigated the participation of soluble extracts prepared from male or female worms or from eggs (E-Asc) in these immunological events. Extracts from either sex (1 mg/animal) or E-Asc (0.35 or 1 mg protein/animal) suppressed the delayed-type hypersensitivity (DTH) reaction (60-85%), proliferative response (50-70%), IL-2 and IFN-γ secretion (below detection threshold) and IgG1 antibody production (70-90%) of DBA/2 mice to OA. A dose of 0.1 mg E-Asc/animal did not change DTH or proliferation, but was as effective as 0.35 mg in suppressing IL-2 and IFN-γ, and OA-specific IgG1 antibodies. Lymph node cells from DBA/2 mice injected with Asc (1 mg/animal) or a high dose of E-Asc (1 mg protein/animal) secreted IL-4 upon *in vitro* stimulation with concanavalin A. As previously demonstrated for Asc, the cytokine profile obtained with the E-Asc was dose dependent and changed towards Th1 when a low dose (0.1 mg protein/animal) was used. Taken together, these results suggest that adult worms of either sex and eggs induce the same type of T cell response and share similar immunosuppressive properties.

Key words
- *Ascaris suum*
- Immunosuppression
- Adult worms
- Eggs
- Cytokines

Introduction

Helminth parasites induce a predominant Th2-type response in the host, which is associated with both protective immunity and chronic states of disease (1-3). It was observed in some infections that the development of Th2 cells is specific for certain stages of the parasite life cycle and can down-regulate the Th1 response. In murine schistosomiasis only egg antigens stimulate IL-4, IL-5 and IL-10 secretion (4,5), while mice exposed to larvae or adult worms in unisexual infections produce mainly IL-2 and IFN-γ. Furthermore, after the onset of egg deposition the Th1 response to larval stage is inhibited (6) as also is the response to non-parasite antigens like sperm whale myoglobin (7) and HIV glycoprotein (8,9). In contrast, adult worms and infective larval form (L3) from species of *Brugia* elicit a high production of IL-4, whereas IFN-γ secretion is sustained throughout infection in response to microfilariae, with IL-4 being secreted

later on (10,11). A Th2 response to *Brugia malayi*, previously induced by continuous stimulation, alters the typical Th1 response to mycobacterial antigen purified protein derivative, inducing IL-4 and IL-5 production, besides IFN-γ (12). In addition, in concurrent infections the expansion of the Th2 response changes the course of disease mediated by Th1 cytokines. For example, AKR mice become resistant to *Trichuris muris* when infected with *Schistosoma mansoni* (13) and attenuated L3 of *B. pahangi* down-regulates the murine susceptibility to cerebral malaria induced by *Plasmodium berghei* in BALB/c mice (14).

We have demonstrated that adult *Ascaris suum* body extract, prepared from male and female worms (with stored eggs), impairs the humoral and cell-mediated immune responses to the unrelated antigen ovalbumin (OA). High doses of adult worm extract preferentially induce a Th2 response to its own antigens, with high levels of IL-4 and IL-10 (15). The extract was previously fractionated by gel filtration and three peaks, named PI, PII and PIII, were obtained. PI components were responsible for the suppression of the OA-specific response, whereas PIII components were not suppressive and stimulated anti-adult worm extract IgE antibodies (16,17). *In vivo* treatment with anti-IL-4 and anti-IL-10 monoclonal antibodies in OA plus adult worm extract-immunized mice abolishes the effects of adult *A. suum* body extract on the Th1-related parameters of the immune response to OA. These results indicate the important role of these cytokines in the down-regulation of this response (18).

Although the suppressive effects of adult *A. suum* body extract have been quite well established, it is not clear which of its components is responsible for this immunosuppression. Therefore, in the present study we worked with soluble extracts prepared from *A. suum* male or female worms or eggs obtained from female uteri (M-Asc, F-Asc and E-Asc, respectively) and assessed their effects on the OA-specific response and the pattern of cytokines and antibody isotypes elicited by them. Our results indicate that adult worms and eggs possess similar immunosuppressive properties.

Material and Methods

Animals

Seven- to eight-week-old male DBA/2 mice were used for immunization. The animals were bred in the animal house facilities of the Department of Immunology, ICB/University of São Paulo, São Paulo, SP, Brazil.

Antigens and antibodies

OA (grade II and V) was obtained from Sigma (St. Louis, MO, USA). Adult worm *A. suum* extracts: live male and female worms were separated by sex and the extracts prepared as previously described (19). The extracts contained 50% protein. Egg extract: briefly, uteri obtained from female worms were homogenized in an Ultra-Turrax apparatus (Janke and Kunkel, Staufen, Germany). After centrifugation at 13,000 *g* for 1 h, the pellet was frozen in liquid nitrogen and macerated in the presence of glass powder. This mixture was diluted in borate-buffered saline, pH 8.0 (equal volumes), stirred overnight at 4ºC, and then centrifuged at 13,000 *g* for 1.5 h. The supernatant was filtered through a 22-µm membrane (Millipore Co., Bedford, MA, USA), dialyzed against distilled water, centrifuged again, aliquoted, and lyophilized. Biotinylated isotype-specific goat anti-mouse antibodies were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA). Monoclonal antibodies and recombinant cytokine standards for cytokine assays were obtained from hybridomas or kindly provided by Dr. R.L. Coffman, DNAX Research Institute, Palo Alto, CA, USA.

Immunization

Groups of 5-7 mice were injected subcutaneously (*sc*) with 100 µg OA plus 1 mg F-Asc or M-Asc emulsified in complete Freund's adjuvant (CFA) (Sigma) on both sides of the base of the tail (0.2 ml/animal). Another group received only OA in CFA. For E-Asc, 100 µg OA plus 1, 0.35 or 0.1 mg protein/animal emulsified in CFA were used. The experiments were repeated three times.

Hypersensitivity reactions

Hypersensitivity reactions were elicited 8 days after immunization by injection of 30 µl of 2% aggregated OA (1 h, 70ºC) into one of the hind footpads and the same volume of saline in the other. Nonimmunized mice were equally injected and used as test control for nonspecific swelling. Footpad swelling was monitored periodically from 3 to 24 h using a pocket thickness gauge (Mitutoyo Mfg. Co. Ltd., Tokyo, Japan) and is reported as the increase in thickness relative to the saline-injected paw. The results are reported as the arithmetic mean ± SEM for each group. Two-way analysis of variance followed by the multiple comparisons Tukey test (20) was used to compare the differently immunized groups.

Proliferation assay

Nine days after immunization, cell suspensions from inguinal and periaortic lymph nodes (LN) of 5 mice were prepared in RPMI 1640 (Sigma) supplemented with 10 mM HEPES, 50 µM M2-mercaptoethanol, 216 mg L-glutamine/l, and 5% FCS. Triplicate preparations of cells (5 x 10^5/well) were distributed into 96-well flat-bottomed microplates (Costar, Cambridge, MA, USA) and incubated with OA, F-Asc, M-Asc (100 µg/ml each), E-Asc (50 µg protein/ml) or 2.5 µg/ml concanavalin A (Con A; Sigma) in a humidified CO_2 incubator for 62 or 96 h. Cell proliferation was measured by [^{3}H]-thymidine incorporation (2 Ci/ml; DuPont, Boston, MA, USA). One µCi/well was added 18 h before cell harvesting and incorporated [^{3}H]-thymidine was determined by liquid scintillation spectrometry. The SD of mean count/min incorporated by triplicate cultures was less than 10%. The results are reported as stimulation index (mean count/min of [^{3}H]-thymidine incorporated by stimulated LN cells divided by mean count/min incorporated by unstimulated cells).

Cytokine assays

The cell suspensions prepared for the proliferation assay were added to 24-well tissue culture plates (Costar) at a final concentration of 10 or 6 x 10^6 cells/ml. The cells were stimulated with OA, F-Asc, M-Asc (500 µg/ml each), E-Asc (250 µg protein/ml) or Con A (5 µg/ml). Supernatants were harvested after 24 h (cultures of 10 x 10^6 cells) or 72 h (cultures of 6 x 10^6 cells) and assayed for cytokine content. All cytokines were measured by specific two-site sandwich ELISA using the following monoclonal antibodies: XMG 1.2 and biotinylated AN 18 for IFN-γ; JES6-IA12 and biotinylated JES6-5H4 for IL-2, and BVD-1D11 and biotinylated BVD6-24G2 for IL-4. Binding of the biotinylated antibodies was determined using the streptavidin-peroxidase conjugate (Sigma) and ABTS (2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid; Sigma) solution in 0.1 M citrate buffer plus H_2O_2. The plates were read (410 nm) in an automated ELISA reader (Dynatech MR 5000). Samples were quantified by comparison with standard curves of purified recombinant cytokines.

Detection of antibody isotypes

Plasma obtained 9 days after immunization was tested for IgG1 and IgG2a antibodies using antigen-coated 96-well plates (20

μg/ml OA, 10 μg/ml F-Asc or M-Asc, and 1 μg/ml E-Asc) and biotinylated goat anti-mouse monospecific antisera. The reactions were developed with the streptavidin-peroxidase conjugate (Sigma), *o*-phenylenediamine and H_2O_2. The results are reported as the mean absorbance of samples/group (± SEM) at various plasma dilutions. Analysis of variance followed by the multiple comparisons Tukey test was employed to compare the antibody response among groups (20).

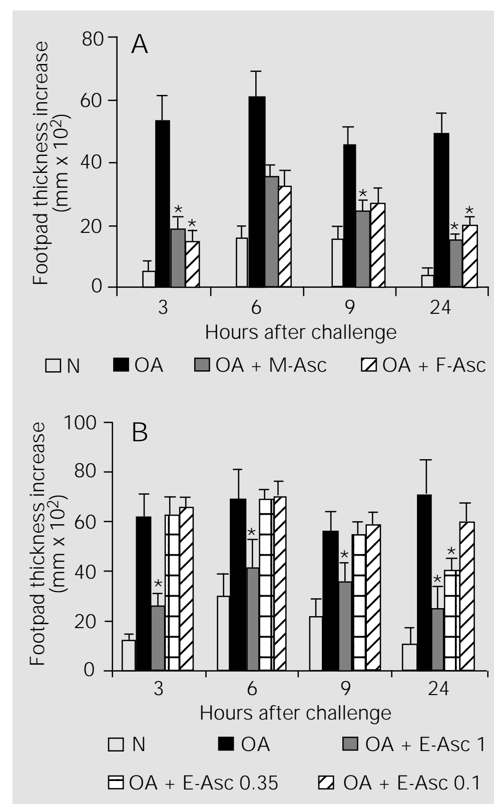

Figure 1. Hypersensitivity reactions in mice immunized with ovalbumin (OA), OA + Ascaris suum female (F-Asc) or male (M-Asc) extract (A), or egg (E-Asc) extract (B) at different protein doses (1, 0.35 and 0.1 mg). The mice were challenged 8 days later with aggregated OA in the footpad. Nonimmunized mice (N) were also challenged in the same way for nonspecific swelling. The results represent the mean ± SEM for 5-7 animals/group. *P<0.05 compared with the OA-immunized group (Tukey test).

Table 1. Proliferative response of lymph node cells from mice immunized with ovalbumin (OA) or OA plus female or male adult worm extract.

Cells	OA	F-Asc	M-Asc	Con A
OA	7.9*	1.9	2.5	3.3
OA + F-Asc	1.0	13.2	14.4	5.2
OA + M-Asc	1.1	10.3	11.8	3.6

Inguinal and periaortic lymph node cells from mice immunized 9 days before with OA, OA + F-Asc or M-Asc in complete Freund's adjuvant were stimulated with 100 μg/ml OA, 100 μg/ml F-Asc or M-Asc, or 2.5 μg/ml Con A for 96 h. F-Asc, M-Asc = female and male worm extract, respectively; Con A = concanavalin A.
*Stimulation index calculated as described in Material and Methods.

Results

Effect of worm and egg extracts on OA-specific hypersensitivity reactions

The development of hypersensitivity reactions in mice immunized with OA alone, OA plus adult worm extracts or different doses of E-Asc after challenge with aggregated OA in the footpad were compared on day 8. As shown in Figure 1A, the immediate (3 h) and delayed-type hypersensitivity (DTH) (24 h) reactions to OA were reduced in animals that received OA + F-Asc or M-Asc compared with OA-immunized mice. Regarding E-Asc (Figure 1B), the group immunized with OA + 1 mg E-Asc showed a reduced immediate reaction and a DTH response that did not differ significantly from that of the nonimmunized group. Mice immunized with OA + 0.35 mg E-Asc showed only a reduced DTH reaction to OA. The hypersensitivity reactions of mice immunized with OA + 0.1 mg E-Asc were similar to those obtained in mice injected with OA alone.

Effect of worm and egg extracts on lymphoproliferative response to OA

The proliferation of LN cells from mice immunized with OA and the different *A. suum* extracts was then measured after *in vitro* stimulation with the antigen or mitogen. The results presented in Table 1 show that simultaneous immunization with OA + F-Asc or M-Asc prevented LN cells from responding to OA *in vitro* when compared with the response of cells from mice immunized with OA alone. The proliferation of cells from the former groups when stimulated with Con A for 96 h was low. The same suppressive effect was caused by E-Asc, irrespective of the protein concentration of

the extract used for immunization (Table 2). In this experiment, the cells were stimulated for 62 h and, therefore, the proliferation of OA-primed cells was lower in response to OA and higher in response to Con A. Consequently, a dose-dependent suppression could also be observed after Con A stimulation. The stimulation index of LN cells from mice immunized with OA + F-Asc or OA + M-Asc was similar when cell cultures were restimulated *in vitro* with either F-Asc or M-Asc. In comparison, the nonspecific proliferation of cells from OA-immunized mice when *in vitro* restimulated with these extracts was negligible (Table 1). The same was true for E-Asc that stimulated just the proliferation of OA + E-Asc-primed cells (Table 2).

Effect of worm and egg extracts on cytokine and antibody production in response to OA

LN cells from the above groups were also cultured with OA or Con A for 24 or 72 h and the supernatants collected for quantification of secreted cytokines. Cytokine assays for adult worm-injected groups and for egg-injected groups were not performed at the same time. Cultures from mice immunized with OA + F-Asc, M-Asc or different doses of E-Asc did not have any detectable IL-2 or IFN-γ when restimulated *in vitro* with OA compared to those from OA-immunized mice (Table 3). The levels of these cytokines were also reduced upon stimulation with Con A, but the lowest dose (0.1 mg) of E-Asc was less suppressive. Regarding IL-4, high levels of this cytokine were only detected in cul-

Table 2. Proliferative response of lymph node cells from mice immunized with ovalbumin (OA) or OA plus Ascaris suum egg extract.

Cells	OA	E-Asc	Con A
OA	3.0*	0.0	37.0
OA + E-Asc (0.1 mg)	1.9	13.0	30.0
OA + E-Asc (0.35 mg)	1.5	11.0	21.0
OA + E-Asc (1 mg)	1.6	11.0	19.0

Inguinal and periaortic lymph node cells from mice immunized 9 days before with OA or OA + E-Asc at different protein doses were stimulated with 100 µg/ml OA, 50 µg protein/ml E-Asc or 2.5 µg/ml Con A for 62 h. E-Asc = Ascaris suum egg extract; Con A = concanavalin A.
*Stimulation index calculated as described in Material and Methods.

Table 3. Synthesis of IL-2, IFN-γ and IL-4 by lymph node cells from mice immunized with ovalbumin (OA) or OA plus female, male or egg extract.

Cells	IL-2 (ng/ml)		IFN-γ (ng/ml)		IL-4 (pg/ml)	
	OA	Con A	OA	Con A	OA	Con A
A.						
OA	0.7 ± 0.1	46.2 ± 2.7	25.7 ± 0.8	44.4 ± 2.3	<78.0	<78.0
OA + F-Asc	<0.19	15.3 ± 1.4	<1.56	27.3 ± 2.8	<78.0	597.0 ± 74.0
OA + M-Asc	<0.19	18.6 ± 1.1	<1.56	16.8 ± 0.4	<78.0	433.0 ± 28.0
B.						
OA	0.8 ± 0.1	23.8 ± 0.3	8.8 ± 1.4	37.1 ± 0.3	<31.2	<31.2
OA + E-Asc (1 mg)	<0.19	5.4 ± 0.5	<1.56	9.4 ± 0.6	<31.2	80.7 ± 14.0
OA + E-Asc (0.35 mg)	<0.19	6.8 ± 0.1	<1.56	10.3 ± 0.8	<31.2	<31.2
OA + E-Asc (0.1 mg)	<0.19	14.2 ± 0.2	<1.56	19.8 ± 0.5	<31.2	<31.2

Inguinal and periaortic lymph node cells from mice immunized 9 days before with OA, OA + F-Asc, M-Asc or E-Asc in complete Freund's adjuvant were stimulated with 500 µg/ml OA or 5 µg/ml Con A. IL-2 was quantified in supernatants harvested after 24 h and IFN-γ and IL-4 in supernatants obtained after 72 h. The results represent the mean ± SD of duplicate cultures. Non-stimulated cells produced <0.19 ng/ml of IL-2, <1.56 ng/ml of IFN-γ and <31.2 or 78.0 pg/ml of IL-4. For abbreviations, see legends to Tables 1 and 2.

tures from OA + F-Asc- or M-Asc-immunized mice stimulated with Con A (Table 3A). IL-4 was also detected in the supernatant of LN cells from mice immunized with the highest dose (1 mg) of E-Asc after Con A stimulation (Table 3B).

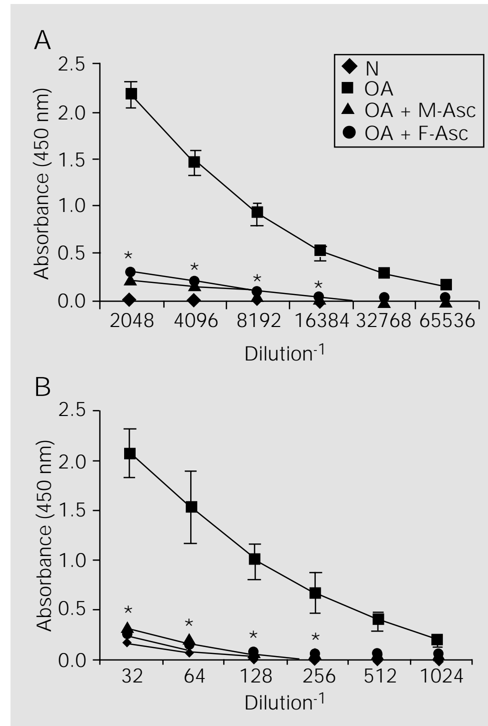

Figure 2. Ovalbumin (OA)-specific IgG1 (A) and IgG2a (B) antibodies produced by OA or OA + Ascaris suum female (F-Asc)- or male (M-Asc) extract-immunized mice. Isotype levels were titrated by ELISA in normal (N) or immune plasma obtained after 9 days of immunization using monospecific antisera. The results represent the mean ± SEM absorbance for 5-7 animals. *P<0.01 compared with the OA-immunized group (Tukey test).

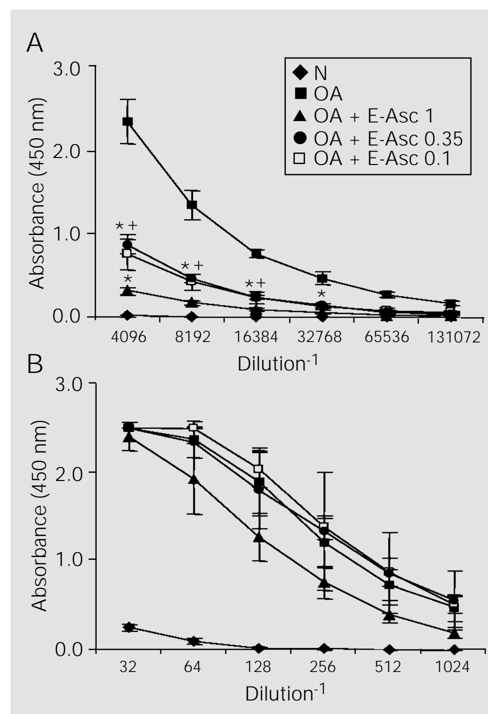

Figure 3. Ovalbumin (OA)-specific IgG1 (A) and IgG2a (B) antibodies produced by OA or OA + Ascaris suum egg (E-Asc) extract-immunized mice (1, 0.35 and 0.1 mg). Isotype levels were titrated by ELISA in normal (N) or immune plasma obtained after 9 days of immunization using monospecific antisera. The results represent the mean ± SEM absorbance for 5-7 animals. *P<0.05 compared with the OA-immunized group. +P<0.05 compared with the OA + E-Asc (1 mg)-immunized group (Tukey test).

Antibody levels were also assessed in plasma obtained from all groups on day 9. IgG1 and IgG2a antibody production was strongly inhibited in mice immunized with OA plus adult worm extracts (Figure 2). The egg extract also had a suppressive effect on anti-OA IgG1 antibody production, with the highest dose being more effective (Figure 3A). IgG2a levels were not significantly different among the immunized groups, although the group injected with 1 mg of E-Asc repeatedly responded less than the other two (Figure 3B).

Profile of cytokines and antibody isotypes in response to adult worm and egg extracts

Cytokines were also measured in supernatants of LN cell cultures from mice injected with the different adult worm extract preparations after *in vitro* restimulation with the respective extract. Table 4 shows that F-Asc induced more IFN-γ and IL-4 than M-Asc. In addition, more IL-2 and IFN-γ, and less IL-4 were produced as the immunizing dose of E-Asc was reduced.

To analyze the isotypes elicited by each extract the ELISA plates were coated with the respective antigens. F-Asc and M-Asc induced the same amounts of antigen-specific IgG1 and IgG2a antibodies (Figure 4). A dose-dependent production of IgG1 antibodies was observed in mice immunized with E-Asc (Figure 5A). IgG2a antibodies were mostly induced by the highest doses of E-Asc (Figure 5B).

Discussion

Unisexual infections have been an important experimental model to study the type of immune response induced by helminths (5,10). In the present study, immunization with extracts of *A. suum* prepared from adult worms of either sex or from eggs allowed us

to show that male and female body components and egg contents elicit the same type of T cell response and possess similar suppressive properties on the immune response to an unrelated antigen.

The male and female extracts exerted an identical suppressive effect on OA-specific immediate and DTH reactions, proliferative response, IL-2 and IFN-γ secretion, and IgG1 and IgG2a antibody production. Extracts of females prepared from worms without a uterus had the same effect, indicating the presence of suppressive components in the female body in the absence of eggs (21).

Mice injected with the egg extract displayed a dose-dependent inhibition of hypersensitivity reactions and IgG1 antibody response to OA and of LN cell proliferation and of cytokine secretion after *in vitro* stimulation with Con A. In addition, the LN cells from the groups of mice injected with different doses of E-Asc did not secrete detectable IL-2 or IFN-γ after *in vitro* restimulation with OA compared to the group immunized with OA alone, indicating a dramatic effect on these Th1-type cytokines. When these LN cells were restimulated with E-Asc, the profile of cytokines detected in cell cultures was similar to that obtained after immunization with *A. suum* extract prepared from adult worms (15). The highest dose of E-Asc, which was more suppressive, induced more IL-4 and less IL-2 and IFN-γ, whereas the opposite was true for the lowest dose that was less suppressive.

These results indicate that the suppressive mechanisms induced by egg contents seem to be similar to those induced by adult worm components, being mediated by the same cytokines (18). However, E-Asc seems to contain less suppressive components than F-Asc or M-Asc, since the latter had a more drastic effect when 500 µg of protein was used compared with 1 mg of the former.

In *S. mansoni* infection, the Th1 response present during the larval stage is inhibited by the Th2 response induced after egg deposition (6). The Th2 response to egg antigens, however, develops through an early and transient Th0 stage during which IL-2, IFN-γ, IL-4, IL-5 and IL-10 are produced (22,23). Fractionation of egg antigens revealed also the presence of IFN-γ inducers among the protein fractions (24).

It is interesting to note that a monoclonal antibody prepared against a high molecular weight suppressive component from adult worm body extract was able to react with

Table 4. Cytokine profile in response to adult worm or egg extract.

Cells	IL-2 (ng/ml)	IFN-γ (ng/ml)	IL-4 (pg/ml)
OA + F-Asc	1.8 ± 0.2	13.4 ± 0.8	170.0 ± 10.0
OA + M-Asc	2.4 ± 0.3	6.4 ± 0.2	96.0 ± 7.0
OA + E-Asc (1 mg)	1.8 ± 0.1	4.2 ± 0.5	104.8 ± 6.7
OA + E-Asc (0.35 mg)	2.1 ± 0.1	7.8 ± 0.2	83.0 ± 6.9
OA + E-Asc (0.1 mg)	4.5 ± 0.1	14.9 ± 0.7	55.7 ± 6.5

Inguinal and periaortic lymph node cells from mice immunized 9 days before with OA + F-Asc, M-Asc or E-Asc in complete Freund's adjuvant were stimulated with 500 µg/ml F-Asc or M-Asc or 250 µg protein/ml E-Asc. IL-2 was quantified in supernatants harvested after 24 h and IFN-γ and IL-4 in supernatants obtained after 72 h. The results represent the mean ± SD of duplicate cultures. Non-stimulated cells produced <0.19 ng/ml of IL-2, <1.56 ng/ml of IFN-γ and <31.2 pg/ml of IL-4. For abbreviations, see legends to Tables 1 and 2.

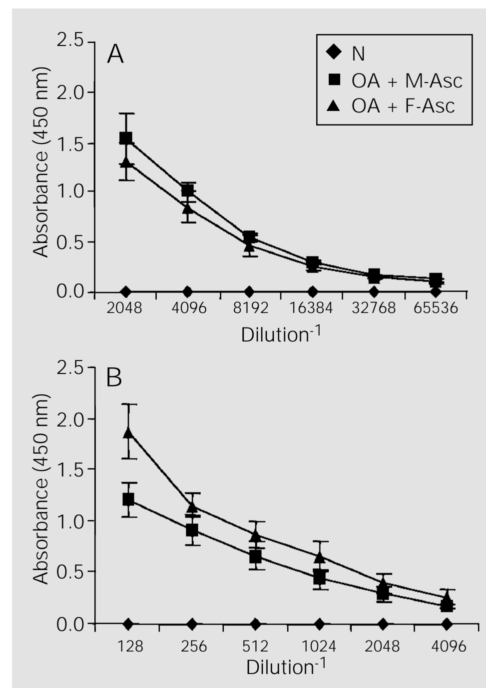

Figure 4. Female or male *Ascaris suum* (Asc)-specific IgG1 (A) and IgG2a (B) antibodies produced by mice immunized with ovalbumin (OA) + female (F-Asc) or male (M-Asc) extract. Isotype levels in normal (N) or immune plasma were titrated by ELISA using monospecific antisera. The results represent the mean ± SEM absorbance for 5-7 animals.

embryonated eggs, L1, L2 and L3/4 larvae, indicating that the suppressive protein was present in different stages of *A. suum* life-cycle (Enobe CS, Oshiro TM and Macedo-Soares MF, unpublished data). Therefore, this component could be responsible for the immunosuppression obtained with the male, female and egg extract. Indeed, the presence of similar high molecular weight components in E-Asc was confirmed when this extract was fractionated on a Sephacryl S300 chromatography column and the suppressive effect of the first peak was demonstrated on the IgE antibody response to OA (21). Using the same experimental protocol, additional suppressive components were also identified in the third peak which contains low molecular mass proteins (29 kDa), in contrast to the results obtained with PIII isolated from the adult body extract (16,17). The presence of egg-specific antigens, not found in adult worms, had been already reported in *A. suum* by Justus and Ivey (25). We also noticed by SDS-PAGE that proteins with molecular masses between 27.2 and 19 kDa were present only in E-Asc (21).

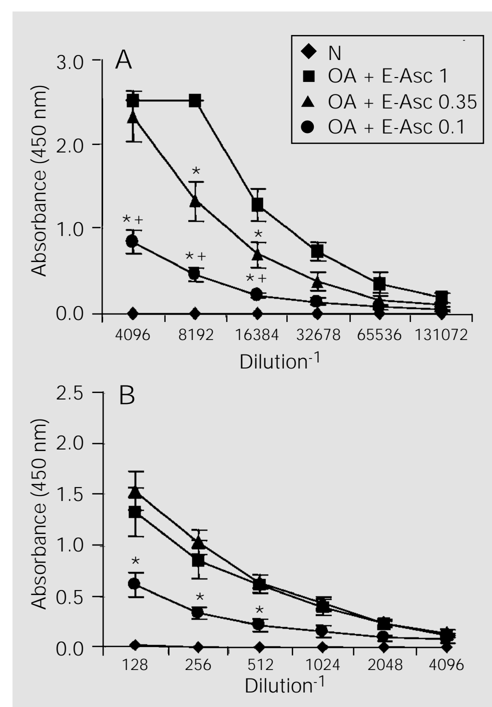

Figure 5. Ascaris suum egg (E-Asc)-specific IgG1 (A) and IgG2a (B) antibodies produced by mice immunized with different doses of E-Asc (1, 0.35 and 0.1 mg). Isotype levels in normal (N) or immune plasma were titrated by ELISA using monospecific antisera. The results represent the mean ± SEM absorbance for 5-7 animals. *P<0.05 compared with OA + E-Asc (1 mg)-immunized group (Tukey test). +P<0.05 compared with OA + E-Asc (0.35 mg)-immunized group (Tukey test).

The different antigenic profiles of E-Asc might also explain why the production of egg-specific IgG2a antibodies and the levels of IFN-γ obtained in response to different doses of this extract were not correlated. It is well known that IFN-γ can enhance antigen-specific as well as polyclonal IgG2a responses, but is not required for the induction of this isotype in all murine antibody responses (26). B cells can also switch to IgG2a producers when stimulated by Th2 clones, although much less (about 20-fold) than when activated by Th1 clones (27). Thus, some egg-specific antigens could be stimulating an IgG2a antibody response in a dose-dependent, but IFN-γ-independent, way.

In conclusion, *A. suum* egg content shares immunosuppressive properties with adult worm components, but also contains other constituents with biological activities that deserve further characterization.

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