

Effectiveness of intranasal vaccination against *Angiostrongylus costaricensis* using a serine/threonine phosphatase 2 A synthetic peptide and recombinant antigens

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ARTICLE INFO

Article history:

Received 17 November 2009

Received in revised form 20 May 2010

Accepted 28 May 2010

Available online 15 June 2010

Keywords:

Intranasal immunization

Angiostrongylus costaricensis

Serine/threonine phosphatase 2

Vaccination

ABSTRACT

Intranasal immunization was assayed in C57BL/6 mice against *Angiostrongylus costaricensis* using a synthetic and a recombinant peptide belonging to the catalytic region of the serine/threonine phosphatase 2 A (PP2A) of the parasite. Immunization was carried out with the synthetic peptide (SP) polymerized either with itself or with the β fraction of the cholera toxin (CTB) and then enclosed in nanocapsules of phosphatidyl choline, cholesterol and Quil A (ISCOM). Another group of mice was immunized with recombinant peptide. Immunization consisted of two intranasal inoculations at two-week intervals, and the challenge with L3 larvae was made one month after the last vaccination.

The effectiveness of immunization was evaluated 30 days after infection by analysis of the number of parasites in the arteries of the immunized mice, as well as by measuring spleen sizes in the experimental groups. The response induced was determined by identifying the isotypes of IgG as well as the IgE and IgA specific antigen response. The interleukins produced by the splenocyte culture of the different groups were assessed after exposing them to the peptide used in the immunization.

From our results, 60%, 80%, and 100% protection against the *A. costaricensis* challenge was achieved in mice immunized with polymerized synthetic peptide in ISCOM, synthetic peptide polymerized with the CTB in ISCOM and inclusion bodies respectively. Splenomegaly was found to be less evident in the immunized mice than in the controls. A significant increase in IFN γ and IL-17 levels was observed in the group with 100% protection.

The results showed that vaccination through the nasal mucosa may constitute a useful method of immunization and result in a protective immune response against *A. costaricensis*.

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1. Introduction

Angiostrongylus costaricensis is a nematode parasite carried by rodents such as *Sigmodon hispidus*, [1] *Oryzomys* spp. [2] and others from the Americas. The adult parasite is found in the mesenteric arteries of the definitive host, in which the female lays eggs and from whence they are carried to the intestinal mucosa, where they hatch into stage L1 larvae, penetrate the mucosa in the intestine and are eliminated with the feces. To continue their life cycle L1 larvae must be ingested by mollusks, where they develop as stage L3 infective larvae. Thereafter they escape via mucus secretions of the invertebrate host, thus infesting both soil and plants. Definitive hosts are infected with the stage L3 larvae either by ingesting contaminated secretions or the infected mollusks themselves [3,4].

Humans act as accidental hosts, giving rise to a serious disease known as abdominal angiostrongylosis [1]. The disease is characterized by a granulomatous inflammatory reaction of the intestinal wall, acute abdomen and mesenteric ischemia, where it is accompanied by a considerable infiltration of eosinophils [5,6]. The disease is fatal in 7.4% of cases if the affected parts of the intestinal zone are not surgically removed [7,8]. Cases of this disease have been described in the Americas from the southern USA to northern Argentina [9,10].

The absence of any effective pharmacological treatment against the adult parasite and possible side effects, such as an increase in mobility that induces the migration of arterial adult worms, make it advisable to avoid the administration of antihelminthic drugs in cases of abdominal angiostrongylosis [11], which currently implies resorting to surgical treatment.

Previous immunological studies have shown the immunological changes caused by *A. costaricensis* in the infected animals. Thus, Pinto et al. [12] concluded that the parasite does not affect IL-6 lev-

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els whilst IL-1 β and the number of bronchoalveolar lavage cells is lower in infected rats than in healthy ones. The same author described in a later work (85) how extracts from the adult parasite contained an immunomodulatory factor capable of diminishing the eosinophilic response in mice sensitized to ovalbumin, just as has also been described for a similar extract from *Ascaris* (64). The appearance of intestinal granulomas in the host occurs after sexual maturity of the parasite in the arteries and the subsequent liberation of its eggs. The granulomas are formed by an infiltration of eosinophils, giant cells and plasma cells, which surround the parasite eggs, accompanied by a significant rise in IgE levels. At the same time IFN γ levels also increase whilst IL-4, a Th2 response marker, remains low (52). Susceptibility and the different pathologies arising from infection depend very much upon genetic factors in the host and the type of immune response [13–16].

Discovering a vaccine against helminth parasites, which affect domestic animals as well as humans, thus adding economic damage to human suffering, constitutes a major challenge for researchers. Some recently published reviews on this subject describe the antigens used, their effectiveness and their manner of administration [17–19].

The efficacy of a vaccine is determined not only by the type of antigen used but also by the adjuvant and the way of administration. Papers describing different adjuvants have also been published recently [20], among which are ISCOMs, nanoparticles formed by saponin (Quil A), phospholipids, and cholesterol, which induce a Th1 response [21–23]. Antigens are enclosed in nanocapsules or can be inoculated together with empty ISCOMs (ISCOMATRIXs) [24,25]. Their particulate nature or hydrophobic properties allow the antigens to be taken up efficiently by the antigen-presenting cells (APC) [26,27]. Other adjuvants include those of bacterial origin, such as cholera toxin and especially the β subunit (CTB) [28], or even LPS from bacterial walls or bacterial ghosts [29]. CTB as adjuvant induces a Th2 response [30].

The immunization route is another key factor to be taken into account in the development of vaccines [31]. Most vaccines are administered intramuscularly or subcutaneously, although the administration of vaccines through the mucosa offers a number of major advantages, such as easy administration, the reduction of adverse effects and the possibility of repeating the dosage of the vaccine. Furthermore, the fact that many pathogenic agents invade the host via mucous barriers renders the activation of immunity in the mucosa an attractive subject for further study (49). Mucosal immunization techniques have been assayed against intestinal protozoan parasites such as *Giardia* and *Entamoeba*, and hematic and tissular protozoans such as *Plasmodium*, *Leishmania*, *Toxoplasma* and *Trypanosoma cruzi* [32–39]. It has also been assayed in vaccines against intestinal helminths and the larvae of tissue-dwelling helminths [40–47].

Serine/threonine protein phosphatase (PP2A) is an enzyme involved in many cell processes, especially in embryogenesis and differentiation [48]. The catalytic subunits of PP2Ac and PP2Aa constitute the constant structural subunits of the enzyme [49,50]. These characteristics make the catalytic region of PP2Ac a good candidate for an antigen to be used in vaccines, especially against those parasites that must undergo morphogenic processes in the definitive host before reaching maturity.

We describe here the results of intranasal immunization against *A. costaricensis* with a synthetic peptide corresponding to the catalytic subunit of pph 1 serine/threonine phosphatase of the parasite, either polymerized and/or linked to CTB, with these antigen/adjuvant complexes contained in ISCOMs. We also describe the results of intranasal immunization with inclusion bodies of a recombinant protein of the gene (EMBL AM041130) of *A. costaricensis*. Intranasal immunization with the antigens and adjuvants studied induced an immune response capable of drastically reduc-

ing the number of adult nematodes after immunized mice were infected with L3 larvae.

2. Materials and methods

2.1. Parasite

The life cycle of *A. costaricensis* was maintained in *Biomphalaria glabrata* as intermediate host and in *Meriones unguiculatus* (Mongolian gerbils) as definitive host. Snails were orally infected with stage L1 larvae obtained from the feces of gerbils infected during the previous cycle. Stage L3 larvae were obtained 20 days after infection by digesting the snails with 1% pepsin and 1% hydrochloric acid (pH 1.5). Gerbils were infected with these L3 larvae, and adult worms were recovered 30 days post-infection from the circulatory blood vessels following the methods described elsewhere [15,51].

The strain used in these experiments was kindly provided by Prof P. Morera of the Laboratory of the Pathology Service, San Juan de Dios Hospital, San José, Costa Rica.

2.2. Cloning of the antigens and immunoscreening

A cDNA library of *A. costaricensis* adult worms was constructed in a λ Triplex2 vector with the A SMART cDNA Library Construction kit (Clontech, Mountain, USA) using adult *A. costaricensis* total RNA extracted with a Perfect RNA Mini kit (Eppendorf, Hamburg, Germany). The library was screened with a pool of 15 sera from patients from Costa Rica diagnosed at the Faculty of Medicine of the University of Costa Rica as suffering from abdominal angiostrongylosis. The pool of sera had previously been absorbed by a lysate of *Escherichia coli* and wild λ phages and also absorbed by the glutathione transferase (GST) of the expression vector to increase specificity. Several clones were obtained from the immunoscreening of 10^5 independent clones. Plasmid DNA was extracted from the resulting colonies with the NucleoSpin Plasmid QuickPure kit (Macherey-Nagel, Düren, Germany), and both strands of the inserts were sequenced in an ABI PrismTM BIGDYE (Applied Biosystem, Foster, USA) using the forward primer (5'-TCCGAGATCTGGACGAGC-3') and reverse primer (5'-CCCTATAGTGAGTCGTATTA-3').

Sequence CT2-2, corresponding to the catalytic region of gene pph-1 serine/threonine protein phosphatase (315 bp), was deposited at EMBL as gene pph-1; serine/threonine protein phosphatase (EMBL: AM041130).

2.3. Synthetic peptide and antiserum production

To design the synthetic peptide to be used in the immunizations we analyzed the sequence of amino acids of the recombinant protein expressed by CT2-2 serine/threonine phosphatase pph-1 protein with the "antigenic" program of the EMBOSS software package (97), available at <http://emboss.sourceforge.net/>. According to the output of the antigenic program most of the antigenic portion corresponded to the amino-acid sequence ANFIIFRPVV, between positions 64 and 73. The synthetic peptide (SP) was produced by the Proteomic Unit of the Severo Ochoa Molecular Biology Center at the Autonomous University of Madrid (Spain).

The antiserum against the SP was prepared by intraperitoneal injection of Balb/c mice with 50 μ g of peptide in complete Freund's adjuvant, followed by injections two and four weeks later with incomplete Freund's adjuvant. The mice were bled two weeks after the final injection. The sera were checked by indirect ELISA in multi-well plates coated with 10 μ g/well of the SP in 0.1 M bicarbonate coating buffer (pH 8.6). Sera with titers higher than 1:800 were pooled and stored at -80°C until used.

2.4. SDS/PAGE and Western blotting

Immunoblots were made to determine the specificity of the antibodies in the immune serum. Protein samples from *A. costaricensis* adult homogenates (male and female) were subject to electrophoresis in 12.5% SDS/PAGE (GE Healthcare, Buckinghamshire, UK) using PhastSystem (Pharmacia LKB, Uppsala, Sweden). After electrophoresis the separated proteins were transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) using PhastTransfer (GE Healthcare), following the standard procedures of Towbin et al. [52]. After blocking the non-specific free sites of the membrane with 2% non-fat dried milk and 0.2% Tween 20 in 0.125 M PBS (PBS-T), the membrane strips were incubated for 30 min at 37 °C with the immune serum at dilutions of 1:50. A non-immunized mouse serum was used as negative control. The membranes were washed five times with PBS-T. The strips were incubated for 30 min at 37 °C with Fc specific anti-IgG mouse serum labeled with peroxidase (Sigma, St Louis, USA) diluted to 1:1800 in PBS-T. After the final incubation the strips were washed and incubated for 15–30 min at 37 °C with 0.5 mg/ml of the peroxidase substrate 3,3-diaminobenzidine diluted in 0.1 M Tris/HCl (pH 7.4) containing 1:5000 H₂O₂. The membranes were finally washed with distilled water.

2.5. Purification of the inclusion bodies and bacterial walls

After verification of the correct cloning of the fragment (CT2-2) and the reading frame, the positive plasmids were used to transform *E. coli* XL1-Blue MRF' once the insertion was subcloned in the pGex2TK vector (Amersham). Purification was carried out from the isolated colonies in agar 2xYT/ampicillin (100 µg/ml), incubated at 37 °C for 12–15 h, and IPTG was added to the culture to a final concentration of 0.5 mM to induce the expression of the recombinant protein, which was incubated for 3 h and then centrifuged at 4000 × g for 10 min. The pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0; 500 mM NaCl; 10 mM EDTA; 5 mM β-mercaptoethanol; 0.35 mg/ml lysozyme) and incubated at room temperature for 30 min before being sonicated and centrifuged at 12,000 × g for 30 min [53]. Both the supernatant and the pellet were analyzed by immunoblotting using an immune serum obtained against the SP as described above at a dilution of 1:100. In a similar way, non-transformed bacteria were used to obtain bacterial walls.

Protein concentration was determined by Bradford's method [54].

2.6. Polymerization of the peptide and formation of chimeras with the β subunit of the cholera toxin (CTB)

The SP was polymerized both with itself and with the CTB following the technique described by Chan and Ho [55]. To polymerize the SP, 2 mg of SP and 2 mg of CTB (Sigma C 9903) were mixed and dissolved in 20 ml of 50 mM sodium phosphate buffer (pH 7.5), to which 40 µl of a 25% aqueous solution of glutaraldehyde was added, and incubated under continuous stirring at room temperature for 6 h. The linking was stopped by the addition of 1 ml of a 1 M solution of glycine. The mixture was kept at room temperature for 2 h and dialyzed overnight at 4 °C against 4 l of a solution of 0.1 M ammonium acetate before being lyophilized for storage. The result of polymerization was confirmed by electrophoresis in 20% SDS-PAGE.

2.7. ISCOM preparation

The antigens in ISCOMs were prepared and encapsulated following the method of Myschik et al. [56]: 30 mg of cholesterol, 30 mg of phosphatidylcholine and 400 mg of decanoyl-*n*-methyl-

glucamide (SIGMA) were mixed in 2 ml of distilled water and stirred at room temperature until completely dissolved. 0.8 ml of this solution was mixed with 0.6 ml of a 100 mg/ml solution of Quil A and distilled water was added to a final volume of 12 ml. This solution was mixed until it became transparent. The polymerized peptides were added to this solution at a proportion of 1:1 [57]. The remaining mixture of ISCOMs was used to make the ISCOM-MATRIX used as control. The preparations were dialyzed for 48 h against water with four changes at 12 h intervals. After dialysis the ISCOM suspension was centrifuged at 50,000 × g for 18 h. The correct formation of the ISCOMs was confirmed by examining aliquots of the samples stained with 2% uranyl acetate, using a C. ZEISS EM 902 transmission electron microscope.

2.8. Vaccination against infection by *Angiostrongylus costaricensis*

The immunization and bleeding scheme were as follows: 75 female C57BL/6 mice, approximately four weeks old, were divided into five different groups of 15 mice per group. All the groups were anesthetized with 50 mg/kg of thiobarbital (B. Braun) and vaccinated intranasally. Group 1 was immunized with the SP encapsulated in ISCOM (5 µg/mouse); Group 2 was immunized with the SP and CTB encapsulated in ISCOM (5 µg/mouse); Group 3 was immunized with the inclusion bodies of the recombinant protein and the bacterial walls (5 µg/mouse); Group 4 was administered with the same quantity of non-transformed bacterial walls, CTB and ISCOM-MATRIXs as that used in the previous groups; and Group 5 was administered with PBS. The different groups were immunized intranasally on days 1 and 15 of the experiment. On day 45 all the mice were challenged by intragastric inoculation with a total of three *A. costaricensis* L3 larvae from infected *Biomphalaria*, in order to induce a mild form of the infection similar to natural infections and to avoid their premature death.

During the experiment the mice were bled submandibularly every 15 days to evaluate the immune response of each immunized group.

Thirty days after the challenge the mice were killed and any vascular lesions caused by the infection were noted. Adult worms were located and collected from the arteries of the mice. The spleen of each mouse was measured longitudinally with calipers to evaluate the degree of splenomegaly induced by the parasites. The "splenomegaly index" was calculated according to the ratio between the mean size of the problem spleens vs that of the controls. To compare the total levels of immunoglobulins with the non-treated mice, a sixth group of five mice (non-vaccinated and uninfected) were bled to obtain their sera and spleens. The serum samples obtained periodically and at final bleeding when the mice were killed were stored at –20 °C until used. The experiments were repeated three times.

All the experiments undertaken with mice were previously approved by the Ethics Committee of the University of Granada.

2.9. Antibody response

Quantification of the total serum IgG immunoglobulin, total IgE and specific IgA against the synthetic peptide as well as against the homogenate of the parasite, together with the determination of IgG isotypes throughout the experiment, was all done by ELISA. The wells of MaxiSorp™ 96-well microplates (Nalge Nunc Intl., Rochester, NY, USA) were coated with 100 µl of the appropriate antigen. A homogenate from adult male and female *A. costaricensis* or the SP were used as antigens. The antigens were diluted in a 0.1 M NaHCO₃ solution (pH 8.6) to give a concentration of 10 µg/well. Total IgG was determined by ELISA using the Immuno-Tek Mouse IgG ELISA kit (ZeptoMetrix Corporation, NY, USA).

Table 1

The nucleotide and amino-acid sequence of the CT2-2 clone corresponding to the PP2A catalytic region of *A. costaricensis*. The synthetic peptide used in the various assays, corresponding to the antigenic region of the protein, is highlighted in yellow and underlined. The stop codon of this sequence (*) is highlighted in blue. (For interpretation of the references to color in this table legend, the reader is referred to the web version of the article.)

1	gtagttgatgagtctgcacaaatcataacattgatctgatcctcagagcacatcagata	60
1	V V D E F C T N H N I D L I L R A H Q I	20
61	accgcagagatgggtttacggaggttatcgaatatcgtgtggacgcttggtgacgatt	120
21	T A E M V Y G G Y R I F A G G R L V T I	40
121	ttttcgccacctaactaccagaatatgatgaacgatggttgtgtgatgagaattaagaga	180
41	F S A P N Y Q N M M N D G C V M R I K R	60
181	gacttgacggcaaaacttcataatttttcgaccggtcgtcaggcgacattacagcaccaa	240
61	D L T <u>A N F I I F R P V V</u> R R H *	76
241	cagccaagacgccacgtttcacgaaattttcgagttgtcggacttgaatagattaataa	300
301	agggataaattttctc	315

The isotypes of IgG and specific IgA were determined using a Monoclonal Antibody Isotyping Reagents kit (Sigma). IgE measurements were quantified with a Mouse IgE ELISA quantification kit (Bethyl Laboratories, Montgomery, TX, USA).

Total parasite antigens were prepared by disrupting six adult nematodes (males and females) by sonication and centrifuging at $20,000 \times g$ for 20 min. The supernatant was used as total antigens.

2.10. Lymphoproliferation and interleukin determination

Splenocytes were collected aseptically from the spleens of mice immediately after death. Single-cell suspensions were prepared in complete RPMI 1640 medium supplemented with 10% inactivated fetal calf serum (IFCS), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.05 mM β -mercaptoethanol, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Splenocytes (2.5×10^6 cells/ml) from different mice and groups of mice were added to multi-well culture trays to make up 100 μ l per well. The cells were stimulated with 100 μ l of 25 μ g/ml of the SP and allowed to proliferate for 72 h at 37°C in a humid atmosphere with 5% CO₂. After 60 h the medium was collected and used for cytokine analysis. The cells were pulsed with 1.0 μ Ci of ³H-thymidine [6-³H-thymidine (specific activity 26–30 Ci/mmol)] and incubated for 12 h.

Subsequently the cultures were centrifuged and the pellet washed three times in cold medium and precipitated with TCA at 10% for 2 h at 4°C before being measured in a β -Spectrometer (BECKMAN LS 6000TA). The radioactivity in the pellet was recorded as a reflection of the existing radioactivity and incorporated in the precipitable material of the splenocytes.

Splenocyte cultures without antigen or stimulated with 5 μ g/ml phytohemagglutinin (PHA) were taken as growth and positive controls respectively. Proliferation index is equal to DPM of the problem/DPM control if ≥ 2 , is taken to be specific proliferation.

2.11. Cytokine determination

The levels of IL-4, IFN γ , TGF- β , IL-2, IL-10, and IL-17 in splenocyte culture supernatants were determined by ELISA following the manufacturer's instructions (Bender MedSystems, Vienna, Austria). Absorbance measurements were made by Multiskan Spectrum (Thermo Scientific, Madrid, Spain) at 492 nm.

2.12. Statistical analysis

Fisher's test was used to determine the homogeneity of variance between groups. Results from proliferation assays and cytokine production are shown as mean values plus the 95% confidence intervals. The Bonferroni test was applied to estimate the significance of the difference between means. Results of the ELISA tests (antibodies and interleukins) were indicated as mean values $\pm$ S.E.M. of the animals of the different groups at different times during each experiment.

All the experiments were repeated three times. $p \leq 0.001$ was considered to be extremely significant. GraphPad Instat v 3.05 software was used for the statistical test.

3. Results

Several positive clones were identified in the immunoscreenings from the 10^5 independent clones of the cDNA library. Among the positive clones was a sequence of 315 bp, which we called CT2-2. In a search for homologies in WormBase we identified a clone possessing 67% similarity to an EST of *Caenorhabditis elegans*, which corresponded to the catalytic subunit of the serine/threonine protein phosphatase gene Y40H4A.2 (EMBL: NP-506609) (Table 1). The sequence was deposited at EMBL as gene *pph-1*; serine/threonine protein phosphatase (EMBL: AM041130).

The CT2-2 clone sequence was translated to amino acids using the Transeq program of the EMBOSS package [58]. According to the amino-acid sequence found, the first codon stop corresponded to

Table 2

Protection values obtained in the different groups. The total number of mice was 75 (15 per group). Column 1 shows the number of adult worms recovered from all the mice in each group. Column 2 shows the total number of infected mice in each group at the end of the experiment. Column 3 shows the percentage of mice that had not developed adult worms after the challenge.

	Number of worms recovered	Number of infected mice	Percentage of mice with effective vaccination
Group 1	9	6	60
Group 2	3	3	80
Group 3	0	0	100
Group 4, adjuvant control	40	15	0
Group 5, control	45	15	0

nucleotides 229, 230 and 231 (TAA), for which a peptide of 76 amino acids was translated with the sequence (Table 1).

The search in the sequence of amino acids by BLASTp showed that this peptide shares close homology with two domains belonging to the catalytic subunit of the family serine/threonine phosphatase 2A (PP2Ac), cd00144, PP2Ac, protein phosphatase 2A homologs, with a value of $E = 3e^{-15}$ and smart 00156 PP2Ac protein phosphatase 2A homolog, with a value of $E = 2e^{-16}$.

The analysis of the sequence with the Antigenic program of the EMBOSS software package showed several antigenic domains, of which the sequence ANFIIFRPVV (Table 1) was selected to obtain a polyclonal serum to be used afterwards in the immunoprotection assay.

The pool of human serum from patients infected by *A. costaricensis* detected a protein band of 36 kDa in the pellet of the bacteria transformed from clone CT2-2, corresponding to the sum of the molecular masses of the recombinant peptide plus the glutathione transferase from the expression vector. Also, by means of ELISA, the serum from the mice immunized against the SP was capable of recognizing the synthetic peptide at a dilution of 1:1600. This immune serum recognized a single band of some 36 kDa in the homogenates of adult nematodes (Fig. 1).

A sample of the polymerized antigens SP and SP plus CTB was analyzed by SDS-PAGE. CTB weighed 12 kDa, and a range of species appeared with apparent molecular weights of 12–40 kDa for the SP linked to CTB, whilst a range of bands from 8 kDa to approximately 27 kDa was observed for the SP in 20% SDS-PAGE, suggesting that most of the complexes contained several copies of each peptide.

The ISCOMs incorporating polymerized antigens showed the typical box structure and were free of either protein precipitates or lipid micelles.

Protection values obtained after the immunization protocols appear in Table 2, which shows the protection values, the number of worms recovered and the number of mice infected in each of the experimental groups.

All mice were examined for the presence of parasites. In Group 1 nine worms (three males and six females) were recovered from six of the 15 mice infected in the group. The infected mice presented hemorrhages and granulomas in the intestine near the vessels where the parasites were found. In Group 2 one male and two female worms were recovered from three mice that showed intestinal granulomas and hemorrhages. No worms were recovered in Group 3 and none of the mice presented alterations. As far as the control groups were concerned, the adjuvant control rendered a total of 40 worms whilst the infection control rendered 45. All the mice in these latter two control groups showed intestinal alterations, granulomas and hemorrhages. In some cases in the adjuvant control group the worms were recovered from ectopic locations such as the aorta.

The spleen sizes of the different mice are set out in Fig. 2, where it can be seen that there were significant differences between the vaccinated groups and the non-vaccinated ones. The mice in control Groups 4 and 5 presented splenomegaly indices of 2.2 and 2.4 respectively compared to the spleens of the uninfected mice. The

spleens of the mice in Group 3 showed no alterations compared to those of the non-immunized and unchallenged mice.

The determination of total IgG in the sera collected from the mice in the different groups vs the SP and vs the total homogenate from adult *A. costaricensis* is shown in Fig. 3a and b. In Groups 1, 2 and 3 the total immunoglobulin titers vs the SP (1:60–1:6000 in Group 1, 1:60–1:8000 in Group 2, and 1:60–1:6000 in Group 3) and vs the total antigen from day 15 to day 75, when the mice were killed (1:60–1:8000 in Group 1, 1:60–1:10,000 in Group 2 and 1:60–1:13,700 in Group 3), whereas in Groups 4 and 5 the titer of antibodies vs the SP and vs total antigen increased after the challenge.

The levels of antigen-specific IgA in the groups vaccinated intranasally with the combination of the SP and CTB in ISCOM, and the inclusion bodies together with the component of the bacterial walls were higher than those induced in other groups (Fig. 4a), whilst the IgE levels (Fig. 4b) rose only after the challenge. It is noteworthy that in Group 3, in which the mice were immunized with the

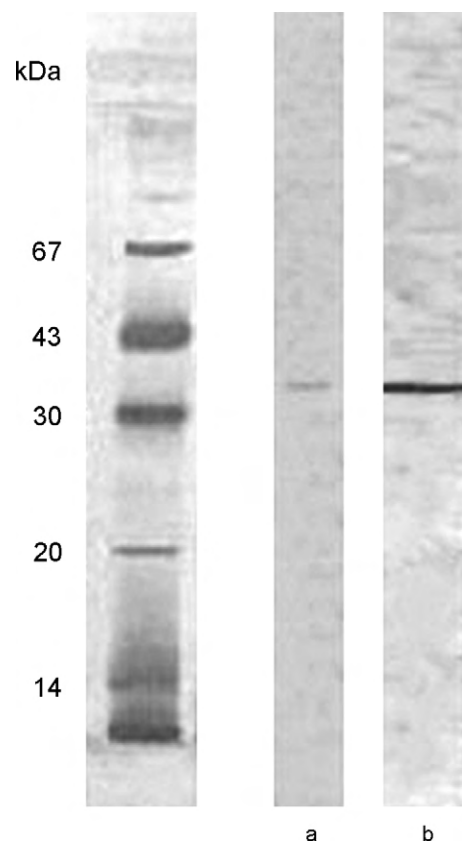


Fig. 1. Immune serum recognition against the synthetic peptide by immunoblotting. (a) CT2-2 recombinant recognition in the pellet of the bacteria transformed with the catalytic gene subunit of pph1 serine/threonine phosphatase. (b) 36 kDa band recognition by the immune serum against the synthetic peptide of a homogenate of adult *A. costaricensis*.

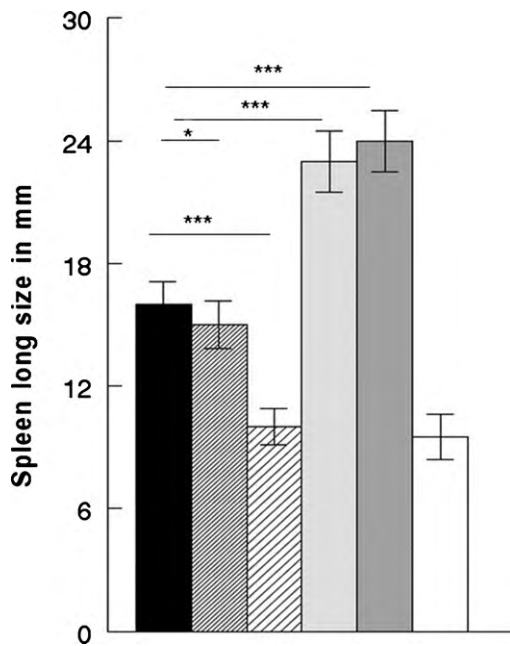


Fig. 2. Spleen sizes in the experimental groups immunized intranasally $\pm$ SEM. Group 1, encapsulated synthetic peptide in ISCOM (black column); Group 2 (finely hatched column), synthetic peptide bound to the CTB encapsulated in ISCOM; Group 3 (broadly hatched column), immunized with recombinant peptide and bacterial walls; Group 4 (light-grey column), control adjuvants; Group 5 (dark-grey column), infected control. Asterisks indicate the statistical significance (***) $p \leq 0.001$; * $p \leq 0.01$). The results are the mean of 3 experiments.

bacterial walls and the recombinant protein, this immunoglobulin did not increase at any time, even after the challenge.

Lymphoproliferation measured as stimulatory index is shown in Fig. 5, indicating that the splenocytes of the mice immunized with either the synthetic or recombinant antigens presented higher lymphoproliferation levels than those in the infection control groups ($p < 0.001$).

The ratio IgG2a/IgG1 indicates that during the immunization period Groups 1 and 3 alone showed a clear Th1 response whilst the remaining groups showed no alteration in the type of response. Fifteen days after the challenge the ratio diminished in Groups 1 and 3 whilst no changes were to be seen in the rest of the groups until 30 days post-challenge, when all the groups showed a change in their Th1 response (Fig. 6). The interleukin levels in the supernatants of the splenocytes from the different mice on exposure to the SP are shown in Figs. 7–9. The results for Group 3 are noteworthy as the levels of IFN γ , IL-2, and IL-17 were higher than in the other

experimental groups. The IL-17 levels in the immunized groups were higher than in the control groups, particularly in those mice which were immunized with inclusion bodies or where CTB was included in the adjuvant.

4. Discussion

Our assays have served to demonstrate the levels of immunological response and protection afforded by vaccination against *A. costaricensis* with synthetic antigens and recombinant peptides belonging to the catalytic region of the serine/threonine phosphatase 2 A (PP2A) of the parasite.

Numerous publications have described protection against reinfection after parasitization by nematodes, leading to attempts to provide immunization with irradiated L3 larvae (4, 8, 46, 66, 112) or excretion components derived from these larvae [59–64], especially when dealing with nematodes whose biological cycle includes an endogenous migratory phase, as in the case of *Ascaris* [44,45,65,66]. Within this context, Watanabe et al. [91] (116) used a massive infection with L3 of *A. costaricensis* to achieve a successful immunoprophylaxis against the challenge after treating the animals with levamisole and eliminating the migratory stages of the parasite, which demonstrates that stages L3–L4 may well be candidates for immunoprophylaxis in this helminth.

The sequence studied in this work (clone CT2-2) corresponds to the subunit C, which carries out the catalytic activity of the enzymes of the PP2A family. The immune serum developed against the recombinant peptide of clone CT2-2 and also a synthetic peptide (SP) recognized a single band of 36 kDa in the homogenate of adult *A. costaricensis* by immunoblotting. This molecular weight has been reported elsewhere for the catalytic subunit of this enzyme [49,67]. Similarly, by dot blotting, the pool of positive human sera recognized the SP immobilized on membranes of cellulose acetate (data not shown).

The catalytic subunits of PP2Ac of 36 kDa and PP2Aa of 65 kDa constitute the constant structural subunits of the enzyme core. Both subunits have only two isoforms [49], but the third subunit, known as subunit “b”, has numerous families, each of which in turn has multiple isoforms [49,50,68]. PP2A is ubiquitous and involved in many cell processes [67]. Furthermore, it participates in the mechanisms of cell signaling [49,69]. The enzyme is also related to the development of different phases of the biological cycle of the protozoan parasites [70–74]. In nematodes, the enzyme has been identified in *Brugia malayi* [75], *Trichinella spiralis* [76], *C. elegans* [77], *Oesophagostomum dentatum* [78], and *Trichostrongylus vitrinus* [79]. In *T. vitrinus* and *O. dentatum* the serine/threonine protein phosphatase has been described as stage-specific and

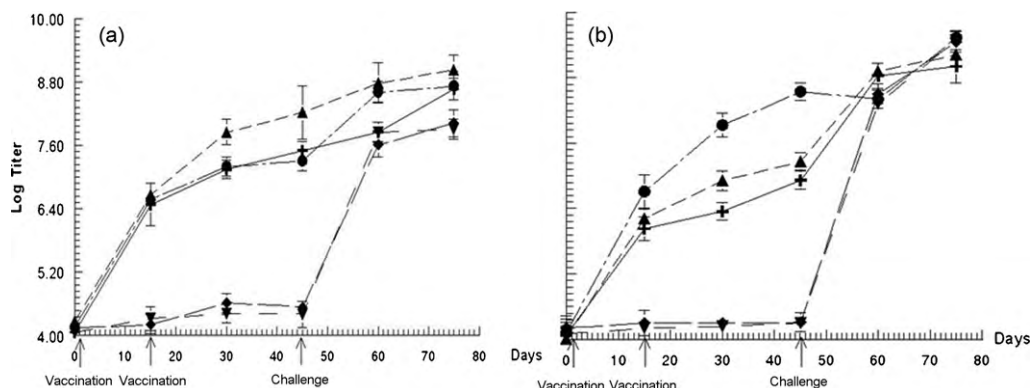


Fig. 3. Humoral immune response in the experimental groups. Titers of the total IgG of the various groups throughout the experiment $\pm$ SEM. (a) Synthetic peptide; (b) total IgG vs total antigen (total homogenate). Group 1: +, synthetic peptide encapsulated in ISCOM; Group 2: ▲, synthetic peptide bound to the β fraction of the cholera toxin in ISCOM; Group 3: ●, immunized with recombinant peptide and bacterial walls; Group 4: ▼, adjuvant control; Group 5: ◆, infection control.

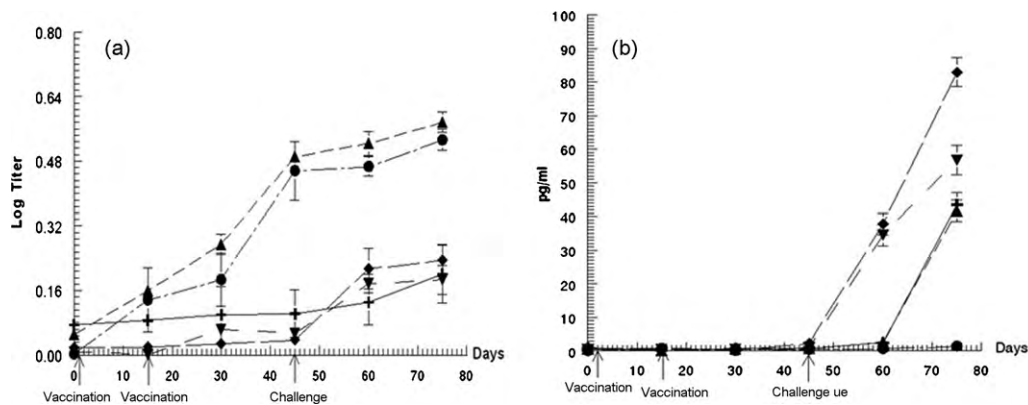


Fig. 4. Humoral immune response in the experimental groups. (a) Titers of the specific IgA levels vs synthetic peptide of the various groups throughout the experiment $\pm$ SEM. (b) Titers of the IgE levels of the various groups throughout the experiment $\pm$ SEM. Group 1: +, synthetic peptide encapsulated in ISCOM; Group 2: ▲, synthetic peptide bound to the β fraction of the cholera toxin in ISCOM; Group 3: ●, immunized with recombinant peptide and bacterial walls; Group 4: ▼, adjuvant control; Group 5: ◆, infection control.

involved in reproductive processes because it participates both in spermatogenesis and sperm mobility [80,81]. In *A. costaricensis*, immunolocalization using the polyclonal antibody against the SP indicates that PP2A is located in the subcuticular epithelium, intestine, and genitals. Apart from this, RT PCR studies have revealed a highly significant level of expression in the larval L1 and L3 stages compared to the stage-specific adult forms, (data in preparation).

As mentioned above, the susceptibility and different immune responses of the host towards *A. costaricensis* is determined genetically by the strain of rodent used in the assays [13–16,82–85]. Geiger et al. [13] reported that mouse strain C57BL/6 resisted the illness best because it showed a more balanced Th1/Th2-type response and so we undertook our assays using this strain, and in some of the immunization groups we used a combination of adjuvants specific both to Th1 and Th2 (ISCOMs and CTB respectively).

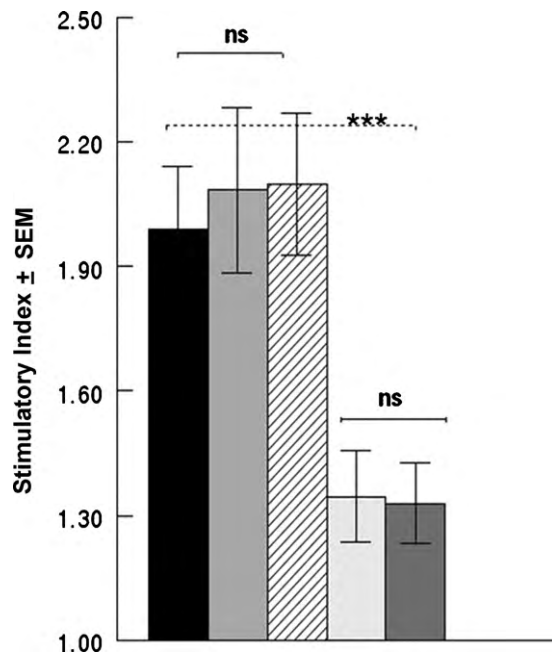


Fig. 5. Stimulatory index $\pm$ SEM. Group 1, encapsulated synthetic peptide in ISCOM (black column); Group 2, synthetic peptide bound to the CTB encapsulated in ISCOM (finely hatched column); Group 3, immunized with recombinant peptide and bacterial walls (broadly hatched column); Group 4, control adjuvants (light-grey column); Group 5, infected control (dark-grey column). Asterisks indicate the statistical significance (***) $p \leq 0.001$; ns = non-significant $p \geq 0.05$). The results are the mean of three separate experiments.

In our experiments none of the mice succumbed to the infection. This may be due to the fact that the animals only contracted a mild form of the infection, similar to natural infections, because of the small number of L3 larvae with which they were infected to avoid any problems in the course of the assays. In the infection control and adjuvant control groups the worms were recovered as adults.

During the post-infection period in the control groups (15 days post-challenge), according to the IgG2a/IgG1 ratio, a Th2-type response was observed, which changed at 30 days post-infection to a Th1 response. This response sequence has been reported elsewhere in studies into the immunological response to *A. costaricensis* in mice [14,85], in which at the outset of maturation and during oviposition the predominant response was of the Th1 type. Ishih et al. [85] suggest that the Th1 response, recognized by the high IFN γ levels observed after the maturation of the parasite, would contribute to a defense mechanism on the part of the host and help stimulate the formation of granulomas surrounding the eggs. It has been reported that in mice IgG2a, IgG2b and IgG3 antibodies are Th1 regulated, whilst the IgG1 response is Th2 dependent [86]. Groups 1 and 3 maintained a clear Th1 response until 15 days after infection, at which time Th1 levels diminished towards a Th1–Th2 response, only to return to Th1 at 30 days. Despite using ISCOMs with Groups 2 and 4, and perhaps as a consequence of using them combined with CTB, the response was Th2-type until 30 days post-

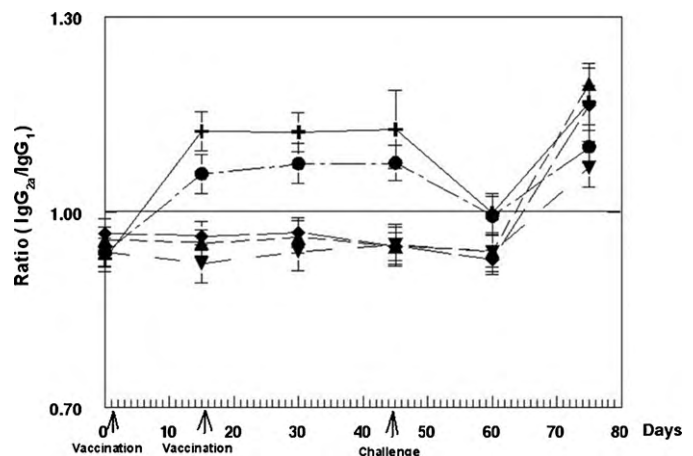


Fig. 6. Ratio of the immunoglobulin isotype (IgG2a/IgG1) $\pm$ SEM. Group 1: +, synthetic peptide encapsulated in ISCOM; Group 2: ▲, synthetic peptide bound to CTB in ISCOM; Group 3: ●, immunized with recombinant peptide and bacterial walls; Group 4: ▼, adjuvant control; Group 5: ◆, infection control. The results are the mean of three experiments.

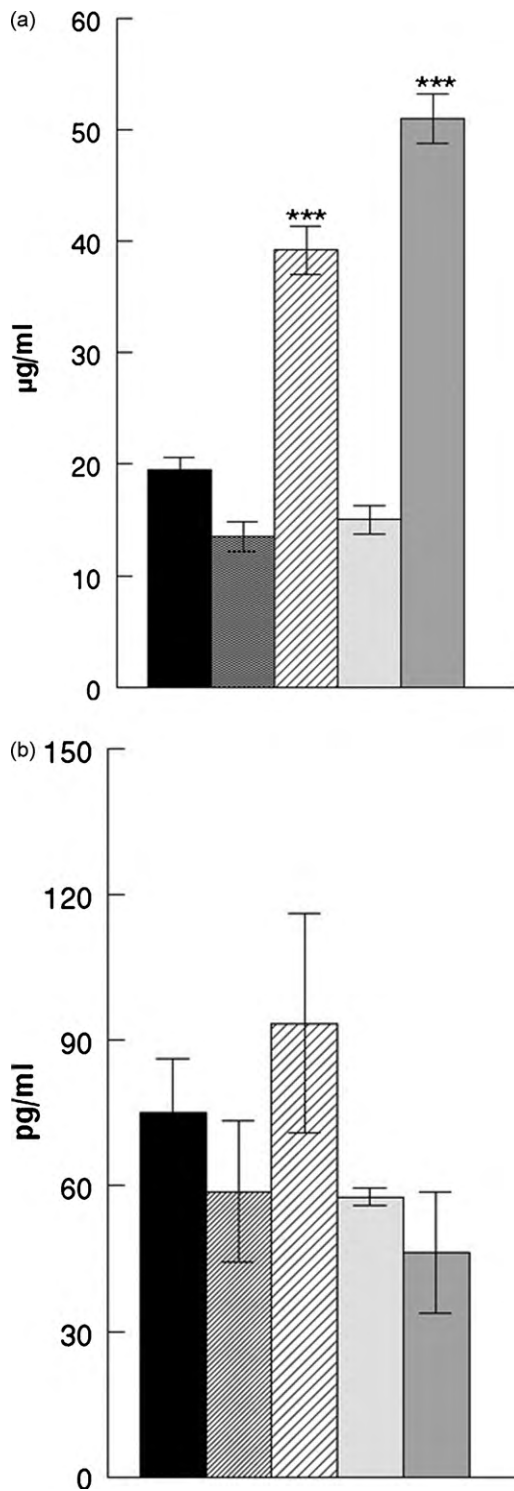


Fig. 7. Th1-type interleukin production by the experimental groups. (a) IFN-γ ± SEM levels in the supernatant of the culture medium produced by splenocytes in the experimental groups at 72 h after stimulation with the synthetic peptide; (b) IL-2 ± SEM levels in the supernatant of the culture medium produced by splenocytes in the experimental groups at 72 h after stimulation with the synthetic peptide. Group 1, encapsulated synthetic peptide in ISCOM (black column); Group 2, synthetic peptide bound to the CTB encapsulated in ISCOM (finely hatched column); Group 3, immunized with recombinant peptide and bacterial walls (broadly hatched column); Group 4, control adjuvants (light-grey column); Group 5, infected control (dark-grey column). Asterisks indicate the statistical significance ($p < 0.001$). The background levels are subtracted. The results are the mean of three separate experiments.

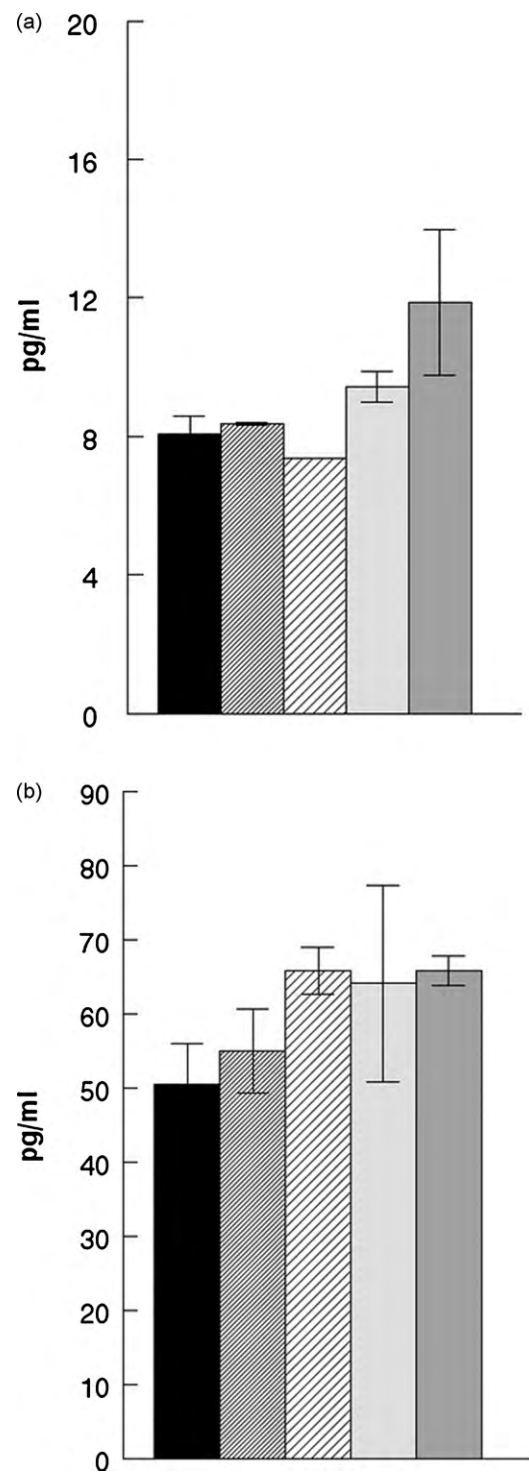


Fig. 8. Th2-type interleukin production by the experimental groups. (a) IL-4 ± SEM levels in the supernatant of the culture medium produced by the splenocytes in the experimental groups 72 h after stimulation with the synthetic peptide. (b) IL-10 ± SEM levels in the supernatant of the culture medium produced by the splenocytes in the experimental groups 72 h after stimulation with the synthetic peptide. Group 1, encapsulated synthetic peptide in ISCOM (black column); Group 2, synthetic peptide bound to the CTB encapsulated in ISCOM (finely hatched column); Group 3, immunized with recombinant peptide and bacterial walls (broadly hatched column); Group 4, control adjuvants (light-grey column); Group 5, infected control (dark-grey column). The results are the mean of three separate experiments.

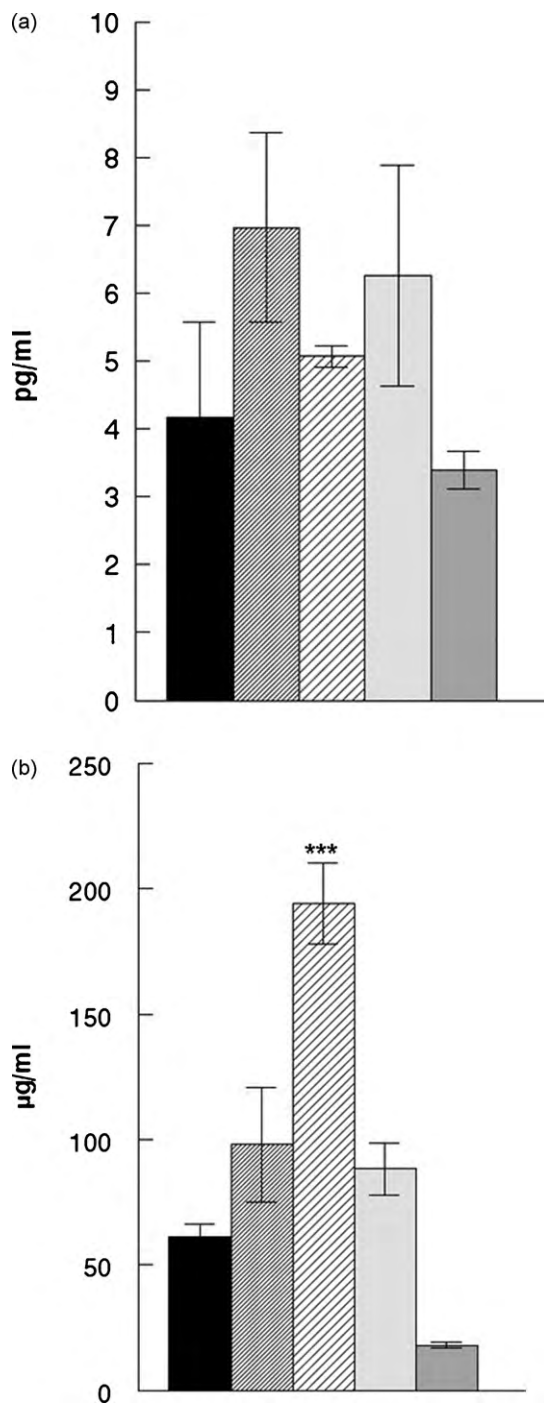


Fig. 9. TGF- β and IL-17 interleukin production in the experimental groups. (a) TGF- β $\pm$ SEM levels in the supernatant of the culture medium produced by the splenocytes in the experimental groups 72 h after stimulation with the synthetic peptide. (b) IL-17 $\pm$ SEM levels in the supernatant of the culture medium produced by the splenocytes in the experimental groups 72 h after stimulation with the synthetic peptide. Group 1, encapsulated synthetic peptide in ISCOM (black column); Group 2, synthetic peptide bound to the CTB encapsulated in ISCOM (finely hatched column); Group 3, immunized with recombinant peptide and bacterial walls (broadly hatched column); Group 4, control adjuvants (light-grey column); Group 5, infected control (dark-grey column). Asterisks indicate the statistical significance ($p < 0.001$). The background levels are subtracted. The results are the mean of three separate experiments.

challenge, at which time it changed to Th1. Some authors have demonstrated how CTB is capable of inhibiting the production of the Th1-type markers IL-12 and IFN- γ [30]. In Groups 1 and 3, immunized with ISCOMs and with inclusion bodies respectively, the Th2 response, typical of the prematuration phase of *A. costaricensis*, is clearly inhibited. *A. costaricensis* induces the Th2 response during the intestinal phase and the migration phase through the mucosa as most intestinal nematodes [87].

In our study we determined the efficiency of the vaccination by the reduction in the number of worms recovered (Table 2) and diminished splenomegaly (Fig. 2). The significant decrease in splenomegaly in the immunized groups compared to the non-immunized controls is the result of the lower parasitic burden in the former. The size of the spleens in the mice with maximum protection (Group 3) showed no alteration compared to the normal mice. In Groups 1 and 2, although the mice suffered slight spleen enlargement ($p \leq 0.05$ and $p \leq 0.001$ respectively compared to the non-vaccinated – uninfected normal control group), this was considerably less than in the infected and adjuvant control groups. Ishih and Nishimura (1997) [15] described how at 28 days post-challenge the spleens of SM/j and A/J mouse strains infected with 10 or 20 L3 larvae were 5–6 times larger than those of uninfected mice, showing that the absence of splenomegaly in vaccinated animals is a marker of the vaccination's capacity to inhibit the development of the parasites. According to our data the level of splenomegaly in mice infected with *A. costaricensis* is proportional to the presence of the parasites in the blood stream in the prematuration and/or adult stages.

Total IgG levels against the SP and against the total antigens of the worm showed increases, whilst in the control groups this increase occurred after the first week of the challenge with L3 larvae. It should be noted that the levels of specific IgA increased in Group 2, in which the antigen was linked to CTB. It is known that CTB induces mucosal response, Treg cells and the production of IgA, which would explain the increase in the levels of this immunoglobulin [88]. Furthermore, it is also known that nasal immunization with bacteria can induce local and systemic antibody responses to elicit strong antigen-specific IgG and IgA responses [89,90]. In Group 3, in which immunization was carried out with the recombinant protein and bacterial walls, both IgA and IgG levels were high from the moment of the first immunization. In contrast to specific IgA, IgE remained at undetectable levels in all the groups during the entire vaccination protocol, whereas 14 days post-challenge it was found to be significantly higher in both control groups. In Groups 1 and 2 this increase was delayed until 30 days post-challenge, showing that they exhibited partial protection. The increase in IgE levels at this time after the challenge coincided with the maturation of the worms in a similar way to that described elsewhere [13,85,91–93]. In Group 3, however, in which vaccination gave 100% protection, IgE levels remained undetectable until the end of the experiment, which confirms that IgE levels are closely related to the presence of adult worms and maturation. The IgG levels compared to both total homogenate and SP showed that intranasal immunization was effective in all cases. The nasal cavity and mucosa harbor less proteolytic activity than the intestinal lumen, in which the antigenic proteins can be degraded, and therefore intranasal inoculation of small quantities of antigens can be more effective and capable of triggering a mucosal immune response [94]. The nasal-associated lymphoid (NALT) identified in rodents [95] is equivalent to Waldeyer's rings in humans [96] and represents the oropharyngeal lymphoid tissue of the upper respiratory tract. This tissue consists of follicles covered by a specialized epithelial cell layer similar to the M cells of Peyer's patches and a layer of lymphoid cells that include CD4+ and CD8+ T cells.

Bacterial ghosts promote the capture and maturation of dendritic cells, thus encouraging a strong Th2 response when the

administration is mucosal [90]. Therefore, antigens when administered intranasally are absorbed and trigger an immune response in the mucosa, thus opening a very promising future for their use in vaccines [97]. Intranasal inoculation has been used in studies and tests of vaccine formulas against parasitic nematodes such as *T. spiralis* [98] and *Ascaris suum*, in which the authors also bound the antigen to a cholera toxin [43–45], achieving a high level of protection. Geiger et al. [14] studied the production levels of the different interleukins by C57BL/6 mice infected with *A. costaricensis* and reported that the splenocytes stimulated with total antigens of the parasite produced high IL-10 and IFN γ levels and low IL-4 and IL-2 levels 28 days post-infection. Our results indicate that the IL-10 levels found in Groups 1 and 2 immunized with ISCOMs as adjuvant were lower than in the rest of the groups but without significant differences (Fig. 8b). It is noteworthy that in the group of maximum protective effectiveness (Group 3) the level of this interleukin was similar to the two control groups. IL-10 was considered to be an interleukin marker of the Th2 response capable of inhibiting the production of IFN γ , although it is currently related both to the Th2 and Th3 response, playing an important role in the susceptibility and resistance of the host to intestinal nematode parasites. Mice deficient in this cytokine do not reject *Trichuris muris* whereas normal mice do [99]. The production of IL-4, the other Th2 interleukin (Fig. 8a and b), showed that in all the immunized groups production levels were appreciably lower than in the control infection group. IFN γ levels in the various experimental groups indicated that the mice in Group 3 and the infection control group expressed significantly more IFN γ (difference $p \leq 0.001$) than Groups 1, 2 and 4. The rise in IFN γ in Group 3 may have been due to the use of the bacterial component used as adjuvant [100] rather than the normal development of the parasite in the mice [14], whereas in Group 4, which should theoretically have responded in a similar way to the infected group (Group 5), the induction of Th2, controlled by CTB, was possibly responsible for the low responses of the interleukin Th1-response markers IFN γ and IL-2 (Fig. 7b). The production of IL-2 was high in Group 3. The low production of IL-2 in the control infection group is noteworthy when compared to the high production of IFN γ in this group, indicating that IFN γ production in the mice in this group does not originate from a Th1 response but stems from some other type of response. It has been shown recently that the production of IFN γ may not depend exclusively on the levels of IL-12 and the Th1 pathway. CD8 T cells can produce IFN γ and IL-17 in an dependent IL-23 form [101]. A study of IL-17 production revealed that it reached higher levels in the immunized groups, especially Group 3, than in the control groups (Fig. 9b). The values of the other vaccinated groups were higher, although they were not significant compared to those of the control groups. The production of TGF- β was higher in the immunized groups and in those treated with the adjuvants than in the control group (Fig. 9a). The activation of a Th17 response in mice has been determined in naïve T cells by their activation with IL-1 β or TGF- β -1 as well as IL-6, and IL-21, which, by the activation of Stat3 in the Treg cells, induce the receptor expression of the transcription factor of the orphan receptor to the retinoic acid (ROR γ mat). Meanwhile, in humans, it appears that TGF- β is inactive, with IL-23 playing a role in the activation in this type of response [102,103]. Dendrite cells activated after the uptake of antigens by the Toll-like receptors (TLR) produce TGF- β , IL-6 and IL-23, prompting the proliferation of a Th17 response [101]. These cells in turn secrete IL-17A, IL-17F and IL-6, promoting the recruitment of polymorphonuclear leukocytes in acute infections, whilst IL-17 encourages the production of GM-CSF, IL-6 and TNF α , which sustain the Th17 lymphocytes that participate in the auto-immune inflammatory response or that caused by microorganisms [104]. Research into the Th17 response has enabled us to identify the three independent and exclusive pathways of the inflammatory responses: IL-12/IFN γ , IL-4/IL-5/IL-

13 and IL-23/IL-17 [105]. IL-4, a marker of the Th2 response, acts as a strong inhibitor of the Th17 response [106]. Our results demonstrate that, especially in the group for which the immunization was made with the recombinant peptide, the induction of a specific Th1-Th17 response, i.e. inflammatory, is capable of blocking the development of the infective L3 larvae responsible for maintaining the cycle in the definitive natural hosts.

Our study has revealed new possibilities with regard to immunization against parasitic helminths in which protective immunity is determined by a Th1-Th17 inflammatory response and the antigen used and manner of inoculation may constitute a model for those parasitic diseases whose infective stage must undergo a process of alterations in their biological cycle and penetrate the intestinal mucosa before maturation.

Acknowledgements

This research was financed by a Spanish Ministry of Science and Technology grant (AGL2007-60123/GAN). The authors thank Prof. Pedro Morera from the Pathology Service, San Juan de Dios Hospital, San José, Costa Rica for providing us with the strain of *A. costaricensis* used in these studies and Dr. J. Trout for correcting and revising their English text.

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